The Development of Aptamers for use with Surface Plasmon Resonance (SPR) Sensing as a Novel Detection Method for *Legionella pneumophila* (*Lp*)

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LIST OF ABBREVIATIONS

4PL	Four parameter logistic
AEGIS	Artificial expanded genetic information systems
AFBI	Aptamer fluorescence binding and internalization
AFNOR	Association Française de Normalisation
Au	Gold
AYE	ACES buffered yeast extract
BSA	Bovine serum albumin
cApt	complementary aptamer sequence
CB	Colorimetric based
CCD	Charge coupled device
CDC	Centre for Diseases Control and Prevention
cDNA	complementary DNA
CE	Capillary electrophoresis
CL	Chemiluminescence based
CPM	Counts per million
CYE	ACES buffered Charcoal yeast extract
DALYs	Disability Adjusted Life Years
ddPCR	digital droplet PCR
DNA	Deoxyribonucleic acid
E phase	Exponential phase
ECDC	European Centre for Disease Control
EDA	Ethidium monoazide
ELISA	Enzyme linked immunosorbent assay
EtBr	Ethidium dibromide
EtOH	Ethanol
EV-FO	Evanescent field-based fibre optics
FB	Fluorescence based
FISH	Fluorescence in situ hybridization
FITC	Fluorescein 5-isothiocyanate
FP	Forward primer
GFP	Green fluorescence protein
GVPC	Glycine, Vancomycin, Polymyxin, Cycloheximide
HPLC	High performance liquid chromatography
HTS	High throughput sequencing
Icm/Dot	Intracellular multiplication/defective organelle trafficking
IM	Inner membrane
IMS	Immunomagnetic separation
ISO	International Standard Organization
ITC	Isothermal titration calorimetry
LB	Lysogeny broth
LCV	Legionella Containing Vacuole
LD	Legionnaires Disease

LED	Light emitting diode
Leg	Legioaminic acid
Lp	*Legionella pneumophila*
LFA	Lateral flow assay
LOD	Limit of detection
LPS	Lipopolysaccharide
LSPR	Long range SPR
MIF	Mature infectious form
Mip	Macrophage infectivity potentiator
MLST	Multi locus sequence typing
mOMP	Outer membrane pore forming protein
MPN	Most probable number
NA	Nutrient agar
NB	Nutrient broth
nESI-MS	nano-electrospray ionization mass spectrometry
NMR	Nuclear magnetic resonance
OD	Optical density
OM	Outer membrane
OMP	Outer membrane proteins
OPPP	Opportunistic premise plumbing pathogens
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activation inhibitor 1
PAO1	*Pseudomonas aeruginosa*
PBS	Phosphate buffered saline
PE phase	Post Exponential phase
PEG	Polyethylene glycol
PEX	Cross-linked polyethylene
PMA	Propidium monoazide
PVC	Polyvinyl chloride
qPCR	Quantitative Polymerase Chain Reaction
RI	Refractive index
RNA	Ribonucleic acids
RP	Reverse primer
rRNA	Ribosomal ribonucleic acid
SARS CoV 2	Severe acute respiratory syndrome coronavirus 2
SELEX	Systemic Evolution of Ligands through Exponential enrichment
Sg	Serogroup
SPR	Surface plasmon resonance
SSC	Sodium saline citrate
ssDNA	single stranded DNA
T4SS	Type IV secretion system
VBNC	Viable but non culturable
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

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ABSTRACT

Legionella pneumophila are water-borne pathogenic bacteria responsible for severe respiratory disease known Legionnaire's disease (LD). Engineered water systems such as cooling towers and plumbing are leading sources of the bacteria, therefore rapid detection of *Lp* in these systems is required to prevent future LD outbreaks. Infections occur when the bacteria are aerosolized, and the contaminated bio-aerosols are inhaled. Current standard ISO certified detection strategies for *Lp* are the standard plate count method and qPCR which entail either long processing times or multiple, laborious and costly processing steps. Biosensors would help overcome the limitations of such traditional microbial detection methods. SPR biosensors specifically are considered a gold standard in optical biosensing due to their sensitivities as well as ability to be label free and use samples in their native state. To adapt the SPR system for *Lp*, biorecognition elements such as antibodies or aptamers need be used. Aptamers are single stranded short DNA or RNA oligonucleotides that fold into specific structures and bind to a wide variety of targets ranging from small molecules to cells. Their chemical make-up gives them numerous advantages over traditional biorecognition molecules i.e antibodies, in terms of cost and stability. Prior to this work no aptamers and thus no aptamer-based technology existed for the detection of *Lp*.

In the first study, Systemic Evolution of Ligands through EXponential enrichment (SELEX) was, therefore, used to identify aptamers that bound specifically to *Lp*. Ten rounds of positive selection and two rounds of negative selection against two *Pseudomona*s species were performed. which lead to the identification of two aptamers binding specifically to *Lp* with K_D in the 100nM range. These two aptamers were characterized using flow cytometry and fluorescence microscopy.

In the second study, the aptamer R10C5 was optimized for use in a Surface Plasmon Resonance imaging (SPRi) sensing platform to develop a titration assay, where the concentration of *Lp* was determined by quantifying the amount of unbound aptamers. The combination of aptamer titration assay with SPR enabled specific detection of *Lp* to an LOD of $10^{4.3}$ CFU/ml, without the use of labelling or signal amplification strategies. The SPR based assay also showed how different media-namely PBS, model tap water (Fraquil) and SSC, affects the ability of the aptamers to bind to *Lp*. In terms of specificity, the assay shows minimal detection of *Pseudomonas*, a common inhabitant of water systems.

In the third study, a single round of four parallel selections were used in combination with high throughput sequencing to identify higher-order aptamers binding to cells of *Lp* Serogroup 1. The combination of HTS and parallel selections enabled the evaluation of aptamer pools that evolved in the presence of different *Lp* variants as well as non-*Lp* water bacteria. The pools were then examined for sequences that were enriched in the presence of target *Lp* and depleted in the presence of non-target bacteria. HTS data revealed that following one round of selection, only a small fraction of sequences was enriched. Analyzing the enrichment of these sequences in the presence of target *Lp* versus non-target bacteria revealed four structurally complex, candidate aptamers of which only three are specific to the clinically dominant, *Lp* serogroup 1.

RESUME

La *Legionella pneumophila (Lp)* est une bactérie pathogène d'origine hydrique responsables d'une maladie respiratoire sévère connue sous le nom de maladie du légionnaire (ML). Les infrastructures d'eau tels que les tours de refroidissement et les réseaux de plomberie sont les principaux lieux où prolifèrent ces bactéries. Par conséquent, une détection rapide de *Lp* dans ces systèmes est nécessaire pour prévenir de futures épidémies de ML. Les infections se produisent lorsque les bactéries sont aérosolisées et que les bio-aérosols contaminés sont inhalés. Actuellement, la culture sur plaque et la qPCR qui impliquent soit de longs temps de traitement, soit des étapes de traitement multiples, laborieuses et coûteuses, sont les méthodes certifiées ISO considérée comme stratégies standard pour la détection de *Lp*. Les biocapteurs aideraient à surmonter les limites de ces méthodes traditionnelles de détection microbienne. Les biocapteurs SPR sont spécifiquement considérés comme une référence en matière de biodétection optique en raison de leurs sensibilités ainsi que de leur capacité à fonctionner sans étiquette et à utiliser des échantillons dans leur état natif. Pour adapter le système SPR à la *Lp*, des éléments de bioreconnaissance tels que des anticorps ou des aptamères doivent être utilisés. Les aptamères sont des oligonucléotides d'ADN ou d'ARN courts simple brin qui se replient dans des structures spécifiques et se lient à une grande variété de cibles allant des petites molécules à des cellules entières. Leur constitution chimique leur confère de nombreux avantages par rapport aux molécules de bioreconnaissance traditionnelles que sont les anticorps, notamment en termes de coût et de stabilité. Aucun aptamère et donc aucune technologie à base d'aptamère n'a été publié jusqu'à présent pour la détection de *Lp*.

Dans la première partie de nos études, l'évolution systémique des ligands par enrichissement EXponentiel (SELEX) a donc été utilisée pour identifier les aptamères qui se lient spécifiquement à *Lp*. Dix cycles de sélection positive et deux cycles de sélection négative contre deux espèces de Pseudomonas ont été effectués qui ont conduit à l'identification de deux aptamères se liant spécifiquement à *Lp* avec un K_D d'une valeur de l'ordre de 100nM. Ces deux aptamères ont été caractérisés par cytométrie en flux et microscopie à fluorescence.

Dans la deuxième partie de nos études, l'aptamère R10C5 a été optimisé pour une utilisation dans une plate-forme de détection d'imagerie par résonance plasmonique de surface (SPRi) pour développer un test de titrage, où la concentration de *Lp* a été déterminée en quantifiant la quantité

d'aptamères non liés. La combinaison du test de titrage aptamère avec SPR a permis une détection spécifique de *Lp* à une limite de détection de $10^{4,3}$ UFC/ml, sans l'utilisation de stratégies de marquage ou d'amplification du signal. Le test basé sur la SPR a également montré comment différents milieux, à savoir le PBS, l'eau du robinet (Fraquil) et le SSC, affectent la capacité des aptamères à se lier à *Lp*. En termes de spécificité, le test montre une détection minimale de *Pseudomonas,* un habitant commun des systèmes d'eau.

Dans la troisième partie de nos études, un seul cycle de quatre sélections parallèles a été utilisé en combinaison avec un séquençage à haut débit (SHD) pour identifier des aptamères d'ordre supérieur se liant aux cellules du sérogroupe *Lp* 1. La combinaison de SHD et de sélections parallèles a permis l'évaluation des pools d'aptamères qui ont évolué en présence de différentes variantes de *Lp* ainsi que de bactéries aquatiques autres que *Lp*. Les contenus des pools en séquences enrichies en présence de Lp cible et appauvries en présence de bactéries non-cibles ont ensuite été examinés. Les données SHD ont révélé qu'après un cycle de sélection, seule une petite fraction des séquences était enrichie. L'analyse de l'enrichissement de ces séquences en présence du *Lp* cible par rapport à des bactéries non-cibles a révélé quatre aptamères candidats aux structures complexes, dont trois se sont avéré spécifiques au sérogroupe 1 cliniquement dominant chez *Lp*.

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CONTRIBUTION TO KNOWLEDGE

The body of work developed in this thesis contributes to the current knowledge of bacterial detection methods by investigating aptamer based SPR biosensing for *Legionella pneumophila* (*Lp*) detection. This thesis does this by

1. Reporting the identification of the first two *Legionella pneumophila (Lp)* aptamers. This is the first time SELEX has been done using *Lp* cells as a target.

2. Reporting the use of aptamers in a SPRi based titration assay where the concentration of *Lp* cells is determined by measuring free or unbound aptamers. The study shows for the first time the use of aptamers in such a titration assay and illustrates a strategy to overcome the optical limits of SPR for bacterial detection.

3. Reporting the use of a parallel selection method (branched SELEX) in conjunction with high-throughput sequencing (HTS), using bacterial whole cells representative of source environments to identify aptamers. No study to date has utilized the branched SELEX method for whole bacterial cells. Additionally, the study highlights, for the first time, some important limitations to using the branched SELEX method. Finally, by generating sequencing data, the study contributes to the field since there is currently a lack of SELEX-HTS datasets.

4. Identifying and reviewing properties of waterborne microbes that would act as potential determinants towards optimal aptamers and aptamer-based biosensor development.

CONTRIBUTION OF AUTHORS

Chapter 2:

I am the first author of the manuscript in chapter 2. I reviewed the literature and compiled the information reported for the chapter and wrote the first draft of the manuscript. Sebastien Faucher and I edited the manuscript.

Chapter 3:

I am the first author of the manuscript in chapter 3. I optimized and performed the SELEX experiments, the cloning and sequencing experiments, as well as the first replicates for the selectivity assays. Furthermore, I analyzed the data generated, wrote the first draft of the manuscript, and edited it. I designed the experiments while Deanna Chinerman performed several of the characterization assays listed in the study specifically the K_D characterization assays, selectivity assays with the *Pseudomonas* strains as well as several cooling tower bacterial isolates. Maryam Tabrizian supervised and helped with data analysis and manuscript editing. Sebastien Faucher supervised and helped with the experimental design, data analysis and editing of the manuscript.

Chapter 4:

I am the first author of the manuscript in Chapter 4. I conceptualized and performed the majority of the experiments, analyzed the data generated and wrote the first draft of the manuscript followed by editing. Francisco Rafael Castiello helped conceptualize experimental parameters and performed preliminary assays. He also aided in data analysis and editing the manuscript. Sebastien Faucher supervised, helped review and edit the manuscript. Maryam Tabrizian supervised, provided input on experimental design, datasets and helped review and edit the manuscript.

Chapter 5:

I am the first author of the manuscript in Chapter 5. I designed and performed the branched SELEX experiments and the selectivity assays. I also prepped the SELEX pools for sequencing and analyzed all the data. Finally, I wrote the first draft of the manuscript and edited it. Maryam Tabrizian supervised and edited the manuscript. Sebastien Faucher supervised, provided input on experimental design, datasets and helped review and edit the manuscript.

INTRODUCTION: RATIONALE AND OBJECTIVES

Legionella pneumophila (*Lp*) is a pathogenic, Gram-negative bacterium responsible for two types of respiratory diseases, namely the severe Legionnaires Disease (LD) and a milder form of flu-like pneumonia known as Pontiac fever (McDade, Shepard et al. 1977). The water-borne bacterium occur in both natural and man-made water systems (Kozak, Lucas et al. 2013). In natural water systems the bacterium is present in low concentrations; however, its preference for increased temperatures (above 25∘C) and assimilating in biofilms make it one of the most prevalent water-based pathogens in engineered water systems (Abdel-Nour, Duncan et al. 2013, Kozak, Lucas et al. 2013).

Lp infections occur when the bacteria are aerosolized from engineered water systems and the contaminated bio-aerosols are inhaled by humans (Parr, Whitney et al. 2015). Man-made water systems provide optimal transmission conditions for *Lp* by generating aerosols. Leading sources of infection are aerosols produced from cooling towers, hot water distribution systems, humidifiers, misters, showers, fountains, spa pools and evaporative condensers (Parr, Whitney et al. 2015).

Outbreaks of LD occur consistently globally and have increased in recent years (PHAC 2019, (ECDC) 2021, CDC 2021). The true incidence rates of *Lp* infection however are often underreported (PHAC 2018, Cassell, Gacek et al. 2019). *Legionellosis* has high population and individual disease burdens (Cassini, Colzani et al. 2018).

Most *Legionellae*-associated outbreaks are instigated by management failure of man-made water systems such as allowing water to stagnate, lack of regular disinfection protocols as well as variability in monitoring strategies (Parr, Whitney et al. 2015, Proctor, Rhoads et al. 2020). As with any infectious agent, preventive maintenance is key to keeping *Lp* presence and transmission under control. Routine monitoring and surveillance strategies for the bacterium are critical to evaluate risk, initiate treatment of water sources, and prevent future outbreaks (Ashbolt 2015, Ramírez-Castillo, Loera-Muro et al. 2015). Detection of *Lp* is critical for monitoring and surveillance programs.

Current ISO certified standards to detect *Lp* from environmental matrices include (i) plate count culture methods (AFNOR NF T90-431, ISO 11731), and (ii) qPCR (AFNOR NF T90-471, ISO/TS

12869) (ISO 2017, ISO 2019). Plate count methods are the gold standard for *Lp* detection (Whiley and Taylor 2016). This method selects for *Lp* by exposing an environmental sample to various physical factors such as high temperatures and low pH followed by cultivation on selective media and the enumeration of bacterial colonies showing *Lp* specific morphology (Bopp, Sumner et al. 1981). These culture methods are extremely time consuming and often require extensive material and specialized labour. The culture method can take up to 15 days before obtaining a result with regards to an environmental sample (Trudel, Veillette et al. 2014, Whiley and Taylor 2016). Another limitation is the presence of viable but non culturable (VBNC) *Lp* cells. VBNC cells are problematic because since they cannot be cultured, true *Lp* cell counts are often underestimated (Whiley and Taylor 2016, Wang, Bédard et al. 2017).

qPCR methods quantify *Legionella* specific genes. In comparison to plate count methods, they have more rapid turn-around times, high sensitivities and specificities, lower limits of detection, as well as the ability to detect viable but non culturable cells (VBNC) cells (Díaz-Flores, Montero et al. 2015, Whiley and Taylor 2016). However, given that the method detects both VBNC and dead cells, a caveat is an overestimation of microbial burden (Whiley and Taylor 2016). Additionally, environmental samples are often complex matrices that may have inhibitors such as humic or fulvic compounds which would adversely impact the qPCR reactions and thus microbial recovery rates (Gentry-Shields, Wang et al. 2013). Finally, the multiple sample processing steps for DNA/RNA extraction increase the overall costs and complexity of this method.

Neither of these methods can be developed into rapid, cost-effective, sensitive tests that would identify the whole *Lp* bacterium in real-time, in situ, without any additional processing steps. Biosensors are promising detection technology that would provide a solution to these problems.

Biosensors are analytical devices that measure the presence or concentration of a bioanalyte such as protein, a cell, etc (Turner 2013). They can provide a quick, cost-effective, sensitive, real-time method of bacterial detection while eliminating the need for specialized labour (Ahmed, Rushworth et al. 2014). Surface plasmon resonance imaging sensors (SPRi) are optical biosensors whose properties make it attractive for bacterial detection (Dudak and Boyacı 2009). SPRi sensors are sensitive to changes in the refractive index (RI) of a media and thus can measure changes in RI that occur as a result of target-bioreceptor binding. This means samples can be used in their native state without any labelling (Dudak and Boyacı 2009). This sensor characteristic eliminates

the need for arduous processing steps while concurrently providing real time, continuous measurements vital for detection and analysis (Dudak and Boyacı 2009). To ensure the SPRi sensor is selective for specific microbial targets, biorecognition elements are required (Kumar, Hu et al. 2018). Antibodies are currently the most widely used bioreceptors in biosensor development and research, but aptamers are rapidly becoming an increasingly attractive alternative (Morales and Halpern 2018, Saad and Faucher 2021).

Aptamers are antibody analogues (Bunka and Stockley 2006). They are short single stranded DNA or RNA oligonucleotides that can bind to a wide range of targets, from small molecules to whole cells, with high affinity and specificity (Bunka and Stockley 2006, McKeague, De Girolamo et al. 2015, Davydova, Vorobjeva et al. 2016). They form stable complex structures to interact with their targets via shape complementarity, hydrogen bonding, electrostatic interactions or stacking interactions (Mayer 2009). A key characteristic of an aptamer is that it can be generated in vitro in a wide array of conditions, unlike antibodies which require strict physiological conditions (McKeague, McConnell et al. 2015). Due to their chemical make-up aptamers are easily modifiable, heat-labile and can be cost effectively, chemically synthesized in a high throughput manner (Mayer 2009). Prior to this work, no aptamers and thus no aptamer-based technology existed for the detection of *Lp*.

Considering their advantages, we hypothesized that aptamers could serve as viable bioreceptors on an SPRi sensing platform to detect whole *Lp* cells. Given the hypothesis, our research objectives for this thesis were to (i) generate candidate aptamers that bind to *Lp* (ii) validate and characterize the *Lp* binding aptamers, (iii) develop and optimize the SPR sensing system for use with aptamers, and (iv) evaluate the aptamer based SPRi biosensing system as a method to detect *Lp* in complex water matrices.

Prior to delving into the details of how each research objective was achieved, the next chapter (Literature Review) aims to address in detail the multiple elements involved in this study so as to equip the reader with the necessary background knowledge required to gain insight on this body of work.

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CHAPTER 1: BACKGROUND AND LITERATURE REVIEW

1.1 <u>Introduction</u>

Air. The subject of breathing pure sanitary air has frequently been hijacked by politicians for reasons other than biology and health (Selin and VanDeveer 2003, Gonzalez 2012, Zheng and Chen 2020). With good reason, as it is an indicator for quality of life and so serves as a powerful tool for furthering any political or economic agenda. Take for instance the window tax, introduced in England in 1696 (Oates and Schwab 2015). The window tax was a property tax based on the number of windows in one's home. To avoid the tax, many homeowners bricked or boarded their windows. It wasn't until 1851 that the tax was repealed, thanks to pressure from doctors and activists, who argued that the lack of light and fresh air caused many illnesses (Oates and Schwab 2015). Though much time has passed since 17th-19th century England, breathing fresh air remains a polarizing subject, forever caught in a battle between economics, politics, and environment (Oates and Schwab 2015).

The year 2020 brought in another dimension on breathing fresh air. From the "I can't breathe" statement of a dying George Floyd to anti-maskers co-opting the phrase for their agenda during the COVID-19 pandemic, the subject of fresh air and breathing was everywhere (Klinenberg and Sherman 2021). The infectious agent behind the COVID-19 pandemic i.e, the SARS-CoV2 virus, had managed to turn the world upside down by using a basic bodily function, i.e breathing, as a tool for its propagation.

Every exhale meant the release of aerosols and the transmission of the notorious coronavirus. Given the viruses public health significance, much research has been done in recent years on the production and spread of the small microbe containing aerosols (El Baz and Imziln 2020, Singh, Sanghvi et al. 2021, Zaneti, Girardi et al. 2021). However, this viral microbe is not the first pneumonia causing agent transmitted by aerosols. Years before COVID, there was another. A bacterium transmitted by aerosols produced by water systems. This infectious agent is known as *Legionella pneumophila (Lp)*.

1.1.1 General Biology of *Legionella pneumophila*

Legionnaire's disease (LD) is the name of the severe, often deadly, pneumonia caused by bacteria belonging to the genus *Legionella*. To date over 61 species of *Legionella* have been identified, with the majority of them being implicated in human disease (Phin, Parry-Ford et al. 2014, Amemura-Maekawa, Kura et al. 2018, Mondino, Schmidt et al. 2020, Walker and McDermott 2021). The species *Lp* is responsible for most LD cases with serogroup 1 being the most dominant disease-causing variant (Phin, Parry-Ford et al. 2014, Amemura-Maekawa, Kura et al. 2018, Mondino, Schmidt et al. 2020, Walker and McDermott 2021)

Lp is a Gram-negative, aerobic, rod shaped gammaproteobacteria (Edelstein and Lück 2015). The bacteria first came into prominence in 1976 when 182 members of the American Legion who had attended a conference at the Bellevue-Stratford Hotel in Philadelphia, developed a severe pneumonia. Thirty-four conference attendees succumbed to the illness, causing widespread alarm and a rush to identify the etiologic agent. To reflect the patients and clinical disease, the causative agent was thus called *Legionella pneumophila* (Fraser, Tsai et al. 1977, McDade, Shepard et al. 1977). Though the bacterium was identified following the Philadelphia outbreak, the first *Legionella spp.* are believed to have been isolated in 1944 and 1959 (Tatlock 1944, Bozeman 1968)

Lp is an intracellular pathogen. This enables the bacteria to occupy an array of niches making it widely prevalent in both natural and engineered water systems. In natural and engineered reservoirs, the main hosts for *Lp* are protozoa such as *Vermamoeba vermiformis* and *Acanthamoeba castellanii* whereas in humans its host cells are alveolar macrophages and monocytes (Figure 1) (Comas 2016).
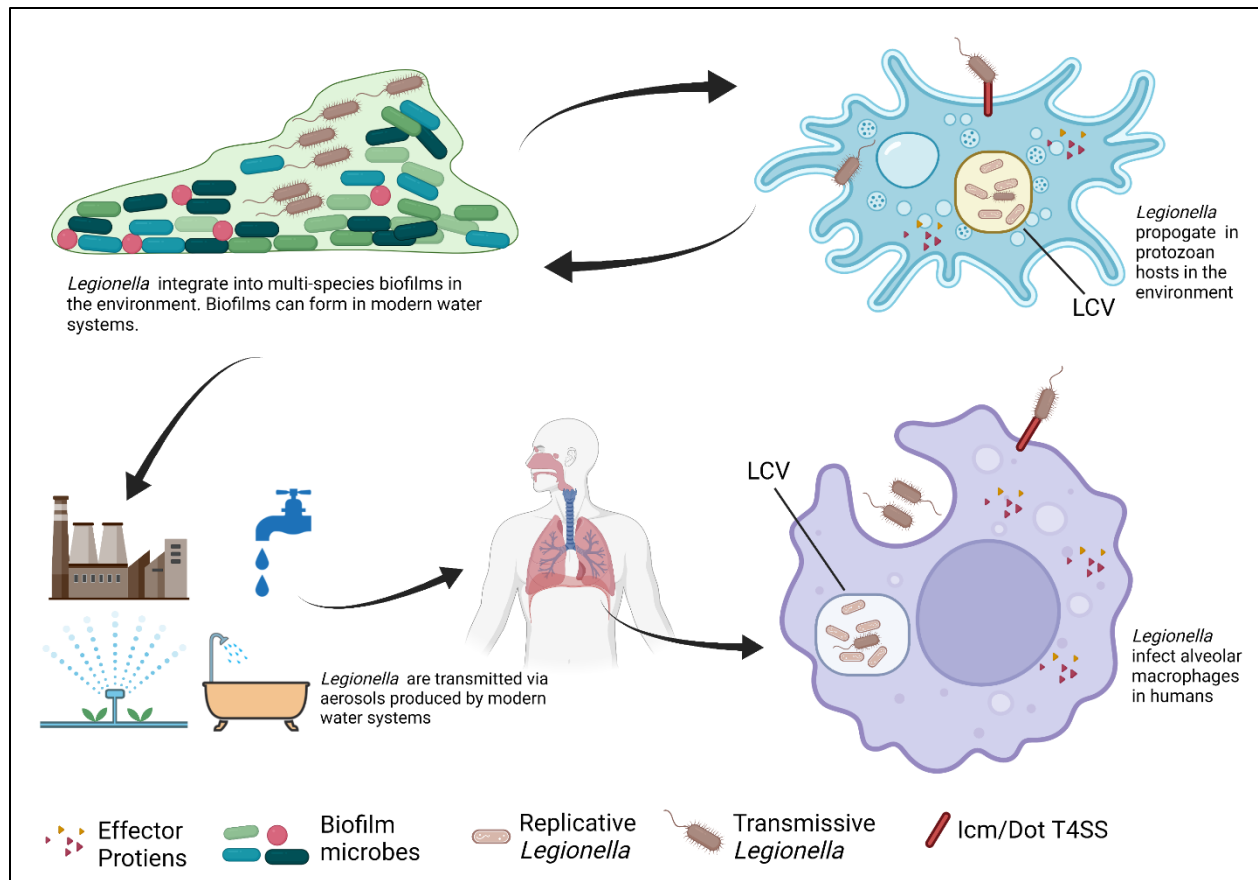
Figure 1. In the environment *Legionella* species infect protozoan hosts and exist in biofilms which can be found in most engineered modern water systems. Infections in humans occur when *Legionella* containing aerosols, produced by modern engineered water systems, are inhaled. In humans, *Legionella* species infect alveolar macrophages. In both protozoa and macrophages, the bacteria replicate and survive with the aid of a *Legionella* containing vacuole (LCV). The Icm/Dot T4SS (Type IV Secretion system) translocates hundreds of effector proteins that help maintain the LCV. Image created in Biorender and adapted from Comas, 2016

Lp replicates and persists intracellularly in multispecies biofilms or in free-living protozoan cells found in environmental (man-made or natural) reservoirs (Declerck 2010, Ashbolt 2015) or as planktonic cells provided the medium is rich with nutrients such as in laboratory conditions (Abdel-Nour, Duncan et al. 2013). The bacteria target hosts when shifting from the replicative to the transmissive phase. During the replicative phase the bacteria are nonmotile and not pathogenic whereas during the transmissive phase the bacteria grow flagella, become motile and consequently become infectious (Albert-Weissenberger, Cazalet et al. 2007). This phase shift from replicative to transmissive forms is governed by depletion of nutrients (Fonseca, Sauer et al. 2008, Fonseca and Swanson 2014). As with most foreign microbes, the host cells engulf the bacteria by

phagocytosis and deliver them to the lysosomal system in order to eliminate them. *Lp*, however, evades elimination by forming a bacterial phagosome known as the *Legionella* containing vacuole (LCV) (Mondino, Schmidt et al. 2020). This LCV protects *Lp* from being recognized by the hosts immune system and provides the bacterium with nutrients for its replication (Fonseca and Swanson 2014, Mondino, Schmidt et al. 2020). The maintenance of this LCV is governed by the Icm/Dot type IV secretion system (T4SS) (Mondino, Schmidt et al. 2020). The latter is a protein complex that traverses bacterial and phagosomal membranes and translocates over 300 effector proteins necessary for the bacterium's survival (Albert-Weissenberger, Cazalet et al. 2007, Gomez-Valero, Rusniok et al. 2019) Genomic sequencing data of over 60 *Legionella* species suggests a diversity of over 18,000 potential effector proteins illustrating the bacterium's adaptive potential ((Gomez-Valero, Rusniok et al. 2019)

Infections from *Lp* occur when aerosols and droplets contaminated with the bacterium are inhaled (Lin, Vidic et al. 1998). Engineered water systems such as cooling towers, hot water distribution systems, humidifiers, misters, showers, fountains, spa pools and evaporative condensers provide optimal transmission conditions by producing aerosols (Lin, Vidic et al. 1998, National Academies of Sciences and Medicine 2020). Infections and outbreaks from natural water systems rarely occur due to low concentrations of the bacteria (National Academies of Sciences and Medicine 2020). Apart from one case reported in Portugal in 2014, human-to-human transmission of *Lp* generally does not occur (Correia, Ferreira et al. 2016).

1.1.2 Epidemiology of *Legionella pneumophila*

Legionella spp., are one of the key water-borne environmental pathogens that contribute significantly to water-borne disease burden, a consequence of which is high human and financial costs (Gargano, Adam et al. 2017, Cassini, Colzani et al. 2018, Greco, Drudge et al. 2020, Collier, Deng et al. 2021). In fact *Legionella* was reported as one of the top five infectious agents in Europe that had a high number of disability adjusted life years (DALYS), which are years lost due to ill-health, disability or early death (Cassini, Colzani et al. 2018). Together with *Pseudomona*s and non-tuberculosis mycobacteria, the total healthcare costs from *Legionella* infections were US 2.39$ billion annually (Collier, Deng et al. 2021).

Outbreaks due to *Lp* occur consistently globally and have increased in recent years with *Lp* serogroup 1 accounting for 90% of global human infections (National Academies of Sciences and

Medicine 2020). The fatality rate for LD ranges between 5-30% depending on the location and environmental setting and can be as high as 50% in hospital and healthcare environments.(Garrison, Kunz et al. 2016). According to the Centre for Disease Control (CDC-USA), incidences of diseases associated with *Lp* infections have increased by nine times between the years 2000 and 2018 (CDC 2021). The ECDC (European Centre for Disease Control) reports a 57% increase in incidence rates of LD from 2015 to 2019 ((ECDC) 2021). Epidemiologic studies from Europe, Australia and the USA indicate that the average incidence rate is about 10-15 cases per million people (World Health Organizaton 2018, National Academies of Sciences and Medicine 2020, (ECDC) 2021). In Canada, Legionellosis incidence rates per 100,000 people have increased by 700% between 2000 and 2019 (Canadian Notifiable Diseases Surveillance System (CNDSS) 2020). The true incidence rates however are much higher and often underreported either due to low rate of testing or a lack of diagnostic tests that can detect other *Legionella* species and *L. pneumophila* serogroups (Collier, Deng et al. 2021). The rise in *Lp* outbreaks is multifactorial and can be attributed to several reasons. One is the aging infrastructure of water distribution systems of most population dense cities (Parr, Whitney et al. 2015). These deteriorating distribution systems are at an increased risk for breaks and corrosion all of which promote *Legionella* colonization (Parr, Whitney et al. 2015, Proctor, Rhoads et al. 2020). Increasing population densities and urbanization also means more exposure to water facilities and devices such as sprinklers, fountains, spas, and cooling towers etc. (National Academies of Sciences and Medicine 2020). Climatic factors such as increased temperatures, rainfall and flooding have been known to increase the number waterborne diseases and *Legionella* infections show a similar pattern (Walker 2018). In fact, many studies have reported that *Legionella* outbreaks tend to occur in the summer or early fall which are characterized as being warmer with high moisture conditions (Walker 2018). The rise of an aging population, which are more vulnerable to infections, is another reason for increased *Lp* associated diseases (Cooley, Pondo et al. 2020). Advances in reporting methods and increased diagnosis and reporting have also contributed to increased incidence rates (Collier, Deng et al. 2021). Risk factors for *Lp* infection include old age, compromised immune systems, smoking and chronic illness which include respiratory cardiovascular and kidney diseases (Cooley, Pondo et al. 2020). Most infections however are primarily due to exposure to contaminated man-made water systems, which can lead to an outbreak (Walker and McDermott 2021).

Outbreaks due to *Lp* can occur either as sporadic cases or major epidemics. Usually, sporadic cases affect few people and are caused by domestic water systems, such as showerheads and premise plumbing (van Heijnsbergen, Schalk et al. 2015). Epidemics, such as the landmark major outbreak in Quebec City in 2012 that resulted in 182 cases and 13 declared fatalities, are caused by larger public water systems, such as cooling towers and recreational water parks (Lévesque, Plante et al. 2014). Most reported infections are sporadic cases (MacIntyre, Dyda et al. 2018).

Legionellae-associated outbreaks are instigated by management failure of man-made water systems. Examples of these failures include keeping water distribution system temperatures below 50°C, allowing water to stagnate, lack of regular cleaning and disinfection protocols as well as lack of consensus on monitoring strategies and protocols (CWWA-ACEPU 2020, Proctor, Rhoads et al. 2020). The recent SARS-CoV2 pandemic is an example of a high environmental risk factor for *Lp* transmission (CWWA-ACEPU 2020, Proctor, Rhoads et al. 2020). The unprecedented global "stay-at-home" orders have led to the closures of buildings, institutions, and offices. These widespread closures and "shut-downs" have reduced water usage and flow, running the risk of stagnation of water (CWWA-ACEPU 2020, Proctor, Rhoads et al. 2020). Stagnation of water leads to a decrease in disinfectant residuals, degradation in water quality, temperature fluctuations that promote microbial growth conditions, as well as the formation of biofilm, all of which are optimal conditions for *Legionella* growth and proliferation (CWWA-ACEPU 2020, Proctor, Rhoads et al. 2020). At the time of writing this thesis, no data has come forth that pandemic related building shutdowns have led to increased Legionellosis incidence rates. This may be in part to strict guidelines released by various institutions and organizations for the safe re-opening of buildings (CDC 2020, CDC 2020).

1.1.3 *Legionella pneumophila* and Engineered Water Systems

The growth of *Lp* to high, pathogenic levels is dictated primarily by two characteristics of an engineered water system. One is the ability of the water system to provide ideal conditions for biofilm formation such as stagnation of water, warm temperatures, low disinfectant residual, type of plumbing material, corrosion of plumbing material, all of which support protozoan growth. Second is the ability of the water system to produce aerosols. Aerosols contaminated with *Lp* can be inhaled by susceptible individuals (Buse, Schoen et al. 2012).

As mentioned previously, given *Lp's* parasitic lifestyle, the bacteria replicate in protozoa which are normally found in biofilms. A biofilm is an aggregation of microbial cells that are attached to a surface and enclosed by an extrapolymeric gel-like substance (Donlan 2002). The microbial communities of a biofilm are often complex containing many species of bacteria but also higher order eukaryotes such amoeba, nematodes, ciliates, etc. (Paranjape, Bédard et al. 2020, Paranjape, Bédard et al. 2020). Biofilms form on all moist surfaces which include engineered water systems and their various components such as filters, gaskets, pipes etc. Specific regions of a water system, and its temperature affect *Lp* concentration levels (Bédard, Fey et al. 2015, Bédard, Paranjape et al. 2019, Bédard, Trigui et al. 2021). For example, distal regions of hot water distribution systems -including taps and showerheads-, where the temperatures are warm to lukewarm and water is prone to stagnation, provide optimal conditions for biofilm formation and thus *Lp* propagation (Boppe, Bedard et al. 2016, Bédard, Paranjape et al. 2019). The material used to fabricate the plumbing or water system also has an impact on *Lp* levels (Wang, Masters et al. 2014). Several studies demonstrated an increased presence of *Legionella* in biofilms grown on copper compared to biofilms grown on cross-linked polyethylene (PEX), polyvinyl chloride (PVC) or unplasticized PVC (uPVC) (Buse, Lu et al. 2014, Gião, Wilks et al. 2015). A recent study investigating to susceptibility of *Legionella* to copper, identified two biofilm-derived *Legionella* isolates with increased expression of copper resistance gene *copA*, suggesting that exposure to copper plumbing leads to adaptation of *Legionella* to local environments (Bédard, Trigui et al. 2021). This local adaptation also provides insight on how other stressors, such as disinfectants, can affect *Lp* levels. Disinfectants and biocides are the primary strategy for treating engineered water systems. Compared to free-living bacterial cells, biofilm integrated microbes are more resistant to disinfectant treatment (Falkinham 2020). *Lp* is just one member of a group of biofilm-associated waterborne pathogens, collectively called opportunistic premise plumbing pathogens (OPPPs). A characteristic of OPPPs is that they are relatively resistant to a common disinfectant used to treat water systems namely chlorine (Falkinham 2020).

Corrosion products can also affect *Lp* levels by consuming disinfectant residuals (Buse, Schoen et al. 2012). During the notorious Flint, Michigan water crisis, a lack of corrosion inhibitors led to an increased level of corrosion products which in turn reduced chlorine disinfectant levels resulting in high levels of *Legionella* in the waters. This led to an outbreak of LD in 2016 (Rhoads, Garner et al. 2017). Corrosion products can also affect *Lp* levels by providing bioavailable metals such as

iron which are important for *Lp* growth (Buse, Schoen et al. 2012, Rhoads, Pruden et al. 2017). Similarly corrosion products can also promote biofilm growth by providing a source of electron donors and hydrogen (Rhoads, Pruden et al. 2017).

There are several types of engineered water systems that could promote biofilm formation and produce aerosols leading to *Lp* propagation. These include building water distribution systems (premise plumbing) ,cooling towers, drinking water treatment plants, wastewater treatment plants that receive warm industrial effluents, and other devices with moderate temperature waters such as hot tubs, humidifiers or sprinklers (van Heijnsbergen, Schalk et al. 2015). Though drinking water systems are the most common source of LD outbreaks, cooling towers are the primary cause behind large community-associated outbreaks, as well as over a quarter of sporadic cases (Llewellyn, Lucas et al. 2017, Walker and McDermott 2021). A cooling tower is a large structure that acts as a heat exchanger in which air and water are mixed to reduce the temperature of water that has previously been increased as a consequence of trapping heat from industrial processes (Mouchtouri, Goutziana et al. 2010). Cooling towers provide an ideal environment for *Lp* growth due to their warm temperatures and large surface areas for biofilm formation (Mouchtouri, Goutziana et al. 2010). Additionally they can produce aerosols that can be dispersed hundreds of kilometers away, increasing the risk of *Lp* infections (Nhu Nguyen, Ilef et al. 2006).

1.1.4 *Legionella pneumophila* Life Cycle and Pathogenesis

Legionella's life cycle and mechanisms of pathogenicity have mostly been studied using *Lp* serogroup 1 as a model. Given its prevalence in a wide array of niches and its ability to propagate itself in the environment and in host cells, it is now well understood that *Lp* is a versatile microbe able to adapt to a range of niches by altering its physiology. *Lp* can exist intracellularly and in multispecies biofilms (Robertson, Abdelhady et al. 2014). Depending on its environment *Lp* can be found in either replicative, transmissive, VBNC (viable but non culturable), filamentous, or MIF (mature infectious forms) forms (Robertson, Abdelhady et al. 2014). Each metabolic state plays a specific role in the bacterium's growth, survival or propagation. (Robertson, Abdelhady et al. 2014).

The replicative state of *Lp* is analogous to exponential phase whereas the transmissive form of *Lp* resemble post-exponential/stationary phase in laboratory conditions (Byrne and Swanson 1998). The shift from replicative to transmissive form results in a change in the transcriptomic and

metabolic profile (Faucher, Mueller et al. 2011, Li, Mendis et al. 2015). In nutrient rich media or in the presence of host cells such as protozoa or macrophages, *Lp* expresses regulatory proteins which in turn inhibits the production of virulence factors such as flagella that would otherwise make the bacterium express transmissive traits and be infectious (Molofsky and Swanson 2004, Trigui, Dudyk et al. 2015). Upon nutrient depletion, signal molecules are produced, in response to starvation, which sets off the "stringent" response (Trigui, Dudyk et al. 2015). The expression of regulatory proteins is inhibited, and *Lp* begin to alter their surface structures, produce flagella, and express a number of virulence factors that enable it to successfully infect a host cell (Molofsky and Swanson 2004, Trigui, Dudyk et al. 2015). In summary during the replicative phase the bacteria focus on growth whereas in the transmissive phase the focus is on escaping and finding a new favorable niche.

MIF of *Lp* occur intracellularly in amoeba or are excreted by ciliate hosts (Faulkner and Garduno 2002). *Lp* in this form are metabolically dormant, thickened cells and observed to be more resistant to a wide range of stressors such as chlorine, pH, and antibiotics. MIF are released as membrane bound vesicles, which enhance the bacterium's airborne viability making it ideal for propagation via aerosols. Both MIF and transmissive *Lp* will switch to a replicative state in response to nutrient rich conditions (Abdelhady and Garduño 2013, Robertson, Abdelhady et al. 2014)

Filamentous forms of *Lp* are observed after exposure to environmental stress such as high temperatures, low level of nutrients, pH or oxidative stress as result of UV light or disinfectants and biocides (Robertson, Abdelhady et al. 2014). These elongated mesh-like forms are thought to aid in attachment, biofilm formation, ability to adhere to host cells as well as inhibiting phago-lysosomal destruction by creating an incomplete vesicle/vacuole (Piao, Sze et al. 2006, Robertson, Abdelhady et al. 2014).

Lp differentiate into VBNC forms in response to prolonged environmental stress due to nutrient, temperature or chemical factors (Li, Mendis et al. 2014). VBNC cells can are dormant, viable cells that can no longer be cultured with routine agar plating methods. Unlike dead cells, VBNC cells have intact membranes and are metabolically active (Li, Mendis et al. 2014). Though they share many characteristics with viable cells, they are morphologically distinct, differing in cell wall and membrane compositions and consequently adhesion and virulence potential. These VBNC forms increase bacterial resistance to chemical and physical stressors but hinder detection of bacterial

cells via culture methods. Though several resuscitation methods exist, VBNC *Lp* are not always easily resuscitated when cultured in rich media, in the presence of host cells or oxidative stress scavengers (Li, Mendis et al. 2014). The molecular mechanisms behind *Lp*'s switch from replicative to VBNC forms or vice versa as well its risk to public health is still poorly understood (Li, Mendis et al. 2014).

During infection, transmissive flagellated *Lp* attach to host cells with the help of flagella, pili and outer membrane proteins such as MIP (macrophage infectivity potentiator), Lcl (*Legionella* collagen-like protein), PilE (pilin E protein) and MOMP (outer membrane pore forming protein). The bacteria are then engulfed by coiling phagocytosis and internalized (Mondino, Schmidt et al. 2020). Once inside the host cell, the bacteria establish a *Legionella* containing vacuole (LCV) with the help of a very important virulence factor - the Icm/Dot Type IVb secretion system (Mondino, Schmidt et al. 2020). The *icm* (intracellular multiplication) and *dot* (defective organelle trafficking) genes encode the Type IVb secretion system, which is a complex that translocates hundreds of effector proteins across the membrane. These effector proteins are critical for maintaining the LCV and inhibiting phagosome-lysosome fusion (Ensminger 2016). Once the LCV is established the bacteria start replicating within 4-8 hours, following infection, and multiply its numbers to the hundreds within the host (Molmeret, Bitar et al. 2004). When nutrients are limited in the host, *Lp* will differentiate into the transmissive phase, lyse the host cell and enter the cytoplasm. *Lp* can also exit the host cells without lysis as MIF (Chen, de Felipe et al. 2004, Bouyer, Imbert et al. 2007). These released MIFs will be in membrane bound vacuoles which will enhance the bacterium's survival and allow it to be highly infectious for several weeks.

1.1.5 *Legionella* spp. Diversity

To date roughly 60 species of *Legionella* have been identified. The *Legionella* genus genome is highly dynamic with diversity due to high rates of recombination and DNA exchange (Khodr, Kay et al. 2016, Gomez-Valero, Rusniok et al. 2019).). This diversity is characterized by (i) the presence of secretion systems e.g the Type 1 secretion system is unique to only *Lp* strains, (ii) a wide repertoire of diverse effectors, (iii) mobile genetic elements and (iv) the presence of various eukaryotic domains containing proteins as well as eukaryotic-like proteins - which may explain why only some species of *Legionella* are pathogenic. A recent study, where representative species of nearly the entire genus of *Legionella* was sequenced and analyzed, showed an effector repertoire

of over 18,000 proteins that contain at least 137 different eukaryotic domains and over 200 different eukaryotic-like proteins (Gomez-Valero, Rusniok et al. 2019). Comparative genomics and evolutionary analyses indicated that *Legionella* species have acquired these eukaryotic domains and eukaryotic-like proteins from either plants, animals, fungi, and archaea (Gomez-Valero, Rusniok et al. 2019).

A pangenome represents the entire set of genes within a species. This consists of a (i) core genome -where sequences are common amongst species individuals- (ii) a "dispensable" or variable genome- where sequences are shared by 2 or more individuals of a species, and (iii) a unique genome-where sequences are unique to species individuals. A comparative pangenomic analysis revealed that amongst these *Legionella* species only 6% of genes were common across species once again illustrating the flux and diversity of the genus (Gomez-Valero, Rusniok et al. 2019). *Legionellas'* high genomic diversity contribute to the bacteria's fitness and adaptability.

Lp subspecies are classified by their serogroups. Sixteen serogroups of *Lp* have been identified with over half having been isolated from patients (Peci, Winter et al. 2016, Byrne, McColm et al. 2018) .Though several of the serogroups have been implicated in causing pneumonia and isolated from clinical patients, serogroup 1 consistently remains the dominant and frequently isolated clinical serotype in the majority of cases worldwide ((ECDC) 2021)

Lp is also the most reported species in building water system outbreaks (Walker and McDermott 2021). Other *Legionella* species account for less than 1% of total LD cases (Kruse, Wehner et al. 2016). In Germany and Hungary 58 to 84 % of the building water system isolates were identified as *L. pneumophila* (Barna, Kádár et al. 2016, Dilger, Melzl et al. 2018) whereas in the US a nationwide study revealed that despite identifying 47% of cooling tower isolate es as non-pneumophila, no cooling tower outbreaks have been attributed to non-pneumophila species (Llewellyn, Lucas et al. 2017) . Similarly in France, 98% of LD cases from cooling tower outbreaks were attributed to *L. pneumophila* (Campese, Bitar et al. 2011).

These studies are just a few that illustrate that the clinical dominance of *Lp* does not reflect its relative environmental abundance. This coupled with the fact that the *Lp* genome is dynamic, necessitates identifying isolates from source environments and matching these environmental strains to clinical isolates. This is done by using molecular methods such as multi-locus sequence typing (MLST). MLST also known as sequence-base typing (SBT) classifies *Lp* into sequence-

types based on the sequences of seven different genes namely *pilE, flaA,asd ,mip, mOmpS, proA* and *neuA*. The combination of this "bar-coding" approach and classical epidemiology has enabled outbreak source identifications as well the study of patterns in spread and proliferation. Worldwide, *Lp* serogroup 1 (ST1) has been identified as the most prevalent sequence type (Harrison, Afshar et al. 2009, Euser, Bruin et al. 2013, Kozak-Muiznieks, Lucas et al. 2014, Qin, Zhou et al. 2014, Al-Matawah, Al-Zenki et al. 2015, Bianchi, Pregliasco et al. 2016, Lévesque, Lalancette et al. 2016). Regional variations do occur with regards to *Lp* spread and infection. An example is a 10-year study of Lp 1 isolates from the province of Quebec, Canada, which identified ST-62, ST-213, ST-1 and ST-37 as the most frequent sequence types from sporadic cases. The study also identified ST-62 as the causal sequence type from an outbreak in 2012 in Quebec City (Lévesque, Lalancette et al. 2016).

1.1.6 *Legionella pneumophila* Surface

The cell envelope of *Lp* is key to its survival and pathogenicity. Like most gram-negative bacteria, *Lp* possess an outer membrane (OM) and inner membrane (IM) separated by a periplasm. Several structures are complexes that traverse either the inner or outer membranes such as the Type 1 secretion system (unique to pneumophila strains) (Fuche, Vianney et al 2015, Qin, Zhou et al 2017) and Type II secretion system which also involves the twin arginine translocation system (Tat) (Cianciotto, 2009). *Lp's* characteristic Type IV secretion system traverses both the inner and outer membranes (Ronaldo, Buchweiser et al 2014). Each secretion system has its respective effectors or proteins that are translocated across the membranes. Other structures that can traverse the membranes and are critical to *Lp* virulence and survival are single polar flagellum as well as pili.

The bacterium *Lp* does not possess a capsule or an extra-polysaccharide layer. The OM of *Lp* is a lipid bilayer. This bilayer is asymmetric with a phospholipid dense inner leaflet and with an outer leaflet containing lipopolysaccharides (LPS) as well key proteins that are necessary for bacterial attachment, adherence and invasion. These include the Mip (macrophage infectivity potentiator), Lcl (legionella collagen like proteins), and mOmp (outer membrane pore forming protein). In addition to these proteins other surface structures that mediate attachment and invasion are pilli and flagella. Several key structures and complexes, such as the Type IV secretion system, have subunits embedded in the OM. The Type IV secretion system of *Lp* has been structurally resolved by cryogenic electron microscopy (Cryo-EM) and cryogenic electron tomography (cryo-ET) in

recent years. It was found to have a dome/outer membrane cap integrated in the outer membrane made up of a core complex of 5 proteins namely DotC, DotD, DotF, DotG, and DotH (Ghosal, Chang et al. 2017, Ghosal, Jeong et al. 2019, Durie, Sheedlo et al. 2020). Δ*dotG* mutants were observed to be lacking the dome/outer membrane cap structure (Durie, Sheedlo et al. 2020). Since there is no exhaustive list of outer membrane proteins and lipoproteins, this section will focus on well-studied surface exposed *Lp* structures found on the outer membrane namely, the LPS, flagella and pili.

1.1.6.1 Legionella pneumophila LPS:

LPS is the dominant molecule on the surface of *Lp* (Figure 2) and contributes significantly to the cell surface properties and thus its interaction with host cells (Gabay and Horwitz 1985, Rietschel, Kirikae et al. 1994) The importance of LPS in attachment and host cell penetration was shown in a study by Palusinska-Szysz et al. where a mutant *Lp* producing a low molecular weight LPS, influenced the membranes lipid and protein composition. The mutant was less efficient in binding and penetration host amoeba cells (Palusinska-Szysz, Luchowski et al. 2019).
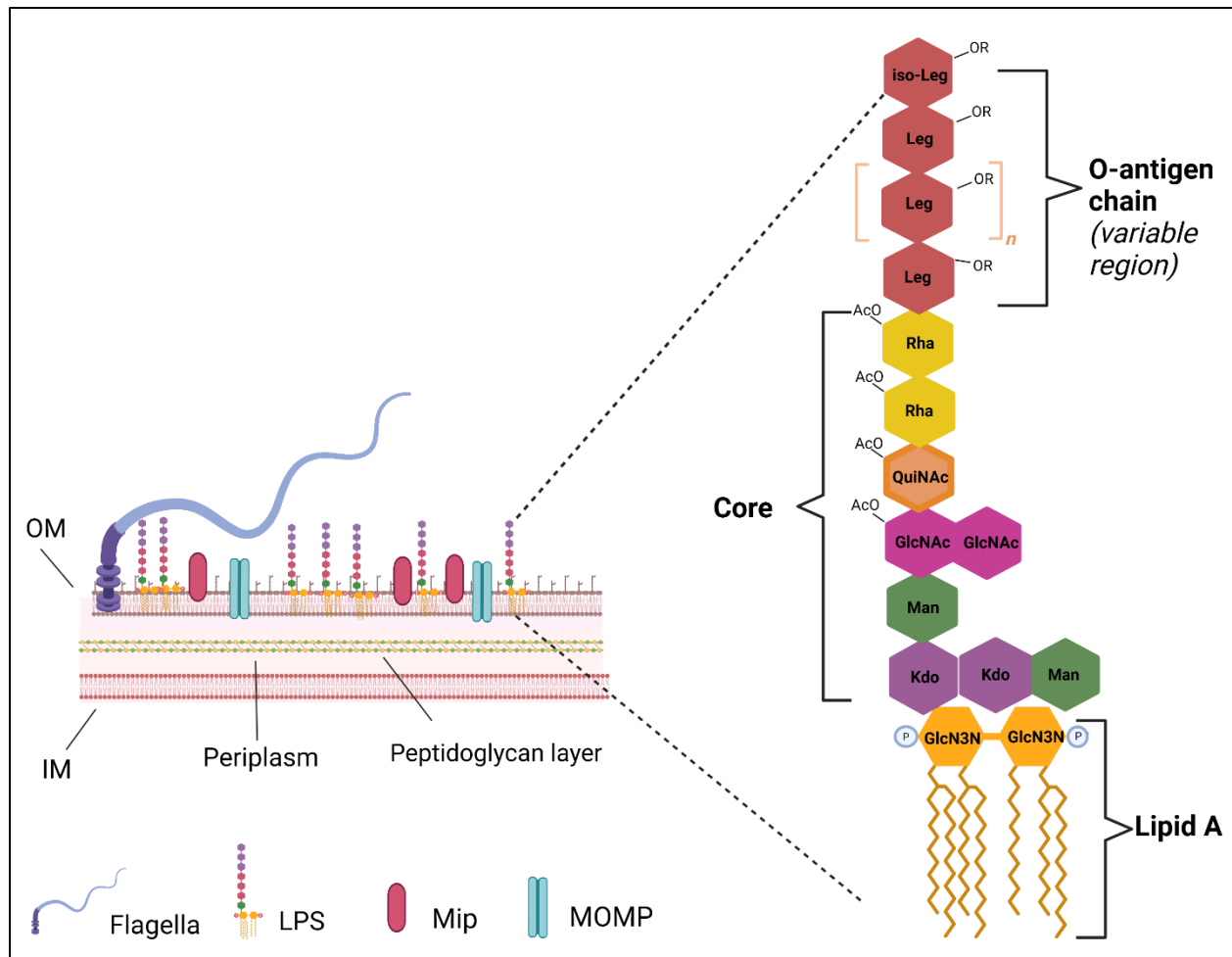
Figure 2: Schematic structure of *Legionella* lipopolysaccharide (LPS). The LPS comprises of 3 regions. The O-antigen region which contains legionaminic acid (Leg). The core region which contains rhamnose (Rha); O-acetyl (OAc); acetylquinovosamine (QuiNAc); acetylglucosamine (GlcNAc); mannose (Man); 3-deoxy-d-manno-oct-2-ulosonic acid (Kdo) and the Lipid A region which contains phosphate (P); and 2,3 diamino-2,3-dideoxy-D-glucose (GlcN3N). Image adapted from (Kowalczyk, Chmiel et al. 2021) and created in Biorender.

Several genes are responsible for the synthesis, modification, and translocation of LPS components. These include *lag1, neuA, wzm,* and *wzt* (Shevchuk, Jäger et al. 2011). The LPS biosynthesis cluster genes are shared by more than 200 serogroup 1 strains (Cazalet, Jarraud et al. 2008) . This suggests that this specific LPS of serogroup 1 plays a role in the predominance of this serogroup in disease (Cazalet, Jarraud et al. 2008).

LPS consists of three regions namely, lipid A, the core-which comprises and inner and outer core-and the O-antigen. Lipid A, also known as endotoxin, is highly branched with long chains and anchors LPS molecules to the outer membrane through hydrophobic interactions with the acyl

fatty acid chains of the phospholipids (Moll, Knirel et al. 1997). These phospholipids make up the inner leaflet of the outer membrane. It was shown that Lipid A of *Lp* is less toxic than other classical endotoxins (Neumeister, Faigle et al. 1998)

The inner core of the polysaccharide core is hydrophilic. It is characterized by a 3-deoxy-d-manno-oct-2-ulosonic acid (Kdo) disaccharide [α-Kdo-(2α4)-α-Kdo-(2α6)] linked to lipid A. The presence of disaccharide as well as the lack of heptoses and phosphate residues are characteristic of the inner core of *L. pneumophila* LPS (Moll, Knirel et al. 1997).

The outer core is hydrophobic and consists of a seven-sugar oligosaccharide composed of rhamnose (Rha), mannose (Man), acetylquinovosamine (QuiNAc), and acetylglucosamine (GlcNAc) (Knirel, Moll et al. 1996, Moll, Knirel et al. 1997). The presence of the N-acetyl groups (QuiNAc and GlcNAc) and methyl groups of 6-deoxy sugars (Rha and QuiNAc) contribute to its hydrophobicity.

The O-antigen chain of *Lp* Serogroup 1 is composed of a homopolymer termed legionaminic acid. This unique sugar lacks free hydroxyl groups, making it extremely highly hydrophobic and consequently more virulent as the hydrophobicity facilitates attachment and invasion of host cells, (Knirel, Rietschel et al. 1994, Zähringer, Knirel et al. 1995).

The O-antigen chain is the target for most immunoassays and shows variations in the types, linkages, and arrangements of its sugar chains (Knirel, Senchenkova et al. 2001). This makes the LPS one of the most variable cell surface components which results in a diversity of serotypes i.e, 16 serotypes, for *Lp*. This diversity in serotypes could be traced to variations in LPS biosynthetic gene clusters amongst the serogroups (Petzold, Thürmer et al. 2013). In fact, these gene variations were used to develop multiplex PCR assays that could identify 15 distinct serogroups of *Lp* based on the LPS biosynthetic sequences (Cao, Tian et al. 2015, Nakaue, Qin et al. 2021). These PCR serotyping assays are an improvement over traditional immune-serotyping assays which are expensive and tedious given they typically require the use of antibodies (Helbig, Kurtz et al. 1997).

1.1.6.2 Legionella pneumophila Pili:

Lp produce long and short pili. The long pili are about 0.8 to 1.5 μm, whereas the short form, measure 0.1 to 0.6 μm. The PilE protein is an integral part of the long pili form. Prepilin peptidase (PilD) is another protein important for the production of the pili (Stone and Kwaik 1998). These

pili mediate attachment, adherence, natural competence, biofilm formation and intracellular replication (Shevchuk, Jäger et al. 2011). A study by Hoppe et al. showed that the fimbrial/pili protein PilY1 was produced by *Lp* in the transmissive stage and that deletion of this protein resulted in low adhesion to macrophages and lung epithelial cells (Hoppe, Ünal et al. 2017). This illustrates the significance of the pili for host cell attachment and infection. Pili also enable *Lp* to move forwards and backwards and slide, a phenomenon known as "twitching motility" (Stewart, Rossier et al. 2009, Hoppe, Ünal et al. 2017).

1.1.6.3 Legionella pneumophila Flagella:

Lp express a single polar flagellum, which traverses the outer and inner membranes as well as the peptidoglycan layer. The flagellum is critical for *Lp* infection and is differentially expressed. It is only present in specific physiological states such as the MIF or transmissive state (Albert-Weissenberger, Sahr et al. 2010). Its expression is thus influenced by environmental factors such as nutrient availability, temperature, and oxidative stress all of which result in transcriptomic changes and expression of specific regulators (Albert-Weissenberger, Sahr et al. 2010, Appelt and Heuner 2017) . The flagellum plays a critical role in *Lp* motility, adhesion, and infection (Heuner and Albert-Weissenberger 2008, Albert-Weissenberger, Sahr et al. 2010).

The production of the flagella is tightly regulated, complex and metabolically expensive, comprising over 50 genes (Albert-Weissenberger, Sahr et al. 2010, Altegoer and Bange 2015, Appelt and Heuner 2017). Flagella are associated with a virulence phenotype since they are expressed when *Lp* are in the infectious states. In response to nutrient depletion, *Lp* become flagellated and do not replicate (Albert-Weissenberger, Sahr et al. 2010). This flagellated form is a characteristic of the transmissive state and enables the bacteria to be motile and find a suitable niche.

The flagella of *Lp* consist of a basal body, hook and filament. Subunits and components are excreted by a type 3 secretion system to assemble the flagella (Altegoer and Bange 2015). Various flagellin proteins are required to synthesize the flagella. *Lp* mutants lacking flagellin proteins- Δ*fliA,* Δ*fliD and* Δ*fliC*- are reported to have straight hooks as opposed to the wildtype curved hooks (Schulz, Rydzewski et al. 2012). The flagellin proteins FliC and FliD are necessary to synthesize the flagellum filament and cap the filament respectively (Heuner and Albert-Weissenberger 2008,

Altegoer and Bange 2015). FliC-also known as FlaA- is the major subunit/protein of the flagella. Absence of FliC affects motility and thus host cell invasion (Dietrich, Heuner et al. 2001).

1.2 *Legionella* Detection

"An ounce of prevention is worth a pound of cure". Benjamin Franklins' adage is pertinent when dealing with infectious agents such as *Legionella pneumophila.* To prevent the spread of *Lp*, it is critical to be able to detect the bacteria. Current strategies to detect *Lp* from environmental matrices include (i) conventional culturing methods, (ii) Nucleic acid-based methods and (iii) Phenotypic assays.

1.2.1 Conventional Culturing Methods

These are the gold standard for detecting *Lp* and involve the selection of *Lp* from an environmental sample via exposure to various physical factors such as high temperatures and low pH followed by cultivation of *Lp* on selective media and the enumeration of bacterial colonies showing *Lp* specific morphology. To elaborate, environmental samples (from cooling towers, water distribution systems, potable water etc.) must first be heat and/or acid treated to reduce the amount of non-legionella microbial growth. Heat treatment is typically performed between 50–59 °C for 30 minutes given that *Lp* can grow at temperatures above 63°C (CDC 2005, ISO 2019). Acid treatment is performed by initially concentrating a sample via centrifugation or filtration. The concentrate is then treated at a pH of ~2.2 for approximately 3-15 mins. Both pre-treatment methods take advantage of *Lp*'s tolerance to these conditions (CDC 2005, ISO 2019).

To cultivate *Legionella,* various complex media formulations exist to select for various *Legionella* species. These media can be supplemented with a range of amino acids (L-cysteine, serine, isoleucine, arginine etc.), trace elements (iron, zinc, magnesium , cobalt, manganese etc.) and antibiotics (polymyxin, vanomycin) depending on the desired species (CDC 2005, ISO 2019). Buffered charcoal yeast extract BCYE is the standard media for *Legionella* culturing and maintenance but most *Legionella* will require supplementation with L-cysteine and iron for growth (ISO 2019). Examples of specific growth requirements are *L. micdadei* and *L. bozemanii* who require BCYE with 1% bovine serum albumin (ABCYE) (CDC 2005, ISO 2019). Current ISO 11731:2017 and CDC protocols require the use of BCYE containing L-cysteine to culture *Lp*.

Selective GVPC agar (BCYE supplemented with glycine, vancomycin, polymyxin B, cycloheximide) and PCV agar (BCYE supplemented with polymyxin B, cycloheximide, and vancomycin) are also listed in standard operating protocols to enhance *Legionella* recovery. The CDC uses BCYE, PCV and GVPC whereas under ISO the medias listed are BCYE and GVPC. Treated environmental samples are plated on BCYE or PCV without L-cysteine to serve as a negative control.

There are several caveats to conventional culture methods for *Lp* detection. The primary limitation is that these techniques are not rapid (National Academies of Sciences and Medicine 2020). The growth of culturable *Lp* takes at least 5-7 days which can translate into <u>15 days</u> before obtaining a result with regards to an environmental sample. This delays timely interventions which is detrimental especially during an outbreak (Trudel, Veillette et al. 2014). The long growth time could also result in competitive microbial growth which in turn would confound cell counts (Bopp, Sumner et al. 1981). A second major limitation is the presence of viable but non culturable (VBNC) *Lp* cells. VBNC cells are problematic because since they cannot be cultured, true *Lp* cell counts are often underestimated (Li, Mendis et al. 2014, Epalle, Girardot et al. 2015). Consequently, conventional culturing techniques often fail to provide a comprehensive picture of the bacterial hazard (Li, Mendis et al. 2014). Heat and acid pre-treatment can further affect *Lp* cultivability by producing VBNC cells (Epalle, Girardot et al. 2015, Cervero-Aragó, Schrammel et al. 2019). Indeed, a study by Epalle et al. (2015) showed that heat treatment at 70C for 30 min of various *L. pneumophila* strains resulted in 10–40% of VBNC cells (Epalle, Girardot et al. 2015). The presence of monochloramine in treated water systems can also induce VBNC cell formation (Casini, Baggiani et al. 2018). Additionally, the low nutrient conditions typically found in a man-made water system also contribute to VBNC cell formation (Dietersdorfer, Kirschner et al. 2018, Schrammel, Cervero-Aragó et al. 2018). An approach to improve cultivability and resuscitate VBNC cells is by co-culturing with amoeba (Ducret, Chabalier et al. 2014). This method however adds more labour and time to an already tedious culturing process. The tedious multiple steps of culture methods also cannot distinguish between pathogenic or non-pathogenic strains, which is problematic in risk assessment analysis (National Academies of Sciences and Medicine 2020). A third problem with conventional culturing techniques, as with most detection strategies, is the variation across labs in sampling strategies, (frequency, location, preservation) pre-treatment methods, cultivation methods and incubation times (Wang, Bédard et al. 2017). An example that

can illustrate this is the 2011 study conducted by the Environmental *Legionella* Isolation Techniques Evaluation (ELITE) Program in the USA. Environmental samples were sent to 20 different U.S. laboratories for *Legionella* detection and compared with data obtained from the (CDC) reference laboratory. 37% of these samples were identified incorrectly as negative when compared to the CDC reference laboratory results. In general culture recovery rates are affected by many factors such as concentration/enrichment method, sample type (bulk water or biofilm), sampling sites, sampling frequency, sample concentration, sample volume, holding time and temperature (Wang, Bédard et al. 2017).

In recent years a simpler culture method using the most-probable-number (MPN) approach (IDEXX Legiolert™) has been developed. Legiolert, makes use of an enzyme uniquely produced by *Lp* which reacts with a substrate present in the provided Legiolert reagent. The detection limit for the Legiolert test is 10 cfu/100ml with results obtained in 7 days. Large scale comparative studies show that the strength of the method is equivalent to standard ISO certified plate culture methods but yields generally higher numbers of bacteria since it is a MPN approach (Sartory, Spies et al. 2017, Petrisek and Hall 2018, Rech, Swalla et al. 2018, Spies, Pleischl et al. 2018, LeChevallier 2019). Though the studies listed here did not confirm results with molecular methods there is recent work that shows Legiolert numbers align with *Lp* estimates from qPCR (Monteiro, Robalo et al. 2021). Additionally, the test is very user-friendly. Like standard culture, Legiolert is not rapid and cannot distinguish between *Lp* nor non-pneumophila species, but its primary drawback is that the isolated colonies are not readily available for further molecular testing. Isolation of *Lp* however is comparatively quick.

The limitations with culture methods have led to a shift towards molecular methods either as a confirmatory or primary method for microbial detection (Wang, Bédard et al. 2017, National Academies of Sciences and Medicine 2020). These include PCR, qPCR, FISH, MLST (multi-locus sequence typing), pyrosequencing, High Throughput Sequencing (HTS), and Immunoassays such as ELISA, immunochromatography, lateral flow assays etc.

1.2.2 Nucleic Acid (NA) Based Methods

These involve the manipulation and analysis of *Legionella* DNA and RNA to quantify *Lp* present in environmental samples. PCR, qPCR, viable qPCR, ddPCR and HTS) are examples of nucleic acid-based methods for *Lp* detection (National Academies of Sciences and Medicine 2020). The

advantage of said techniques, in comparison to conventional culture techniques, are their rapid turn-around times, high sensitivities and specificities, lower limits of detection, as well as their ability to detect VBNC cells (Whiley and Taylor 2016). PCR entails the amplification of *Legionella* specific genes such as 16S and/or macrophage infectivity potentiator (*mip*) which serves as a biomarker for *Lp*. The presence/absence of *Legionella* is verified if a band is visible on an agarose gel. This method, however, only confirms if there are any bacteria present in the sample. qPCR is more useful for determining the quantity of *Lp* cells. Target genes for *Lp* qPCR also include 16srRNA and/or *mip* (Wilson, Yen-Lieberman et al. 2003, Stølhaug and Bergh 2006). The amount of *Lp* determined through qPCR is given as genomic units per litre (GU/L). Given its reproducibility and quantitative abilities, it is a widely used method to quantify *Legionella*. Examples that illustrate this are the presence of commercial *Legionella* detection kits (Legionella iQ check BioRad). Additionally, there are standards in place to detect and quantify said bacterium by qPCR under the the Association Française de Normalisation (AFNOR) NF T90-471 protocol and ISO/TS 12869:2012. When used in conjunction with the culture method, qPCR can serve as a powerful tool. There are however several caveats to using qPCR as a detection and quantification method. Given that the method detects both VBNC and dead bacterial cells, its main limitation is an overestimation of *Lp* burden (Whiley and Taylor 2016). Additionally, environmental samples are often complex matrices that may have inhibitors/compounds which would adversely impact the PCR/qPCR reactions and hence *Lp* recovery rates (Gentry-Shields, Wang et al. 2013, Whiley and Taylor 2016). Though there are kits and buffers that could mitigate the impact of certain compounds, the variation in DNA/RNA extraction techniques can further impact *Lp* recovery rates, while concurrently increasing reagent prep time. qPCR numbers are often several folds higher than culture results (Ditommaso, Ricciardi et al. 2015, Whiley and Taylor 2016). The variations in sampling strategies, DNA/RNA extraction techniques, expression in genomic units/l or ml, as well as its poor correlation with culture methods, has made it difficult to interpret data from qPCR thus adopt it as a stand-alone standard for *Lp* detection.

Viable qPCR is an alternative to method to qPCR to overcome the caveat of discerning between live and dead *Lp* bacteria (Nocker, Cheung et al. 2006). It entails the use of NA intercalating dyes such as propidium monoazide (PMA) or ethidium monoazide (EDA) which traverse damaged cell membranes and bind to target DNA/RNA after photoactivation. This in turn prevents amplification of the dead/membrane compromised cells' nucleic acids (Nocker, Cheung et al. 2006). Several

studies have shown that this method leads to a quantification of *Lp* with numbers higher than conventional culture but lower than qPCR due to the inclusion of VBNC cells (Chen and Chang 2010, Lizana, López et al. 2017, Kontchou and Nocker 2019). This method however is not without its limitations (Kontchou and Nocker 2019). The effectiveness of EMA/PMA-qPCR is contingent upon several factors, amplicon length, such as dye selection and dosage, sample types, target cell concentration, presence of dead/live microbial flora etc (Taylor, Bentham et al. 2014, Ditommaso, Giacomuzzi et al. 2015, Kontchou and Nocker 2019) Therefore, optimization steps would need to be taken prior to application of EMA/PMA-qPCR. Furthermore PMA/EMA dyes cannot easily penetrate and thus target *Lp* cells embedded in biofilm (Taylor, Bentham et al. 2014).. Additionally, the same limitations that make it difficult to interpret qPCR data apply to viable qPCR.

A newer alternative to qPCR for *Legionella* detection ,that is less sensitive to PCR inhibitors and provides rapid absolute quantification, without needing a standard curve is ddPCR (Falzone, Gattuso et al. 2020, Alshae'R, Flood et al. 2021). It can be multiplexed in that it can be applied to more than one genetic marker at a time. The method works by dividing the sample into thousands of individual droplets wherein the PCR reaction occurs; the numbers of positive and negative droplets then provide a most probable number of the concentration.

HTS is a term that encompasses several different modern sequencing technologies that allows rapid and simultaneous sequencing of large numbers of NA fragments. HTS is a powerful tool for understanding the microbial composition, ecology and diversity in a sample water system (Pereira, Peplies et al. 2017). Consequently, it can provide information on the presence of *Lp* in a particular water system (Pereira, Peplies et al. 2017). Universal primers targeting 16S rRNA genes are used to detect the relative abundance of *Legionella*. A major limitation of HTS for environmental monitoring and detection is that sequencing is based on 16S rRNA gene PCR products which have limited taxonomic resolution. This inhibits the ability to differentiate at the species level (Borthong, Omori et al. 2018). Furthermore, the limit of detection (LOD) of *Lp* for most HTS methods are high which is challenging given *Lp* have low relative abundances compared to other microbes in a water system (Borthong, Omori et al. 2018, Dai, Rhoads et al. 2018, Paranjape, Bédard et al. 2020).

In general, nucleic acid methods are more rapid, sensitive, and specific. Their main drawbacks however lie in the multiple processing steps and the use of complex equipment which increases overall costs and times associated with this method.

1.2.3 Phenotypic Assays

These assays involve techniques that measure and detect phenotypic characteristics such as a cells morphological, biochemical, physiological, serological trait. Examples of such assays that exploit *Lp*'s biochemical make-up are the commercially available Click4Tag assay and Legiolert. Click4Tag identifies *Lp* by using species specific metabolic lipopolysaccharide (LPS) labelling (Mas Pons, Dumont et al. 2014). Legioaminic acid is an exclusive *Lp* LPS component. A labelled azido analog of legioaminic acid is incorporated in the bacterial membrane via click chemistry and the analog is then used to detect *Lp* (Mas Pons, Dumont et al. 2014) Legiolert is also a phenotypic assay in that it exploits the use of an enzyme uniquely produced by *Lp*.

Currently, the most widely studied phenotypic assays for *Lp* detection are immunological assays (Bedrina, Macián et al. 2013, Párraga-Niño, Quero et al. 2018). These involve the use of monoclonal or polyclonal antibodies specific to *Lp* antigens. Immunoassays have a wide repertoire of formats ranging from enzyme linked immunosorbent assays (ELISA) and lateral flow assays (LFA) to microscopy, immune-sensors and immunomagnetic bead separation (IMS) (Bedrina, Macián et al. 2013, Albalat, Broch et al. 2014, Congestrì, Morotti et al. 2019). For direct detection such as in flow cytometry and microscopy, antibodies against *Lp* are conjugated with fluorescent tags. After interaction with an *Lp* antigen a fluorescent signal is obtained. Antibodies can also be conjugated with reporter enzymes to give a colorimetric signal as shown with the commercially available lateral flow assay Duopath (Helbig, Lück et al. 2006). IMS is a simple powerful tool to increase capture of *Lp* cells through magnetic beads coated with anti-*Lp* antibodies (Bedrina, Macián et al. 2013). These cells can further be analyzed using techniques such as ELISA, qPCR or flow cytometry. Traditional ELISA detection for *Lp* has detection limits as high as 10^5 CFU/mL. This limit may be improved to 10^3 by concentrating for *Lp* using IMS. An example of how IMS is used to improve detection limits is the Legipid® *Legionella* Fast Detection kit. It combines the use of IMS with an enzyme linked colorimetric assay to obtain a detection limit of 93 CFU/ml for *Lp* (Rodríguez Albalat, Bedrina Broch et al. 2012, Albalat, Broch et al. 2014).   Polyclonal fluorescently tagged *Lp* antibodies were combined with an on-site microfluidic sensor to detect

Lp in the low range of 10^1 -10^3 CFU/mL in cooling tower water samples (Yamaguchi, Tokunaga et al. 2017).

Antibodies serve as useful capture elements that can increase specificity and sensitivity for *Lp* in immunoassays. However, key limitations are the tedious labour and high costs that go into producing an antibody. Biorecognition elements such as aptamers can be viable as well as cost-effective alternatives to antibodies.

Amidst the above-mentioned strategies the most widely accepted and industry standard methods for detecting *Lp* in environmental samples are the standard plate count method (AFNOR NF T90-431, ISO 11731) as well as qPCR (AFNOR NF T90-471, ISO/TS 12869) with Legiolert having recently joined industry certification ranks (AFNOR NF IDX 33/06-06/19). It is prudent to note however that studies from the past ten years show a disconcertingly high lack of agreement between the standard plate count and qPCR methods. Further comparative studies are still underway with Legiolert and qPCR methods. After combining the results of 28 studies, Whiley et al. showed that 72% of environmental samples tested positive for *Legionella* using qPCR while only 34% tested positive using culture-based methods (Whiley and Taylor 2016). Furthermore, culture and qPCR methods showed good agreement for 20 drinking water samples but poor agreement for water samples from 52 cooling towers For *L. pneumophila* SG1 detection specifically, the agreement between both methods was poor for drinking water and only slightly enhanced among cooling tower samples (Toplitsch, Platzer et al. 2018). Generally, qPCR reported 10- to 100-fold higher concentrations than the culture method (Toplitsch, Platzer et al. 2018). A recent study by the same group demonstrated that qPCR was more accurate than culture methods for determining true negatives i.e, no *Legionella,* in water samples that had a high microbial burden (Toplitsch, Platzer et al. 2021) . These numbers illustrate the need for improved standardized detection methods for *Lp*. Additionally, these methods cannot be developed into rapid, cost-effective, sensitive tests that would identify the whole *Lp* bacterium in real-time, in situ/at point-of-care without any additional processing steps**.** An *Lp* biosensor could be a viable solution.

1.3 <u>Biosensors</u>

Biosensors are analytical devices used to quantify or detect a specific biological target or analyte such as proteins and cells (Turner 2013). Extensive research in the field of biosensing has shown

that biosensors can be selective and detect a specific analyte from a mixture (Turner 2013). They are ideal for detection as they can have high sensitivities with low limits of detection, be reproducible and give discrete measurable responses over a wide range of concentrations (Turner 2013). Other advantages are the ability to be regenerative for multiple uses, be miniaturized, multiplex and analyze multiple targets at a time (Turner 2013). They can also be cost-effective, portable and user-friendly (Turner 2013). All these characteristics make them attractive as part of detection strategies for biological agents such as waterborne pathogens (Ahmed, Rushworth et al. 2014, Kumar, Hu et al. 2018).
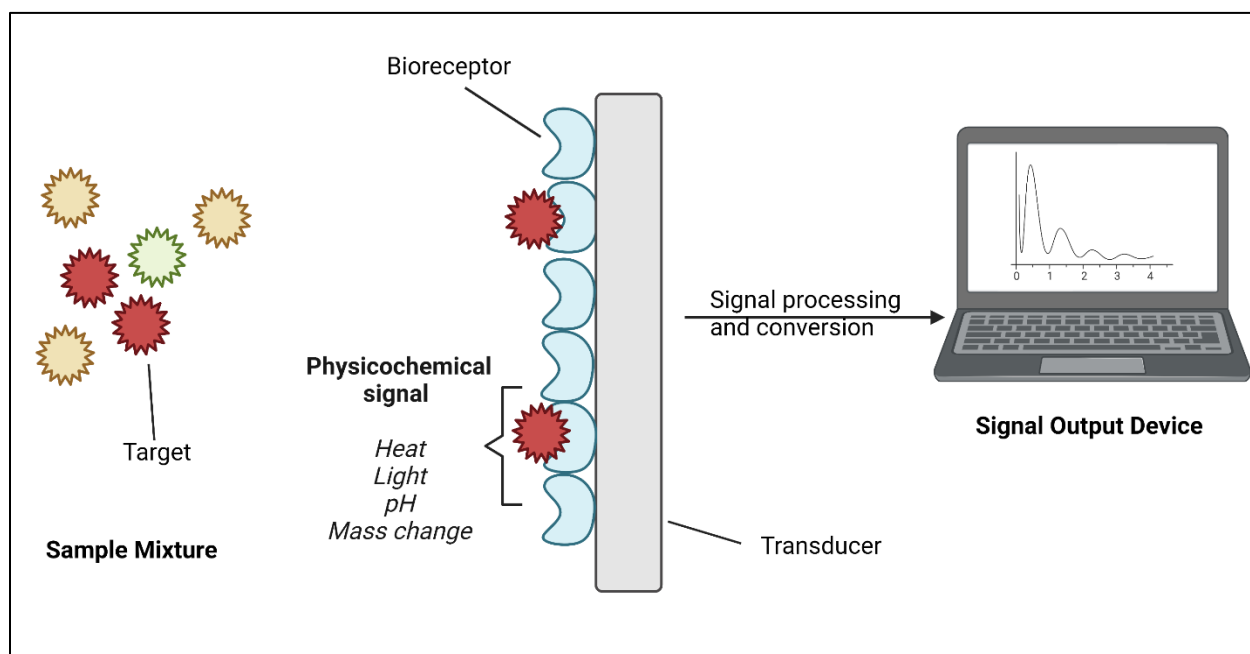
Figure 3. Components of a biosensor. Image created using Biorender.

A biosensor set-up typically consists of three elements (Figure 3); (i) A biorecognition element, which upon interaction with a target, produces a physico-chemical signal. (ii) A transducing element into converts that signal into a form captured by a detection element. (iii) A detection element that takes the converted signal and gives an output readout (Figure 3).

Biosensors can be categorized based on either their transducing element (mechanical, optical, electrochemical) or biorecognition element (affinity, catalytic) (Ahmed, Rushworth et al. 2014, Bhalla, Jolly et al. 2016).

1.3.1 Mechanical Biosensors

Mechanical biosensors consist of Quartz Crystal Microbalance and microcantilevers. These are mass sensitive sensors operating at a specific frequency. For both, analyte binding results in increased mass on the sensor surface, which leads to measurable changes in resonance frequencies (Arlett, Myers et al. 2011) . Though these sensors can be highly sensitive, quick, and require no processing steps a key caveat is the inability to use samples in their native state or in physiological media. This is because the signal resulting from a given analyte varies depending on the surface energy and binding region both of which are influenced by the surrounding medium (Waggoner, Varshney et al. 2009, Kumar, Hu et al. 2018). Consequently, sensitivities in air or fluid are much lower than in vacuum. Furthermore due to damping effects cantilevers cannot function in fluid mediums which means samples would have to be desiccated prior to application (Waggoner, Varshney et al. 2009, Kumar, Hu et al. 2018). These limitations make this set-up difficult for real-time monitoring.

1.3.2 Electrochemical Sensors

Electrochemical biosensors rely on principles of electrochemistry and consist of amperometric, potentiometric and impedimetric sensors (Cesewski and Johnson 2020, Curulli 2021). Due to their low cost, sensitivity, and ability to be miniaturized, they have been very popular for detecting bacterial pathogens although there is an increasing number of viral pathogen detection with such sensors in recent years (Cesewski and Johnson 2020). Amperometric biosensors measure the current created as result of redox reactions from analyte-bioreceptor interaction (Ahmed, Rushworth et al. 2014, Kumar, Hu et al. 2018, Cesewski and Johnson 2020). Potentiometric biosensors measure electric potential between a working electrode and a reference electrode in response to a fixed current (Ahmed, Rushworth et al. 2014, Kumar, Hu et al. 2018, Cesewski and Johnson 2020). This method measures the change in potential that occurs upon analyte recognition at the working electrode. Impedimetric biosensors operate because of change in capacitance and electron transfer resistance across a working electrode where the analyte-bioreceptor interaction occurs. Higher analyte concentration leads to an increase in analyte binding causing the impedance across the electrode surface to change and be detected at a transducer (Ahmed, Rushworth et al. 2014, Kumar, Hu et al. 2018, Cesewski and Johnson 2020). For real-time monitoring electrochemical sensors have several limitations. One is the presence of reactive species causing

unwanted redox reactions at the electrode surface (Cesewski and Johnson 2020). Another is the cost of the electrode material, though carbon is being increasingly studied and used as an alternative to metallic and ceramic electrodes. Additionally, large sized microbes such as protozoa *Cryptosporidium parvum* have a small effect on charge transfer at the electrode-electrolyte interface, adversely affecting readouts (Cesewski and Johnson 2020). Fouling/saturation at the electrode surface is also a concern requiring optimized regeneration strategies (Cesewski and Johnson 2020). Finally, an electrochemical sensor by itself has limited molecular selectivity and is prone to reactions from non-specific binding (Kumar, Hu et al. 2018, Cesewski and Johnson 2020). This necessitates the use of a bioreceptor/probe.

1.3.3 Optical Biosensors

Optical biosensors measure changes in optical properties that occur at the sensor surface/interface, because of analyte-bioreceptor interaction (Ahmed, Rushworth et al. 2014, Chen and Wang 2020). Microbial detection using optical methods can be broadly classified under either vibrational spectroscopy, fluorescence spectroscopy or plasmonic methods (Hu, Bohn et al. 2017). Vibrational spectroscopy includes Surface Enhanced Raman Spectra (SERS) or Infrared (IR) spectroscopy. These methods have the capacity to identify bacteria based on vibration patterns of specific molecules present on the bacteria which creates a spectral fingerprint. For example, Martok et al. were able to identify *P. aeruginosa, K. pneumoniae,* and *E. cloacae* isolates belonging to the same sequence types based on their IR fingerprint (Martok, Valot et al. 2019). Fluorescence spectroscopy for microbial detection requires the use of a fluorophore which have specific excitation and emission spectra. When using fluorescence spectroscopy for specific microbial detection, typically an exogenous synthetic fluorophore is conjugated to a biorecognition element, such as antibodies. The resulting complex labels targets such as bacterial cells enabling their detection via flow cytometry or microscopy. Such fluorophore labelled antibodies were used to detect *Lp* bacteria to a limit of 10^1 to 10^3 cells/ml (Yamaguchi, Tokunaga et al. 2017). Plasmonic methods take advantage of properties of nanometer sized metals. These metals generate various oscillations of electrons in response to incident photons. Surface Plasmon Resonance (SPR) sensors and evanescent field-based fibre optics (EV-FO) are some examples of plasmonic methods to detect bacteria. Examples of SPR for microbial detection are given in further detail below.

In general, commonly employed optical biosensors include fluorescence-based (FB) sensors, chemiluminescence (CL) sensors, Colorimetric-based sensors (CB), Surface Plasmon Resonance (SPR) sensors and evanescent field-based fibre optic (EV-FO) sensors (Ahmed, Rushworth et al. 2014, Chen and Wang 2020).. Fluorescence, chemiluminescence and colorimetric based sensors have been widely used in research and diagnostics with competitive sensitivities and, in the case of colorimetric based sensors, often at low cost. Enzyme linked immunosorbent assays (ELISAs) and gold (Au) nanoparticle aggregation are just some examples of FB and CL and CB sensing. However, these sensing methods require multiple reagents, sample preparation and labelling (Kumar, Hu et al. 2018, Chalklen, Jing et al. 2020). In contrast EV-FO and SPR are sensitive to changes in refractive index (RI) and thus measure changes in RI in response to analyte-bioreceptor binding. This means they require no labelling and samples can be used in their native state (Ahmed, Rushworth et al. 2014, Chen and Wang 2020). Drawbacks for microbial detection are the low sensitivities and high LODs, as a result of the limited range of the electromagnetic field (~ 300-500nm), the similarity of the refractive index (RI) of the microbial cytoplasm and aqueous medium, and the diffusion-limited mass transport of the microbe to the metal-dielectric surface where the resonance phenomenon occurs and thus where surface plasmons are generated (Torun, Boyacı et al. 2012, Galvan, Parekh et al. 2018). However, much research is being done to mitigate these problems with progress in assay design, signal amplification strategies such as the use of nanoparticles and nanorods, photonic technologies such as LSPR, and transducer surface modifications (Gasparyan and Bazukyan 2013, Boulade, Morlay et al. 2019, Castiello and Tabrizian 2019, Chen and Wang 2020). Like electrochemical sensors, optical biosensors also require the use of a bioreceptor/probe to be selective for a microbial target. In the realm of optical biosensors, SPR is considered the gold standard for biosensing (Breitsprecher, Fung et al. 2018).

1.3.3.1 SPR Biosensors and Bacterial Detection:

Between 1902 and 1912, R.W. Woods at the John Hopkins University in Baltimore, MD, USA, noticed that when polarized light is shone on a metal coated diffraction grating, a pattern of light and dark bands appears (Wood 1902, Wood 1912). Although he speculated as to how this phenomenon occurred, he did not obtain a clear answer. Subsequent decades of research would identify and utilize this phenomenon to unveil, Surface Plasmon Resonance (SPR). SPR is an optical phenomenon (Homola and Piliarik 2006, Prabowo, Purwidyantri et al. 2018). Under total

internal reflection conditions-a phenomenon in which waves at the interface of two different media are completely reflected back into the first media- a portion of polarized light penetrates and hits a thin metal film coated on the surface of a glass prism (Homola and Piliarik 2006, Prabowo, Purwidyantri et al. 2018). This small portion of light generates an evanescent wave and transfers energy to free valence electrons of the metal to generate surface plasmons and a "plasmon wave" (Homola and Piliarik 2006, Prabowo, Purwidyantri et al. 2018). At a specific angle of incidence or wavelength, the surface plasmons and evanescent waves can "resonate" with equal frequencies, hence, resonance. This "resonance" is sensitive to changes in the refractive index near the surface of the metal coated prism (Homola and Piliarik 2006, Prabowo, Purwidyantri et al. 2018). This is because the polarized incident light will be absorbed resulting in a subsequent drop in the energy of the reflected light. The lowest "drop" in this reflected light spectrum gives rise to a resonance peak which will vary in response to any changes near the thin metal film (Homola and Piliarik 2006, Prabowo, Purwidyantri et al. 2018). An example of such a change can be the binding of a target analyte to a surface immobilized ligand (Figure 4) (Homola and Piliarik 2006, Prabowo, Purwidyantri et al. 2018).
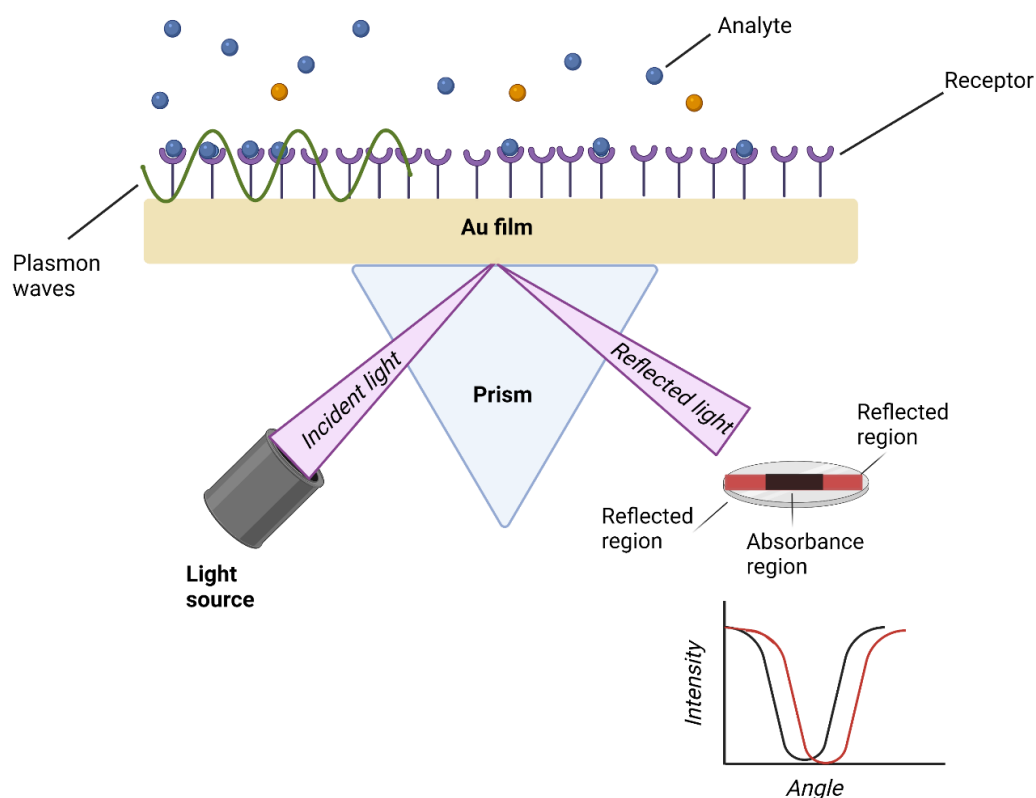
Figure 4. Surface plasmon resonance (SPR). Image created in Biorender.

Biacores development of the first commercial SPR sensor in 1990 (Liedberg, Nylander et al. 1995) led to an impetus towards the studying of many molecular interactions with SPR. One of the earliest whole microbial cells to be detected with an SPR system was *E. coli* O157:H7 (Fratamico, Strobaugh et al. 1998, Fratamico, Strobaugh et al. 1998). In one study the authors used immobilized antibodies or a sandwich assay to detect the bacterium (Fratamico, Strobaugh et al. 1998). In another study, the authors used pre-enriched bacterial cultures as a strategy to detect the *E. coli* cells to the limit of 10^6-10^7 CFU/ml (Fratamico, Strobaugh et al. 1998). These initial studies illustrate the primary challenge of SPR-based microbial detection i.e low sensitivities and high limits of detection.

Over the years many researchers have successfully overcome this limitation and detected as low as <10 CFU/ml of bacteria through a variety of strategies (Ahmed, Rushworth et al. 2014, Kumar, Hu et al. 2018, Bocková, Slabý et al. 2019, Saad and Faucher 2021). For example, instead of whole cells, microbial biomarkers such as 16Sr RNA or specific proteins or antigens were used to determine bacterial cell concentrations indirectly (Wang, Ye et al. 2011, Foudeh, Trigui et al. 2015, Taheri, Rezayan et al. 2016, Melaine, Saad et al. 2017, Sikarwar, Singh et al. 2017, Masdor, Altintas et al. 2019). These strategies minimize steric hindrance as well as the diffusion-limited mass transport of the microbial target to the metal-dielectric surface. Other strategies include transducer modifications, in an attempt to enhance the evanescent field and resonance effect by using nanoparticles, nanorods or long range SPR (LSPR) (Ahmed, Rushworth et al. 2014, Chen and Wang 2020). The extension of this field means the changes in the RI can be better captured. Hu et al used a LSPR system with a gold nano-triangle array to detect whole *Pseudomonas aeruginosa* cells at an LOD of 1 CFU/ml (Hu, Fu et al. 2018). Using gold coated silica nanoparticles on the metal-dielectric surface, Yoo et al were able to detect *Salmonella spp.* down to the level of 30 CFU/ml (Yoo, Kim et al. 2015) .

Regardless of the biosensing system, the development of versatile, stable bioreceptors is critical to improve the sensitivity and specificity of a biosensor. Though there are several bioreceptors namely- antibodies, lecithin's, bacteriophages, and molecularly imprinted polymers (MIPS)- currently antibodies are the most widely used bioreceptors in biosensor development and research (Morales and Halpern 2018). They serve as the gold standard in biorecognition elements due their selective properties and strong affinities to a range of microbial targets, many of which have been

validated over the past few decades (Morales and Halpern 2018). Limitations to the use of antibodies as bioreceptors are cross-reactivity (polyclonal antibodies), high production and purification costs, batch-to-batch variability, stringent handling conditions such as low temperature storage, requirement of an immunogenic target, stability in response to changes in temperature, humidity, pH ,etc (Jayasena 1999, Toh, Citartan et al. 2015, Bauer, Strom et al. 2019). Their relatively larger size also means lower packing densities which reduces sensor surface coverage and thus can impact sensor sensitivity (Morales and Halpern 2018). An increasingly popular alternative to antibodies that addresses these drawbacks are aptamers (Morales and Halpern 2018, Bauer, Strom et al. 2019).

1.4 <u>Aptamers</u>

Aptamers are just one in a series of fascinating nucleic acid elements that have functional properties beyond what is typically associated with DNA and RNA. Nucleic acids have additional functional roles other than being a "blueprint "or code for most life forms. They can fold into complex, 3D conformational structures and thus recognize molecules and cells with high affinities and specificities as well as modulate expression of genes and cellular processes (Savinov, Perez et al. 2014). These structures can be dynamic and form quickly, which is critical for binding and discerning target sites. For example, a slow off-rate and fast on-rate is important for tight, stable binding to targets. Additionally, nucleic acids can fold into complex structures slowly, such as in the case of riboswitches which alternate their secondary structures to better regulate gene expression (Savinov, Perez et al. 2014). Structural diversities are what lend nucleic acids their functional features. (Micura and Höbartner 2020).

There are several types of functional nucleic acid (FNA) elements such as aptamers, riboswitches, RNAzymes and DNAzymes, each with unique physical and chemical properties and thus functionality (Silverman 2009, Micura and Höbartner 2020). The latter three nucleic acid elements all possess an aptamer motif which serves as the target recognition region (Silverman 2009). For brevity and keeping in line with the topic of this thesis, this excerpt will focus on the functional nucleic acid element known as aptamers. Additionally, though aptamers or aptameric regions of FNAs do exist widely in nature, such as in riboswitches, (Silverman 2009, Savinov, Perez et al. 2014), this section focuses on laboratory derived aptamers.

First discovered in the 1990s by the labs of Szostak and Gold ((Ellington and Szostak 1990, Tuerk and Gold 1990), aptamers are antibody analogues. They are short, single stranded DNA or RNA oligonucleotides typically up to 100 nucleotides in length. They can bind to a wide variety of targets such as small molecules, peptides, proteins, whole cells etc. with high affinity and specificity. They can interact with their targets via a wide array of chemical interactions such as shape complementarity, hydrogen bonding, electrostatic interactions and/or stacking interactions (Tan, Acquah et al. 2016, Cai, Yan et al. 2018).

A key characteristic of an aptamer is that it can be generated chemically, *in vitro* to function in desired conditions, unlike an antibody which requires strict physiological conditions for its development (Jayasena 1999, Bauer, Strom et al. 2019). Unlike antibody targets, aptamer targets do not have to be immunogenic (Jayasena 1999, McKeague, De Girolamo et al. 2015, Rozenblum, Lopez et al. 2016). Aptamers' chemical properties make them more thermotolerant and stable, and much easier to modify as compared to antibodies (McKeague, De Girolamo et al. 2015, Röthlisberger and Hollenstein 2018). In addition to their chemical characteristics, their small size (15-30 kDa versus ~150 kDa for a full-size antibody) facilitates functionalization and high packing densities on surfaces which is advantageous for biosensor development (Morales and Halpern 2018). This versatility coupled with decreasing complexities and costs of synthesizing oligonucleotides add to the appeal of aptamers as ideal bioreceptors (Dunn, Jimenez et al. 2017, Komarova, Barkova et al. 2020). Current oligonucleotide synthesizing technology also means that aptamers can be produced in a high throughput manner with minimal batch to batch variation and at low cost (Komarova, Barkova et al. 2020). The fact that they are nucleotides also means that they can be easily integrated with other technologies involving nucleic acid-based systems, such as sequencing technologies, amplification systems and nucleic acid computational modelling (Buglak, Samokhvalov et al. 2020, Komarova, Barkova et al. 2020).

A class of laboratory derived aptamers known as aptasensors has been gaining traction in research. Aptasensors are engineered aptamers that act as stand-alone, ligand-responsive devices which "activate", either by cleavage or altering its configuration, in the presence of a specific cognate analyte. In other words, aptasensors are capable of coupling binding with signaling the concentration of a specific analyte of interest. This has led to an outburst of research aimed at designing sensors and other genetically controlled elements for various applications. Several types

of aptasensors exist and have been reported in the literature e.g structure switching, molecular beacon, target induced strand displacement, and duplex aptamers (Tyagi and Kramer 1996, Yoshizumi, Kumamoto et al. 2008, Feagin, Maganzini et al. 2018, Munzar, Ng et al. 2019). This aspect of aptamer engineering however is beyond the scope of this thesis.

Over the last three decades aptamers have been made against a wide array of targets ranging from small molecules to whole cells (McKeague, De Girolamo et al. 2015, Davydova, Vorobjeva et al. 2016, Saad and Faucher 2021). For example, aptamers have been incorporated as target binders for detection in analytical assays such as ELISA (Lee and Zeng 2017) and lateral flow (Bruno 2014), in therapeutic applications (Ishiguro, Akiyama et al. 2011, Adachi and Nakamura 2019) and in nano-delivery systems or assays (Lao, Phua et al. 2015).

Many aptamers have been developed and used in biosensing platforms to detect many targets such as proteins, molecules such as thrombin and theophylline as well toxins such as aflatoxins and various environmental contaminants (Morales and Halpern 2018, McConnell, Nguyen et al. 2020). Aptamers developed against bacteria, for example, have been used on multiple platforms ranging from lateral flow assays (Bruno 2014, Saad and Faucher 2021) to electrochemical impedance spectroscopy (Brosel-Oliu, Ferreira et al. 2018, Saad and Faucher 2021) to SPR-based systems (Yoo, Kim et al. 2015, Oh, Heo et al. 2017, Saad and Faucher 2021).

Aptamer research has increased exponentially over the years since the discovery of the first aptamer in 1990 (Dunn, Jimenez et al. 2017). In 25 years, aptamers were the subject of 4,795 articles. Among those articles, 3,995 were experimental articles and 800 were review articles (Dunn, Jimenez et al. 2017). A quick search in PubMed database, chosen for its least redundancies, showed that the number of review articles for aptamers increased from 172 in 2015 to 305 in 2020, illustrating the surge of research activity and interest in this domain. This interest has also led to the creation of several aptamer-based companies and corporations (Table 1) (McKeague).

APTAMER COMPANIES
2bind, GmbH, Germany
Altermune LLC, Sandwich, Kent or CA, USA (Acquired by Cantauri Therapeutics)
AM Biotechnologies, LLC, TX, USA
AMS Biotechnology, Abingdon, UK
APTAGEN, LLC, PA, USA
AptaMatrix, NY, USA
Aptamer Group, York, UK
Aptamer Sciences Inc, Korea
Apta Targets Madrid, Spain
Aptabiosciences, UK and Singapore
AptaIT GmbH, Germany
Apterna, UK
Aptitude Medical Systems Inc, USA
AptusBiotech, Spain
Astrazeneca
ATDBio Ltd, Southampton, UK
AuramerBio, New Zealand
Base Pair Biotechnologies, Inc, USA
BBI Group, Cardiff, UK
DSM Biotechnology, The Netherlands
Firefly Bioworks, MA, USA
iba GmbH Germany
IDT DNA, Iowa, USA
Izon Science Oxford, UK
LC Sciences, TX, USA
LFB Biotechnologies, France
Nal von Minden Germany
Neoventures, Ontario, Canada

Novaptech
Noxxon Pharma, AG Germany
Ophthotech NY, USA
OTC Biotech TX, USA
Piculet Biosciences The Netherlands
Pure Biologics Poland
Ribomic Japan
SomaLogic CO, USA
Tobira Therapeutics CA, USA
Tocris Bioscience Bristol, UK
TriLink Biotechnologies CA, USA
Veraptus, China

Table 1. List aptamer companies. List compiled by Dr. Maureen McKeague, Dr. Sarah Shigdar and Dr. Mohammad Sohail. Retrieved from http://aptamersociety.org/aptamer-companies/.

Most of the published research are studies of aptamer-based applications versus aptamer development (Dunn, Jimenez et al. 2017). In studies that report the development of aptamers, most work list proteins as the primary target for aptamers, followed by small molecules and then whole cells (Dunn, Jimenez et al. 2017). A large proportion of these protein targets are of mammalian origin such as thrombin, VEGF etc. whereas the majority of whole cell targets are pathogenic microbes followed by cancer cells (Dunn, Jimenez et al. 2017).

Studies that report the development of aptamers also predominantly use a DNA backbone for selection, followed by the RNA backbone (McKeague, McConnell et al. 2015, Dunn, Jimenez et al. 2017). In initial years, RNA backbone aptamers were primarily selected due to the well established fact that RNA could have functional motifs and fold into complex secondary structures, thus increasing the chances for finding high affinity probes (McKeague, McConnell et al. 2015, Dunn, Jimenez et al. 2017). However, further investigation revealed that DNA secondary structures could have high binding affinities (Lin and Patei 1997). This coupled with the stability of DNA, due the lack of 2'-hydroxyl moiety on the DNA sugar, lead to a shift towards aptamers with DNA backbones (McKeague, McConnell et al. 2015, Dunn, Jimenez et al. 2017, Micura and Höbartner 2020). This stability also contributes to the ease of handling DNA (Amero, Lokesh et

al. 2021). Traditional in vitro selection steps would require the RNA aptamer pool be converted to cDNA followed by reverse transcription to develop the input pool for the next round of selection. Interestingly, aptamer studies with modified or unnatural nucleotides i.e neither a DNA nor RNA backbone, have been increasing over recent years (Dunn, Jimenez et al. 2017). Our own literature review of aptamers against water-borne pathogens, which will be discussed in the next chapter, unveiled similar trends with regards to backbone chemistry. In over 110 studies, only 4% of studies used or developed RNA aptamers (TableS1, Saad et al 2021) (Appendix, FileA1).

Aptamer performance is dependant on the quality of the experimental design of the selection procedure. This selection procedure, known as SELEX (systemic evolution of ligands through exponential enrichment), is discussed in the following section.

1.5 <u>SELEX</u>

The procedure by which an aptamer is created is known as Systemic Evolution of Ligands through EXponential enrichment (**SELEX**). SELEX, also known as *in vitro* selection, is an iterative process which involves repeatedly exposing a target molecule with a large random library of oligonucleotides, up until the final pool of oligos are enriched with sequences that bind to the target with high affinity and specificity (Figure 5).
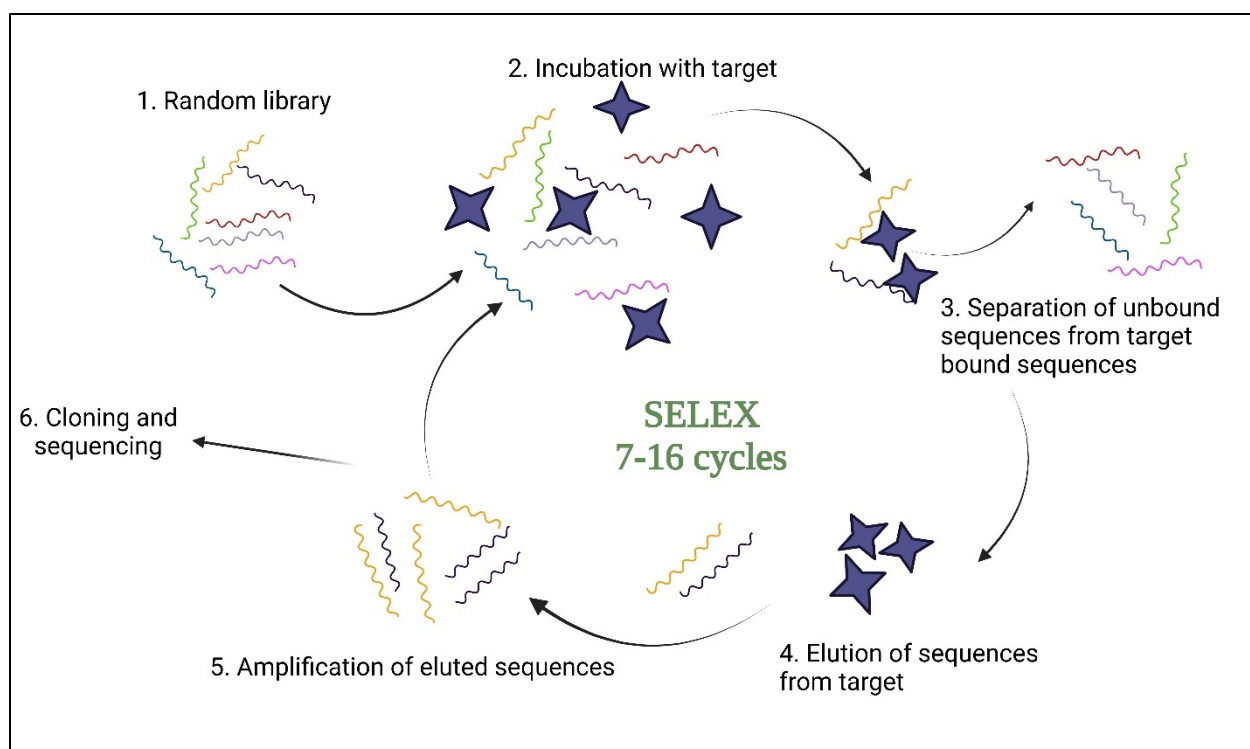
Figure 5. Schematic of SELEX procedure. Image created in Biorender.

The classical SELEX approach involves incubating a target molecule with a large (10^{16}), diverse nucleotide library, separating the target bound sequences from the unbound oligonucleotides and then amplifying the target bound sequences via PCR for the next round of selection (Gold 2015). Generally, the selection rounds are repeated until the oligonucleotide pool is enriched with sequences that bind to the target specifically with high affinity (Gold 2015).

There are multiple steps in the SELEX method. These include library design and selection, target preparation, reaction conditions, partitioning steps, purification, and sequence pool recovery steps (Gold 2015, McKeague, McConnell et al. 2015, Kalra, Dhiman et al. 2018). Given the multiple steps of this technique, the SELEX process lends itself to being modular. This is evidenced by the multitude of SELEX techniques currently present in the literature (Table 2).

Type of SELEX	Reference
Duplexed Aptamer SELEX	(Munzar, Ng et al. 2019)
One-Pot SELEX	(Missailidis, Thomaidou et al. 2005, Jauset-Rubio, Botero et al. 2019)
Cell-SELEX	(Morris, Jensen et al. 1998, Daniels, Chen et al. 2003)
Capillary Electrophoresis SELEX	(Mendonsa and Bowser 2004)
FluMag SELEX	(Stoltenburg, Reinemann et al. 2005)
Differential SELEX	(Cerchia, Esposito et al. 2009)
Branched SELEX	(Dupont, Larsen et al. 2015)
Capture SELEX	(Stoltenburg, Nikolaus et al. 2012)
In silico SELEX	(Ikebukuro, Okumura et al. 2005, Zhou, Xia et al. 2015)
AEGIS SELEX	(Sefah, Yang et al. 2014)
SMART Selex	(Song, Zheng et al. 2019)
Bead based SELEX	(Tok and Fischer 2008)
Complex Target/Deconvolution SELEX	(Morris, Jensen et al. 1998)
DRIVR and CleaveSeq SELEX	(Townshend, Xiang et al. 2021)
Primerless/Tailored SELEX	(Vater, Jarosch et al. 2003)
Microfluidic SELEX	(Lou, Qian et al. 2009)
Genomic SELEX	(Lorenz, Von Pelchrzim et al. 2006)
Toggle SELEX	(White, Rusconi et al. 2001)

Table 2: List of different types of SELEX.

Each step in SELEX must be designed and optimized to maximize the likelihood of a high affinity aptamer for a specific downstream application. For example, by incubating an aptamer library with serum of lung cancer patients and doing negative selection with serum from healthy individuals, Li et al were able to identify 6 DNA aptamers that were highly specific in detecting lung cancer in the serum of 20 lung cancer patients but not in 20 healthy patients (Li, Xiu et al. 2017). Similarly, to identify aptamers that would retain functionality in physiological conditions, Ferreira et al. incubated their aptamer library with metastatic breast cancer MDA-MB-231 cells at a temperature of 37°C during their reaction incubation steps (Ferreira, Barbosa et al. 2021). The latter study is

also one of many examples of a technique adapted from the original SELEX methodology known as Cell-SELEX.

Cell-SELEX utilizes a different target preparation approach by using live whole cells as the target for selection instead of a specific biomarker (Sefah, Shangguan et al. 2010, Kaur 2018). This eliminates the need for prior knowledge of a specific target biomolecule as well as cumbersome purification and separation experiments. Indeed, this adaptation has even led this approach to be called Blind-SELEX by one group (Yoon, Armstrong et al. 2017).

The goal of the SELEX process is to produce specific, high affinity aptamers. These aptamers are characterized as having low equilibrium dissociation constants (K_D) (Tan, Acquah et al. 2016, Plach and Schubert 2020). Therefore, many aptamer development and discovery works report this metric as part of their characterization studies. The flexibility of the SELEX process also means its outcomes can be unpredictable or have a high failure rate (Zhou and Rossi 2017, Komarova and Kuznetsov 2019, Micura and Höbartner 2020). Several parameters must therefore be investigated and analyzed to increase the probability of recovering robust aptamers. We will now briefly describe several parameters affecting the outcome of SELEX.

1.5.1 Library Design

1.5.1.1 Backbone:

As previously mentioned, the use of DNA backbone libraries facilitates handling and manipulation in addition to reducing multiple processing steps that could otherwise contribute to variability in experimental procedures e.g cDNA generation and reverse transcription (McKeague, McConnell et al. 2015, Dunn, Jimenez et al. 2017, Micura and Höbartner 2020). Additionally, the absence of 2'OH group makes the DNA sugar more stable (Röthlisberger and Hollenstein 2018). Though RNA and DNA backbone libraries are purported to result in aptamers with similar affinities (Röthlisberger and Hollenstein 2018, Amero, Lokesh et al. 2021), functional motifs and secondary structures of RNA are more diverse and well-studied (McKeague, McConnell et al. 2015, Komarova, Barkova et al. 2020, Micura and Höbartner 2020). In fact, a lot of robust aptamers come from aptasensor libraries that are typically RNA based due to complex secondary structures and functional features. Examples are riboswitches and ribozymes (Micura and Höbartner 2020, Townshend, Xiang et al. 2021).

1.5.1.2 Modified and Unnatural Nucleotides:

Regardless of backbone, given that only four nucleotides (A, G, C and T) exist in nature, aptamer libraries must be large (10^{15}) and thus diverse enough to increase chances of finding motifs with required functional properties. Binding sites of antibodies can have six segments of variable structures composed of either 110-130 amino acids of 20 different types (Schroeder Jr and Cavacini 2010). The diverse combinations of amino acids also mean that antibodies can have more variety of physicochemical characteristics such as charges, pKa etc. Previously, altering the composition of the library by including more G's and C's instead of using a randomized library has been found to enhance diversity by developing more secondary structures (Vorobyeva, Davydova et al. 2018). One other way to achieve enhanced diversity is by using "artificial" and/or modified nucleotides. The use of such nucleotides/sequence backbones has steadily increased over the past 15 years (Dunn, Jimenez et al. 2017). Some of these modifications are either on the nitrogenous base such as 5'methyluridine or on the sugar backbone such as 2'Fluoro. Locked nucleic acid aptamers (LNA) and SOMAmers are examples of aptamers with modified nucleotides (Schmidt, Borkowski et al. 2004, Brody, Gold et al. 2010). In fact, AEGIS-SELEX (artificially expanded genetic information system – SELEX) is a technique adapted from the original SELEX method by the inclusion two new artificial nucleotides in the library (Sefah, Yang et al. 2014).

1.5.1.3 Presence of primer binding sites/constant regions:

To enable amplification of target bound sequences via PCR, traditional SELEX methods require the presence of primer binding regions flanked on either side of the variable sequence region. These primer binding regions are constant and could, plausibly, contribute to the overall structure of the final aptamer but they also have the risk of non-specific binding and can interfere with the variable sequence region that specifically binds the target (Pan and Clawson 2009). An analysis of more than 2000 structures from AptamerDatabase showed that the constant regions had minimal to no effect on the secondary structure formation as the majority of the structural motifs occurred in the randomized region (Cowperthwaite and Ellington 2008). This data however is not recent and contains several outliers. Several studies have minimized the effect of primer binding sites during SELEX by either replacing the constant sites after a certain number of rounds using restriction enzymes or blocking the primer binding sites with complementary oligonucleotides (Shtatland, Gill et al. 2000, Ouellet, Lagally et al. 2014). Primer less SELEX has also been

developed (Jarosch, Buchner et al. 2006, Pan and Clawson 2009). Several of these strategies involve cumbersome removal, cleavage and re-ligation steps. To tackle these limitations a recent article suggests the use of structure-switching primers coupled with an electrophoretic gradient. Upon changing buffer conditions, the structured primers would unfold and release the target. Variable region sequences that bind to the target would not be altered by the changed buffer conditions and so an electrophoretic gradient could be applied to release the target (Wang 2020).

1.5.1.4 Length of variable region:

Investigation of several SELEX experiments reveals that the length of the randomized or variable region of libraries does not appear to affect the affinities of the final selected aptamers (Wang, Chen et al. 2019). However the majority of libraries in SELEX studies have variable regions sized between 30-90 nucleotides (McKeague, McConnell et al. 2015, Vorobyeva, Davydova et al. 2018). Variable region lengths must strike a balance between diversity and the complexity of secondary structures. Longer sequences may have more secondary structures but could have lower sequence space. For example, a sequence of 30 nucleotides could reach a diversity of 10^{18}, (given 4^n). This empirical high diversity, though desired, means the risk of subsampling the initial library and since each distinct sequence is initially present in only one copy number, this could lead to the "loss" of unique sequences and hence reduced diversity (Pobanz and Lupták 2016). Additionally, longer sequence libraries also have increased costs with synthesis and purification and run the risk of producing PCR artifacts (Vorobyeva, Davydova et al. 2018, Wang, Chen et al. 2019). Variable regions as short as 20nt and 15nt have been successful in producing aptamers (Kupakuwana, Crill et al. 2011, Thiel, Bair et al. 2011).

1.5.2 Temperature

Aptamers can be dynamic and have alternative configurations depending on their environment. Therefore, the temperature most relevant to the target application tends to be used for selecting robust aptamers. The majority of selections are done at 25 °C which mimic ambient conditions (McKeague, McConnell et al. 2015). Other common selection temperatures are 37 °C which represent physiological conditions followed by 4 °C. The latter temperature minimizes cellular uptake of oligonucleotides (Sefah, Shangguan et al. 2010). When DU145 cells were incubated with the fluorophore-labelled DML-7 aptamer at 4 ºC or 37 ºC for 2 hours, fluorescence was only observed inside the cells incubated at 37ºC (Zhang, Sefah et al. 2012, Duan, Long et al. 2016). The

low temperature of 4ºC is also a common preparatory step, following heating, in many SELEX procedures to enable nucleic acid folding. Thiel et al. developed a cell-based aptamer fluorescence binding and internalization (AFBI) assay for high throughput screening of aptamer performance under various conditions (Thiel and Giangrande 2016). Interestingly, through this assay, they determined that most temperature protocols had negligible impact on aptamer function post folding and that aptamer folding is more dependent on buffer components than the temperature protocol (Dickey, Giangrande et al. 2016).

1.5.3 Buffer Conditions (pH, buffering agent, metal cations)

The selection media has significant influence on target properties as well aptamer properties such as structure, charge, protonation and thus affinities (McKeague, McConnell et al. 2015). Protonation can cause different base pairings to occur and thus affect aptamer target binding (Belleperche and DeRosa 2018). For example, an in-depth study of the thrombin aptamer showed varying pH altered the aptamers binding affinities (Hianik, Ostatná et al. 2007). Programmable aptamers that respond to pH have also been developed (Li, Jiang et al. 2018, Thompson, Zheng et al. 2020).

The effect of buffering agent on aptamer affinities is not well studied. Most SELEX reactions use phosphate buffered saline, Tris buffered saline or HEPES buffer, Tris buffered saline seems to be the most widely used buffering agent (McKeague, McConnell et al. 2015). All three buffers are well known in molecular biology for their osmotic properties and compatibility with most biologics. Typically, the same buffer is adapted and used in washing steps to separate unbound oligos from target bound oligos. However some researchers found that by increasing the ionic strength of the "wash" buffer, using a gradient of 0.1 to 1M, the most tightly bound aptamers were eluted by the highest salt concentration, thus enabling the selection of aptamers with different binding characteristics (Hernandez, Flenker et al. 2013). Another research group separated target bound sequences from unbound using a combination of salt gradient with chromatography (Martin, Parekh et al. 2013).

Cations have been observed to influence aptamer K_D. A review of 25 years of published aptamers suggested that higher affinity aptamers (low K_D) were correlated with lower metal ion (K^+, Na^+, Mg^{+2}) concentrations (McKeague, McConnell et al. 2015). However this correlations between metal cation concentration and K_D was stronger for target type versus nucleic acid type suggesting

that when deciding the metal cation composition of the SELEX buffer, the target should be considered more than the nucleic acid type (McKeague, McConnell et al. 2015). The presence of ions, such as K^+ and Mg^{+2}, has been previously reported to highly stabilize key secondary structure elements known as G-quadruplex regions (Bhattacharyya, Mirihana Arachchilage et al. 2016). This however can be problematic during subsequent SELEX rounds which often require PCR amplification steps (Ruggiero and Richter 2018).

1.5.4 Number of SELEX Rounds

Currently the average number of selection rounds for most SELEX experiments is somewhere between 5–15 (McKeague, McConnell et al. 2015).This number depends on many factors and are often a tradeoff between cost, time and throughput. For example, if there are no negative selection steps with non-target molecules then more rounds of SELEX may be needed to achieve aptamers that are highly specific to a desired target (Hamula, Peng et al. 2015). By contrast increasing the number of SELEX rounds would lead to multiple PCR steps. This would result in PCR bias which results in the increase of sequences more amenable to amplification and consequently an extreme loss in the diversity of functional molecules (Gentry-Shields, Wang et al. 2013, Head, Komori et al. 2014, Dunn, Jimenez et al. 2017, Witt, Phung et al. 2017). PCR bias also tends to result in shorter sequences further reducing potential key secondary structural elements (Head, Komori et al. 2014, Witt, Phung et al. 2017). To mitigate the effects of PCR bias researchers have come up with various strategies such as optimizing the number of PCR cycles required, also known as preparative PCR (Sefah, Shangguan et al. 2010), limiting the number of SELEX rounds through parallel selection (Dupont, Larsen et al. 2015) or using emulsion and droplet PCR (Kanagal-Shamanna 2016). Partitioning methods, which involve separating unbound non-specific sequences from target-bound hence specific sequences, also influence the number of SELEX rounds. The more efficient a partitioning method is at separating unbound from bound sequences, the fewer number of SELEX rounds will be required (Hernandez, Flenker et al. 2013, Dunn, Jimenez et al. 2017). To monitor the enrichment and "evolution" of the sequence pools per round of SELEX, researchers typically use either flow cytometry (Sefah, Shangguan et al. 2010) or-more recently due to improved technologies and decreasing costs- sequencing (Dupont, Larsen et al. 2015, Hoinka and Przytycka 2016, Hoinka, Backofen et al. 2018). The advent of high throughput

sequencing has enabled deeper insights into the "evolution" of sequence pools over the course of SELEX rounds.

1.5.5 Partitioning Methods

The separation of target bound sequences from unbound non-specific sequences, is critical to the success of the SELEX procedure. Some more advanced SELEX methods have been published using specialized partitioning methods with the help microfluidic platforms or capillary electrophoresis (Wang, Chen et al. 2019). Washing steps are the most common for the removal of unbound molecules. As mentioned previously, using different ionic buffers with the help of salt gradients or adjusting the number of washing steps can increase stringency of the selection process (Sefah, Shangguan et al. 2010, Hernandez, Flenker et al. 2013, Kim, Song et al. 2013). Fluorescence cell sorting and electromobility shift assay (EMSA) are also some partitioning methods where aptamer-target complexes are separated from the reaction milieu (Mayer, Ahmed et al. 2010, Szeto, Latulippe et al. 2013, Wu and Kwon 2016)..Heat gradients, urea and DNAase have also been used as denaturing agents to separate loosely bound or unbound sequences (Wang, Chen et al. 2019).

1.5.6 Recovery Methods

Following partitioning and/or PCR amplification steps, it is necessary to obtain high integrity pools as the input or template for subsequent rounds of SELEX. Consequently, the efficient recovery of high-quality ssDNA or RNA that retain functional motifs and complex secondary structures, is important. Given the context of the experimental work conducted in this thesis and the increase in research towards developing DNA aptamers, ssDNA recovery methods are described.

There are four primary methods for recovering ssDNA from the dsDNA formed during PCR amplification.

(i) Asymmetric PCR: This involves the preferential amplification of one strand of template DNA by using an unequal ratio of forward and reverse primers. Though it is a low cost and straightforward method, the multiple steps involved with fragment purification from the gel impact the speed and efficiency of this method which can also result in the loss of some sequences (Citartan, Tang et al. 2012, Svobodová, Pinto et al. 2012)..

Furthermore, conditions would need to be optimized for maximum yield and efficiency (Heiat, Ranjbar et al. 2017, Tolnai, Harkai et al. 2019).

(ii) <u>Magnetic bead-based separation:</u> This method makes use of the tight interaction between biotin and streptavidin. Using a biotinylated primer during PCR tags the aptamer pool with biotin. The PCR product – biotinylated dsDNA – is then captured by streptavidin coated magnetic beads and denatured by heat or high alkaline pH. One strand remains on the bead and is separated with the help of a magnet while the supernatant containing the other strand is collected and purified. The most common method, this technique is simple and quick but expensive due to the use of labelling moieties and magnetic beads. Additionally, the denaturing conditions can result in a dissociation of streptavidin from beads, leading to poor quality ssDNA and the presence of another SELEX target. A recent study reviewed SELEX experiments over the past 13 years and noted that alkaline denaturation was a significant factor in low quality aptamer selection (Oteng, Gu et al. 2020). The study also conducted two parallel SELEX to compare the impact of two different methods i.e magnetic bead-based vs, lambda nuclease based, for ssDNA recovery on the efficiency of aptamer enrichment. They discovered that the lambda nuclease method for ssDNA recovery resulted in more aptamer enrichment (Oteng, Gu et al. 2020).

(iii) <u>Lambda nuclease digestion method:</u> This method involves the incorporation of a 5' phosphate group, with the help of specific primer and PCR, in the undesired strand. This will enable digestion by the lambda nuclease (Svobodová, Pinto et al. 2012). This method is relatively simple to do and can have a high recovery rate of ssDNA (Svobodová, Pinto et al. 2012). To minimize non-specific digestion of the non-phosphorylated target strand, the target strand must be labelled with a moiety such as fluorophore (Svobodová, Pinto et al. 2012). Other limitations associated with this technique are the cost of the enzyme, the risk of having by-products, and adversely impacting ssDNA yields following enzyme and buffer removal (Wang, Chen et al. 2019).

(iv) <u>Denaturing polyacrylamide gel electrophoresis (PAGE) separation method:</u> This method involves the use of specially modified primers which results in two strands of PCR products with different lengths. These two strands are then separated by

denaturing gel electrophoresis (Komarova and Kuznetsov 2019). The ssDNA is then extracted and collected. The separation of different lengths and hence different molecular weight bands minimizes chances of collecting PCR by products and thus improves ssDNA recovery. This process however is very tedious and time consuming and can take up to 12 hours to elute the ssDNA from the gel block (Komarova and Kuznetsov 2019).

1.5.7 Negative Selection/Counter SELEX conditions

The incorporation of appropriate negative selection steps can greatly improve SELEX outcomes (Hamula, Peng et al. 2015, Wang, Chen et al. 2019). Not only will negative selection steps greatly minimize the number of SELEX rounds and minimize PCR bias, but it can also lead to a highly specific aptamer with increased functionality in a relevant downstream application. For example, given that the bacterial pathogen, *Campylobacter jejuni*, is found on raw poultry and the gastrointestinal tract and feces of animals (Mughini-Gras, Penny et al. 2016) , Dwivedi et al. used a large number, approximately 20 non-target bacterial species for negative selection. These included other food-borne pathogens, native enteric bacteria, native non-enteric bacteria and lactic acid bacteria (Dwivedi, Smiley et al. 2010). The result was a species-specific aptamer with high affinities in the nanomolar range.

1.5.8 Target Preparation Steps

The nature of the target can dictate the SELEX outcome. The more available binding pockets and forces of attraction the higher the chances of avidity and selecting high affinity aptamers (Kalra, Dhiman et al. 2018). The majority of aptamers currently published in literature bind to proteins (Dunn, Jimenez et al. 2017). Most aptamers targeting small molecules have lower affinities compared to aptamers binding more complex targets (McKeague, McConnell et al. 2015). In the case of larger multimeric complex targets such as cells, multiple factors need to be taken into account to develop efficient aptamers that make sense for specific applications. We will now review, in the next chapter, how target properties can impact SELEX specifically for finding aptamers for water-borne pathogens.

1.6 <u>References</u>

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CHAPTER 2: CONNECTING TEXT

Understanding the context and downstream environment for an aptamer's application is important for aptamer development. Consequently, we examined the literature reporting the development of aptamers against waterborne microbes and identified several properties that would serve as determinants to aptamer development.

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Aptamers and Aptamer-coupled Biosensors to detect Water-borne Pathogens

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2.1 ABSTRACT

Aptamers can serve as efficient bioreceptors for the development of biosensing detection platforms. Aptamers are short DNA or RNA oligonucleotides that fold into specific structures, which enable them to selectively bind to target analytes. The method used to identify aptamers is SELEX. Target properties can have an impact on aptamer efficiencies. Therefore, characteristics of water-borne microbial targets must be carefully considered during SELEX for optimal aptamer development. Several aptamers have been described for key water-borne pathogens. Here, we provide an exhaustive overview of these aptamers and discuss important microbial aspects to consider when developing such aptamers.

2.2 INTRODUCTION:

Access to water that is safe for use and consumption is a basic human right. As a result, most countries have strict guidelines, regulations and standards for managing water sources and water distribution systems to supply high quality water free from chemical and microbial contaminants. In most cases, microbial contaminants must be removed from the water before distribution. These microbes include pathogens that cause gastroenteritis, such as *Cryptosporidium, Giardia, Norovirus, Rotavirus, Campylobacter, and E. coli* (WHO 2017). Other water-borne diseases are caused by pathogens growing inside water distribution systems or within engineered water systems, such as cooling tower, fountains, spas and humidifiers (Wang, Bédard et al. 2017). The latter include *Legionella pneumophila*, *Pseudomonas spp.* and non-tuberculosis mycobacteria. In recent years, several studies have shown a high proportion of water associated deaths and illnesses are due to the aforementioned three environmental water-borne pathogens (Gargano, Adam et al. 2017, Greco, Drudge et al. 2020). In fact, *L. pneumophila*, the causative agent of Legionnaires disease, has become the number one cause of water-borne outbreaks in recent years (McClung, Roth et al. 2017). The presence of coliforms is not indicative of the presence of several key water-based pathogens that are of significance to public health (Payment and Locas 2011). Consequently, specific detection methods are needed to ensure safe water from the source to the point of use.

Monitoring and surveillance of specific water-borne microbes require robust detection methods. Challenges in select current detection methods for waterborne pathogens have been reviewed excellently in detail elsewhere (Ramírez-Castillo, Loera-Muro et al. 2015, Wang, Bedard et al. 2017). In general, traditional microbial detection methods rely heavily on culture methods, which is fraught with several limitations. Culture methods are extremely time consuming and often require extensive material, specialized labour, and time. Culture recovery rates are also adversely affected by many factors such as the presence of competing microbes, the presence of viable but non culturable (VBNC) cells, methods used for concentration of the sample or enrichment of the target microbe and sample type (bulk water or biofilm) (Wang, Bedard et al. 2017). Drawbacks with culture techniques has led to a shift towards the use of molecular methods, including PCR, quantitative PCR (qPCR), high throughput sequencing, and immunoassays such as ELISA, immunochromatography and immuno-lateral flow assays. The most widely used molecular method is qPCR (Ramírez-Castillo, Loera-Muro et al. 2015, Wang, Bedard et al. 2017). The

advantage of qPCR, over conventional culture techniques, is more rapid turn-around times, high sensitivities and specificities, lower limits of detection, as well as an ability to detect VBNC cells. However, by detecting live, VBNC and dead cells qPCR leads to an overestimation of microbial burden. Additionally, qPCR involves multiple sample processing steps which requires specialized labor. qPCR is also inhibited by several compounds routinely found in water samples resulting in possible false negatives (Gentry-Shields, Wang et al 2013).

Biosensors can mitigate some of the problems associated with traditional detection methods (Ahmed, Rushworth et al. 2014). They are analytical devices used to quantify or detect a specific analyte (Turner 2013). Qualities of biosensors includes high specificity, high sensitivity, multiplexing capability, cost-effectiveness, portability and ease of use (Ahmed, Rushworth et al. 2014, Kumar, Hu et al. 2018, Cesewski and Johnson 2020, McConnell, Nguyen et al. 2020). A biosensor set-up typically consists of three elements. A biorecognition element, which upon interaction with a target, produces a physico-chemical signal that is converted by a transducing element into a signal captured by a detection element (Turner 2013). Biosensors are categorized based on either their transducing element (mechanical, optical, electrochemical) or the nature of the biorecognition element (affinity, catalytic) (Ahmed, Rushworth et al. 2014).

A versatile and stable biorecognition element is a critical component of any biosensing platform (Ahmed, Rushworth et al. 2014, Kumar, Hu et al. 2018). Antibodies are the most used bioreceptors in biosensor development and research, but aptamers are an increasingly widespread popular alternative (Song, Wang et al. 2008, Morales and Halpern 2018). Aptamers are single stranded DNA or RNA oligonucleotides that fold into specific complex structures and interact with their targets via shape complementarity, hydrogen bonding, electrostatic interactions and stacking interactions (McKeague, McConnell et al. 2015). Besides having high affinities and selectivity, they can bind to a wide range of targets from small non-immunogenic compounds to whole cells (McKeague, McConnell et al. 2015). Aptamers can be generated in vitro in conditions one can preferentially select making them stable and versatile for a variety of applications (Song, Wang et al. 2008). They are cost-effective to synthesize with minimal batch to batch variation (Strehlitz, Reinemann et al. 2012, McConnell, Nguyen et al. 2020). Their easily modifiable nature facilitates functionalization on sensing surfaces (Song, Wang et al. 2008, McConnell, Nguyen et al. 2020). Their inherent small size also promotes high packing densities during functionalization (Song,

Wang et al. 2008, Crivianu-Gaita and Thompson 2016). In this minireview, we will briefly provide examples of aptamers with potential for detection of water-borne pathogens and discuss microbial determinants for the development of optimal aptamers and thus improved aptamer-coupled biosensors. Examples of aptamers is provided in Table 1 and a complete list of aptasensing platforms is provided in Supplementary Table 1.

2.3 APTAMER DEVELOPMENT:

Aptamers are typically identified by SELEX (Systematic Evolution of Ligands through Exponential Enrichment). SELEX is an iterative process where repeated exposure of a target to a large pool of random oligonucleotides results in the gradual enrichment of specific sequences that bind with the highest affinity to the target. Since the technique's inception in 1990, many variations of the original SELEX method have been published (Darmostuk, Rimpelova et al. 2015). These experimental variations differ based on desired aptamer properties and details have been reviewed elsewhere (Wang, Chen et al. 2019). Of note, cell-SELEX can be used to select aptamers against whole cells in solution, to ensure cell surface target epitopes are in their native state (Kaur 2018). This method is particularly useful for developing aptamers to detect water-borne pathogens. Cell-SELEX may include counter-selection steps to remove sequences binding to non-target microbes thus minimizing cross-reactivity and improving the specificity of the resulting aptamers (see Table 1 for examples).

Several aptamer-coupled biosensing systems or aptasensors have been described for the detection of water-borne pathogens or toxins accumulating in water (Table 1 and Supplementary Table S1) with the majority targeting bacterial pathogens. Nevertheless, none have been officially adopted for routine detection of water-borne pathogens. The development of successful aptamer-coupled biosensors to detect water-borne pathogens requires a multi-pronged approach. Besides intricate knowledge of the sensing system, its transducer, the physico-chemical phenomenon that mediate signal responses, and a deep understanding of aptamer chemistries, careful consideration of the physiology and ecology of the target microorganism is required. This is because physio-ecological factors affect microbial morphologies and surface structures and thus the presence of aptamer targets (Figure 1). Although several works discuss transducing systems and aptamer design and chemistries in detail, relatively fewer studies consider the physio-ecological context of water-

borne microbes for sensing platforms. Since most aptamers and aptasensing systems described in the literature detects water-borne bacterial pathogens, properties of bacteria are discussed in more detail to illustrate the importance of considering the target's microbial characteristics for aptamer and aptasensor development.
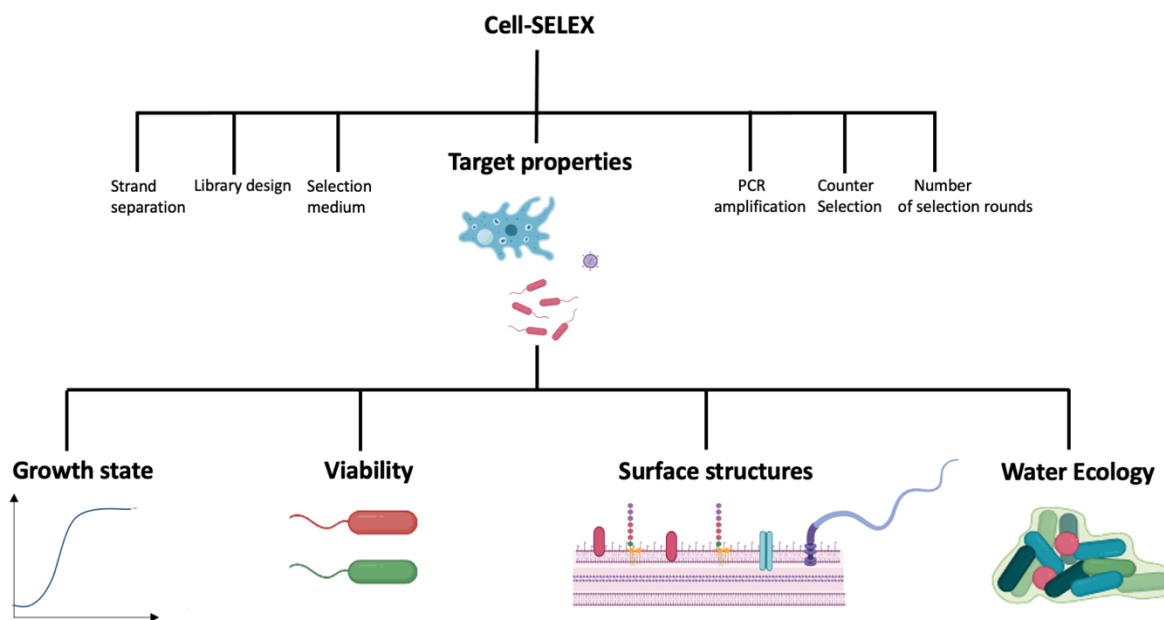
Figure 1. Factors affecting Cell-SELEX and thus the efficiency of aptamers targeting water-borne microbial pathogens. Most factors have been reviewed elsewhere, except the properties of the target, which are the topics of this review. Images were created in Biorender (https://biorender.com).

2.4 APTAMERS TARGETING MICROBES IN SPECIFIC STATES AND GROWTH CONDITIONS:

Protozoan microbes have varying life cycles which can alternate between metabolically active feeding states i.e trophozoites, or inactive, dormant states such as oocysts or cysts (Aguilar-Díaz, Carrero et al. 2011, Jain, Costa Melo et al. 2019). Both oocysts and cysts are infectious forms that persist for long periods of time in environmental waters and resist a wide range of stressors (Omarova, Tussupova et al. 2018). The *Cryptosporidium parvum* oocyst-specific aptamer R4-6 was thus developed using cell-SELEX (Table 1) (Iqbal, Labib et al. 2015). A counter selection

step against *Giardia duodenalis*, another protozoan commonly found in water samples (Ongerth 2013, WHO 2017) was included to enhance aptamer specificity. This aptamer was first used in multiple assay formats on electrochemical biosensing platforms to detect oocysts of *C. parvum* down to 50 oocysts in river and lake water samples (Iqbal, Labib et al. 2015, Iqbal, Liu et al. 2019). Recently, a fluorescence plate assay coupled with magnetic beads labelled with a truncated version of the aptamer R4-6, named Min_Crypto2 achieved a detection limit of 5 oocysts (Hassan, Dixon et al. 2021). The low LOD of this system is promising for oocyst detection in water given that the infectious dose of *C. parvum* is between 10 and 30 oocysts, (Jain, Costa Melo et al. 2019). Aptamer Min_Crypto2 was selective for *Cryptosporiudum* species, despite differences in size amongst species, but did not bind to *Giardia* oocysts. These features combined with its robust performance in water samples highlights its potential for oocyst detection in water.

Bacteria suspended in water are in a different metabolic state than bacteria growing in laboratory media. For example, *L. pneumophila* adopts a specific regulatory program when suspended in water due to starvation (Li, Mendis et al. 2015). Consequently, the aptamers R10C5 and R10C1 were created by cell-SELEX using *L. pneumophila* suspended in water for 24 hrs, to allow the bacterium to adopt the associated metabolic state (Table 1). Counter selection was performed on two *Pseudomonas spp* strains, prevalent in environmental waters (Paranjape, Bédard et al. 2020). Both aptamers have excellent specificity for *L. pneumophila* (Saad, Chinerman et al. 2020).

Water borne bacteria can also be biofilm-associated. These bacteria can gain adaptive traits which make it harder to eliminate or disinfect them. To that end, biofilm-derived *Pseudomonas aeruginosa* cells were used to select aptamers through Cell-SELEX, without counter selection (Soundy and Day 2017). The resulting aptamers were specific for 4 out of 5 clinical *Pseudomonas aeruginosa* isolates, minimally labelled non-*Pseudomonas* bacteria, and bound to both biofilm derived and planktonic *Pseudomonas* cells. The authors created chimeras and generated aptamers St17Lp21, St21Lp17.The chimeric aptamers showed improved binding and enhanced specificity for *Pseudomonas* bacteria as compared to the parent non-chimeric aptamers but were still unable to differentiate between biofilm and planktonic cells. This is not surprising since the biofilm-derived cells were washed and vortexed to release cells and remove alginate and exopolysaccharides. Mechanical stress induced by vortexing can destroy larger surface structures such as fimbriae and flagella. The lack of counter-selection coupled with the vigorous washing

steps may have exposed cell surface structures not unique to the biofilm-derived *Pseudomonas*. Using counter selection could have eliminated sequences that bind to surface structures such as LPS or ubiquitous OMPS that are common in both planktonic and biofilm-derived *Pseudomonas*.

Aptamers against *Yersinia enterocolitica* were generated using Cell-SELEX with bacteria grown at, 26 °C (Shoaib, Shehzad et al. 2020). After counter selecting with several bacterial pathogens, the three aptamers M1, M5 and M7 were isolated (Table 1). *Y. enterocolitica* grown at 37 °C showed reduced binding by the aptamers compared to bacteria grown at 25 °C. Presumably this aptamer is specific for a cell surface component mostly expressed at low temperature. This study illustrates another characteristic of bacteria, which are temperature dependent surface structure and morphological changes. In the case of *Y. enterocolitica* specifically, the bacterium inhibits flagellum synthesis at 37 °C (Kapatral, Olson et al. 1996). Components of the LPS are also temperature regulated (Białas, Kasperkiewicz et al. 2012).

2.5 APTAMERS TARGETING VIABLE CELLS:

The ability to differentiate between dead and viable cells has important implications when assessing the risk or hazard of a microbe. For example, it would be costly and inefficient to administer shutdowns or disinfection protocols for the presence of dead pathogens in a system. The detection of viable cells is also important to determine the efficacy of water disinfection protocols. Some aptamers are able to differentiate between live and dead cells. Aptamer 33, specific for *Salmonella enterica* serovar Typhimurium, does not bind heat-killed cells (Table 1) (Joshi, Janagama et al. 2009). This aptamer might therefore be useful for monitoring the efficiency of heat-based disinfection. This aptamer is described in more detail below. Another example is aptamer ONS-23 created against whole cell *C. jejuni* (Table 1) (Dwivedi, Smiley et al. 2010). This aptamer was developed, using cell-SELEX, against a chicken isolate showing characteristic *C. jejuni* morphology (Thomas, Hill et al. 2002). Given that *C. jejuni* is found on raw poultry as well as in the gastrointestinal tract and feces of animals (Mughini-Gras, Penny et al. 2016), 20 bacterial species were used for counter selection, including food-borne pathogens, enteric bacteria, non-enteric bacteria and lactic acid bacteria. ONS-23 is therefore highly specific to *C. jejuni* strains showing minimal binding to non-*C. jejuni* strains (Dwivedi, Smiley et al. 2010). Furthermore,ONS-23 does not bind non-viable *C. jejuni* (Kim, Kim et al. 2018) indicating that it

is specific for a surface structure only present on live *C. jejuni* cells (Kim, Kim et al. 2018). Though this aptamer was not tested for water application, its selective properties for viable *C. jejuni* makes it promising for monitoring disinfection processes.

2.6 APTAMERS TARGETING SOURCE- OR APPLICATION-SPECIFIC ISOLATES:

Isolates that are representative of the sample source of the downstream application should be used during aptamer development to ensure usefulness of the aptasensor. Aptamers E1, E2 and E10 were generated against a non-pathogenic *E.coli* strain of fecal origin (Crooks strain) using cell-SELEX (Table 1) (Kim, Song et al. 2013). For counter selection a combination of fecal coliform species and two Gram positives were used. The resulting aptamers were better at binding *E. coli* isolates of fecal origin than others and showed low binding to other species including laboratory strains of *E. coli* (Kim, Song et al. 2013, Jin, Wang et al. 2017, Wu, Dai et al. 2017). A detection system using aptamer E2 was able to detect the Crooks strain in spiked tap water, pond water and milk, making it promising for *E. coli* detection in water (Jin, Wang et al. 2017).

2.7 APTAMERS TARGETING SPECIFIC SURFACE STRUCTURES:

Surface structures can be differentially expressed in response to growth states and environment (Justice, Hung et al. 2004, Van Der Woude and Bäumler 2004, Liu, Hu et al. 2012, Fonseca and Swanson 2014, Li, Mendis et al. 2015). If the aptamer surface target is not differentially regulated then aptamers may bind cells in several conditions, including exponential and post-exponential phase. Examples of these are the ST2P aptamer against whole cell *S. enterica* Typhimurium (Duan, Wu et al. 2013, Duan, Wu et al. 2014, Duan, Chang et al. 2016) and the *E. coli* E2 aptamer (Kim, Song et al. 2013, Jin, Wang et al. 2017, Wu, Dai et al. 2017). Instead of whole cells, surface structures related to virulence can also be used as aptamer targets. The pathotype EHEC (*E. coli* enterohaemorrhagic) contains the infamous O157:H7 serotype which is strongly linked to deadly outbreaks from contaminated drinking water (Solomon, Yaron et al. 2002, Ali 2004, Saxena, Kaushik et al. 2015). For detecting this serotype, the specific variant of LPS can be exploited. *E. coli* aptamers a-aptamer and c-aptamer were created against LPS of *E. coli* O157:H7 (Table 1)

(Bruno and Chanpong 2009). These aptamers were used in several aptasensing platforms to detect whole *E. coli* O157:H7 cells with great specificity, showing minimal signals with other serotypes (Wu, Zhao et al. 2015, Díaz-Amaya, Lin et al. 2019, Díaz-Amaya, Zhao et al. 2019, Hao, Yeh et al. 2019, Jiang, Qiu et al. 2020). The aptamers could bind to heat-killed and formalin killed *E. coli* (Hao, Yeh et al. 2019, Jiang, Qiu et al. 2020). This is likely due to the fact that these treatments do not negatively affect the LPS (Gao, Wang et al. 2006, Chafin, Theiss et al. 2013). This approach allowed for very specific aptamers to be developed; however, since the target persists after killing of cells, the aptamers are of limited use for monitoring the efficacy of disinfection programs in water. This illustrates the need for designing aptamers relevant to the downstream application.

Outer membrane proteins (OMP) of Typhimurium were used to create Aptamer 33. Counter selection was done with purified LPS of the *Salmonella* isolate as well as OMPs and LPS from *E. coli*. Aptamer 33 showed pan-serovar specificity, binding to 7 different serovars of *S. enterica* in one study and 4 different *S. enterica* serovars in another study (Joshi, Janagama et al. 2009, Hasan, Pulingam et al. 2018). The aptamer was used in a fluoresence aptasensor to detect whole Typhimurium in water samples from different sources highlighting its potential for detection in water (Duan, Wu et al. 2012). The aptamer does not bind to heat-killed Typhimurium which is to be expected as most OMPs are heat labile (Oh, Heo et al. 2017). The authors also observed that the aptamers could not bind *S.* enterica serovars Tennessee and Muenchen. This suggests that the aptamer may not have broad serovar specificity.

2.8 DISCUSSION:

Aptamer-coupled biosensors are promising systems for the detection of pathogens in water samples but are limited in real-world applications. There are a few things to consider to improve aptamers practicality in aptasensing technology. Many studies do not explicitly report the growth states and conditions used during cell-SELEX or during subsequent testing of the aptamers (Table 1 and supplementary Table1). For example, OD_{600} values are meaningless without details about the growth conditions, including medium, temperature and aeration. We suggest that instead of reporting OD_{600}, the growth phase should be determined and reported, as done by (Zou, Duan et al. 2018), as this would offer insight into an aptamer's potential for specific applications. Regardless, it is important to keep the end goal in mind while developing aptamers. For example,

monitoring efficiency of disinfection program will require discerning viable cells from dead cells. Aptamer ONS23 and Aptamer 33 are able to distinguish between live and dead cells (Dwivedi, Smiley et al. 2010, Kim, Kim et al. 2018) (Joshi, Janagama et al. 2009, Oh, Heo et al. 2017). A cell-SELEX strategy for such an application could use dead cells for counter selection. Another factor to consider is the physio-morphological state of microbes. This ensures that the microbial target possesses traits and characteristics that are representative of what's typically found in the environment that will be sampled. For example, biofilm-derived cells might be used (Soundy and Day 2017), but care must be taken not to remove the biofilm-specific target when preparing the target for cell-SELEX. Alternatively, if the end goal is to detect pathogens in water, then bacteria suspended in water may be used as the target (Saad, Chinerman et al. 2020). Lastly, it is not trivial to select appropriate strains for counter selection. This will impact aptamer affinities for targets in source environments. A possible approach is to use a cocktail of strains for the target species and a cocktail of species typically found in the same environment for counter-selection (Dwivedi, Smiley et al. 2010, Kim, Song et al. 2013). In conclusion, it is necessary to better elucidate the microbial target and the limitation of its cognate aptamer to help push microbial aptasensing platforms to market. As such a collaborative effort is needed between academics and stakeholders (governments, industry, engineers) to develop both transducer and aptamer technologies for specific microbial contaminants in the context of source water, taking into account the particularities of the microbe and its physiological state.

2.9 CONFLICT OF INTEREST

The authors, together with Maryam Tabrizian (McGill University, Department of Biomedical Engineering), are the inventors of aptamers R10C1 and R10C5, subject of patent applications filed in USA, patent application no US 16/850,355; and in Canada – patent application number pending at the time of revised manuscript submission. At the time of submission of the manuscript, the applications were under review. The authors declare no other competing interest.

2.10 AUTHOR CONTRIBUTIONS

MS reviewed the literatures and compiled the information reported here. MS wrote the first draft of the manuscript. MS and SPF edited the manuscript. All authors approved submission of the manuscript.

2.11 FUNDING

Table 1: Aptamers developed against water-borne bacteria

Aptamer Name	Target	Culture condition[1]	OD/ Growth stage[1,2]	Counter-Selex Strains[3]	Type of Sensors	LOD	Reference
Norovirus							
AG3	MuNoV	*NA*	*NA*	feline calicivirus (FCV)	Electrochemical	180 virus particles	(Giamberardino, Labib et al. 2013)
				NA	Optical (colorimetric)	200 virus/ml	(Weerathunge, Ramanathan et al. 2019)
Aptamer 25/SMV-25	SMV	*NA*	*NA*	HuNoV-negative human stool suspension, bead-antibody complex	*NA*	*NA*	(Escudero-Abarca, Suh et al. 2014)
	Non-toxic norovirus GII capsid recombinant			*NA*	Optical (Chemiluminescence)	80 ng/ml	(Kim, Chung et al. 2018)
Aptamer 21/SMV-21	SMV	*NA*	*NA*	HuNoV-negative human stool suspension, bead-antibody complex	*NA*	*NA*	(Escudero-Abarca, Suh et al. 2014)
	Norovirus Group II (recombinant VLP)			*NA*	Electrochemical	100 pM	(Chand and Neethirajan 2017)
C.parvum							
R4-6	Oocysts	*NA*	*NA*	*Giardia duodenalis* cysts	Electrochemical	100 oocysts	(Iqbal, Labib et al. 2015)
				NA	Electrochemical	50 oocysts	(Iqbal, Liu et al. 2019)
Min_Crypto2	Oocysts	*NA*	*NA*	*NA*	Optical Fluorescence	5 oocysts	(Hassan, Dixon et al. 2021)
Acinetobacter							
Aci49	whole-cell-*A. baumannii* (ATCC 19606)	BHI broth, 37 °C, overnight	0.4/E	*Acinetobacter lwoffii*, *Acinetobacter calcoaceticus*, and 11 species	Optical (colorimetric)	10^3 CFU/ml	(Rasoulinejad and Gargari 2016)
				NA	Optical (Fluorescence)	3 CFU/ml	(Li, Yang et al. 2020)
				NA	Optical (Fluorescent)	10 CFU/ml	(Yang, Guo et al. 2020)
AB aptamer	whole-cell *A. baumannii*	**NR**	**NR**	**NR**	Optical (Colorimetric)	450 CFU/rxn	(Wu, Wang et al. 2018)
				NA	Optical (Fluorescence)	10^5 CFU/ml	(Su, Tsai et al. 2020)
				NA	Optical (Fluorescence)	100 CFU/ml	(Su, Tsai et al. 2020)

				NA	Optical (Fluorescence)	300 CFU/ml	(Bahari, Babamiri et al. 2021)
Aeromonas							
Apt1	whole-cell (*A. hydrophila*)	LB,37 °C, 18hrs	**NR**	**NR**	Optical (Fluorescence)	1.5 CFU/ml	(Zhu, Zhang et al. 2019)
Campylobacter							
Aptamer C2 and Aptamer C3	surface protein (*C. jejuni*)	**NR**	**NR**	**NR**	Optical (Fluorescence)	2.5 CFU/ml	(Bruno, Phillips et al. 2009)
				NA	Optical (colorimetric)	5-10 CFU/ml	(Bruno and Sivils 2017)
ONS-23	whole-cell (*C. jejuni* A9a)	BBL brucella broth, 42 °C, 48 hours, microaerophillic conditions	PE*	20 strains (enteric,non-enteric, lactic acid)	*NA*	*NA*	(Dwivedi, Smiley et al. 2010)
				NA	Optical (colorimetric)	10 CFU/ml	(Dehghani, Hosseini et al. 2018)
				NA	Optical (colorimetric)	7.2×10^5 CFU/ml	(Kim, Kim et al. 2018)
CJA1	whole-cell (*C. jejuni*)			**NR**	Optical (colorimetric)	10 CFU/ml	(Chen, Teng et al. 2020)
Cyanobacteria							
ATX8	anatoxin-a (ATX)	*NA*	*NA*	ATX free beads	Electrochemical	0.5nM	(Elshafey, Siaj et al. 2015)
MC-LR aptamer/AN6	Microcystin-LR	*NA*	*NA*	blank sepharose beads	Electrochemical	10pM	(Ng, Chinnappan et al. 2012)
				NA	Optical (Fluoresence)	0.002 ng/ml	(Lv, Zhao et al. 2017)
E.coli							
L9F	O111-LPS (*E. coli* O111:K58)	35 °C, TSB, overnight	**NR**	**NR**	*NA*	*NA*	(Bruno, Carrillo et al. 2008)
				NA	Electrochemical	112 CFU/ml	(Luo, Lei et al. 2012)
Eco4R/ECAII	Outer membrane protien (OMP) - *E. coli* 8739	37 °C, blood agar, overnight	**NR**	**NR**	*NA*	*NA*	(Bruno, Carrillo et al. 2010)
				NA	Electrochemical	**NR**	(Queirós, de-los-Santos-Álvarez et al. 2013)
Eco4F	OMP- *E. coli* 8739	37 °C, blood agar, overnight	**NR**	**NR**	*NA*	*NA*	(Bruno, Carrillo et al. 2010)

				NA	Optical (colorimetric/fluoresence)	300 CFU/ml	(Bruno 2014)
Eco3R/ECAI	OMP- *E. coli* 8739	37 °C, blood agar, overnight	**NR**	**NR**	NA	NA	(Bruno, Carrillo et al. 2010)
					NA	Electrochemical	**NR**	(Queirós, de-los-Santos-Álvarez et al. 2013)
				NA	Optical (colorimetric/fluoresence)	300 CFU/ml	(Bruno 2014)
				NA	Optical (Evanescent wave fiber optics)	0.1nM	(Queirós, Gouveia et al. 2014)
E1	whole cell (*E. coli* fecal isolate)	NB, 37 °C	0.45/E	*E. coli* (non-fecal isolate), other fecal isolates	NA	NA	(Kim, Song et al. 2013)
E2	whole cell (*E. coli* fecal isolate)	NB, 37 °C	0.45/E	*E. coli* (non-fecal isolate), other fecal isolates	NA	NA	(Kim, Song et al. 2013)
				NA	Optical (fluorescence)	3 CFU/ml	(Jin, Wang et al. 2017)
				NA	Electrochemical	100 CFU/ml	(Wu, Dai et al. 2017)
E10	whole cell (*E. coli* fecal isolate)	NB, 37 °C	0.45/E	*E. coli* (non-fecal isolate), other fecal isolates	NA	NA	(Kim, Song et al. 2013)
E1+E2+E10 (pooled)				NA	Electrochemical	371 CFU/ml	(Kim, Chung et al. 2014)
AptB12	whole cell (*E. coli* ETEC K88)	LB	E	ETEC K99, *S. enteritidis, S. aureus*,	Optical (Fluorescence)	1.1×10^3 CFU/ml	(Peng, Ling et al. 2014)
RNAaptamer	**NR**	LB, 37 °C, 2-3 hrs	**NR**	NA	Electrochemical	**NR**	(So, Park et al. 2008)
				NA	immunomagnetic separation and RT-PCR	10 CFU/ml	(Lee, Kim et al. 2009)
				NA	Electrochemical	6-26 CFU/ml	(Zelada-Guillén, Bhosale et al. 2010)
Aptamer I-1	O-antigen LPS (*E. coli* O157:H7)	Brucella broth,37 °C ,24 h (+0.04% formaldehyde)	**NR**	*E. coli* K12	NA	NA	(Lee, Han et al. 2012)
				NA	Electrochemical	4 CFU/ml	(Burrs, Bhargava et al. 2016)
Ec3(31)	whole cell (*E. coli* DH5α)	LB	0.4	*B. subtilis*	Electrochemical	2×10^4 CFU/ml	(Dua, Ren et al. 2016)

P12-31	whole cell (*E. coli* O6)	37 °C, LB	0.3	**NR**	*NA*	*NA*	(Marton, Cleto et al. 2016)
AM-6	whole cell (*E. coli* O157:H7)	LB	0.6	*E. coli* strains O42, K12, Top10, DH5α, *S. flexneri, S. Typhi*	*NA*	*NA*	(Amraee, Oloomi et al. 2017)
S1	whole cell (*E. coli* O157:H7)	BHI, 37 °C	E	*S. aureus, S. Typhyimurium, L. monocytogens*	Mechanical (Quartz Crystal Microbalance-QCM)	1.46 × 10³ CFU/ml	(Yu, Chen et al. 2018)
Apt-5	whole cell (*E. coli* O157:H7)	LB, 37 °C	**NR**	*E. coli* ETEC and 3 other species	*NA*	*NA*	(Zou, Duan et al. 2018)
a-aptamer/E-17F72*	O157:H7 LPS	LB, 37 °C	**NR**	**NR**	*NA*	*NA*	(Bruno and Chanpong 2009)
c-aptamer/E-18R72*	O157:H7 LPS	LB, 37 °C	**NR**	**NR**	*NA*	*NA*	(Bruno and Chanpong 2009)
a-aptamer, c-aptamer				*NA*	Optical (colorimetric)	10 CFU/ml	(Wu, Zhao et al. 2015)
a-aptamer, c-aptamer				*NA*	Optical (colorimetric)	25 CFU/ml	(Díaz-Amaya, Zhao et al. 2019)
a-aptamer, c-aptamer				*NA*	Optical (Surface Enhanced Raman Spectroscopy - SERS)	100 CFU/ml	(Díaz-Amaya, Lin et al. 2019)
c-aptamer				*NA*	Optical (fluorescence)	100 CFU/ml	(Hao, Yeh et al. 2019)
				NA	Optical (fluorescence)	80 CFU/ml	(Jiang, Qiu et al. 2020)
Helicobacter pylori							
Hp-Ag aptamer	recombinant Hp surface antigen	**NR**	**NR**	BSA	*NA*	*NA*	(Gu, Yan et al. 2018)
Hp4	recombinant Hp surface antigen	blood agar, 37 °C, 3 days	**NR**	BSA	*NA*	*NA*	(Yan, Gu et al. 2019)
Legionella							
R10C5, R10C1	whole cell (*Lp* 120292)	CYE agar plate, 37 °C, 3 days followed by AYE media,37 °C, 24hr	2.5/PE	*Pseudomonas putida* KT2440, *Pseudomonas fluorescens* LMG1794	*NA*	*NA*	(Saad, Chinerman et al. 2020)
NTM							
BM2/N31	ManLAM, *M.bovis* (BCG)	L-J medium	E	**NR**	Optical (ELONA)	10⁴ CFU/ml	(Sun, Pan et al. 2016)
					Electrochemical	**NR**	(Sodia, Poch et al. 2020)
Pseudomonas aeruginosa							

F23	whole cell (*P. aeruginosa* clinical isolate)	Mueller-Hinton (MH) media, 37 °C, 24 hrs	**NR**	*S. maltophilia ,A. baumannii*	Optical (fluoresence)	**NR**	(Wang, Zeng et al. 2011)
				NA	Optical (fluorescence)	100 CFU/ml	(Gao, Zhong et al. 2018)
				NA	Optical (Long range Surface Plasomon Resonance-LSPR)	1 CFU/ml	(Hu, Fu et al. 2018)
				NA	Optical (Fluoresence)	1 CFU/ml	(Zhong, Gao et al. 2018)
				NA	Electrochemical and Optical (colorimetric)	60 CFU/ml	(Das, Dhiman et al. 2019)
				NA	Electrochemical	33 CFU/ml	(Roushani, Sarabaegi et al. 2019)
				NA	Mechanical (piezoelectric quartz crystal)	9 CFU/ml	(Shi, Zhang et al. 2019)
St17Lp21, St21Lp17, St08Lp17	biofilm-derived whole cells (PA 692 /ATCC 14502)	LB broth, 37 °C, 16 hrs followed by 22 °C, 42 hours to make biofilm.	E	**NR**	*NA*	*NA*	(Soundy and Day 2017)
F23+St08Lp17 (pool)				*NA*	Optical (Fluoresence)	1 CFU/ml	(Zhong, Gao et al. 2020)
Salmonella							
aptamer 33	OMP (*S. tyhpimurium* PT10)	BHI, 37 °C, 2-3 hrs		*E. coli* OMP and LPS, *Salmonella* LPS	Magentic bead based pull down assay and qPCR	10-100 CFU/ml	(Joshi, Janagama et al. 2009)
				NA	Optical (Fluoresence)	5 CFU/ml	(Duan, Wu et al. 2012)
				NA	Electrochemical	3 CFU/ml	(Ma, Jiang et al. 2014)
				NA	Electrochemical	55 CFU/ml	(Hasan, Pulingam et al. 2018)
				NA	Optical (LSPR)	30 CFU/ml	(Yoo, Kim et al. 2015)
				NA	Optical (LSPR)	10^4 CFU/ml	(Oh, Heo et al. 2017)
ST2P	whole cell (*S. typhimurium* ATCC 50761)	BBL-BHI, 37 °C, overnight	0.3/E	*L. monocytogenes, E. coli, S. aureus, S. pneumoniae, V. parahemolyticus, C. sakazakii*	Optical (Fluoresence)	25 CFU/ml	(Duan, Wu et al. 2013)

					Optical (Colorimetric, SERS)	10 CFU/ml	(Duan, Chang et al. 2016)
				NA	Optical (Fluorescence)	25 CFU/ml	(Duan, Wu et al. 2014)
S8-7	whole cell (*S. typhimurium* S913)	TSB-amp, 37C, overnight	**NR**	*L. monocytogenes* Scott A *E. coli O157: H7, B. cereus, E. faecalis*	*NA*	*NA*	(Dwivedi, Smiley et al. 2013)
C4	whole cell (*S. typhimurium*)	BHI, 35 °C, overnight	**NR**	*E. coli, S. enteritidis, S. aureus*	*NA*	*NA*	(Moon, Kim et al. 2013)
Apt22	whole cell (*S. paratyphi* A)	NB,37C	2.1/E	*S. Enteritidis, S. Typhimurium, S. Cholerasuis, S. Arizonae*	Optical (chemiluminescence)	1000 CFU/ml	(Yang, Peng et al. 2013)
S25	whole cell (*S. enteriditis-*multiple)	TSB, overnight	**NR**	*Salmonella* serovars-multiple			(Hyeon, Chon et al. 2012)
SAL26	whole cell (*S. typhimurium* ATCC14028)	TSB,37 °C, overnight culture followed by TSB,37 °C, 3 hours then fixing with methanol	E	4 *Salmonella enterica* serovars and 9 bacterial species.	Optical (Colorimetric)	100 CFU/ml	(Lavu, Mondal et al. 2016)
SAL1	whole cell (*S.paratyphi-*A ATCC 9150)	LB broth, 37 °C	E	*S. Typhimurium, S .flexneri, E. coli O157:H7, Yersinia enterocolitica*	Optical (fluorescence)	10 CFU/ml	(Rm, Maroli et al. 2020)
B5	whole cell (*S. typhimurium* ATCC14028)	BHI broth, 37 °C	PE	*S. aureus, L. monocytogenes, E. coli O157:H7*	Mechanical (QCM)	1000 CFU/ml	(Wang, Wang et al. 2017)
Shigella							
Aptamer S 1	whole cell (*Shigella dysenteriae*)	LB	E	*S .aureus, S. typhimurium, E. coli, L. monocytogenes, V. parahaemolyticus*	Optical (Fluorescence)	50 CFU/ml	(Duan, Ding et al. 2013)
				NA	Electrochemical	1 CFU/ml	(Zarei, Soleimanian-Zad et al. 2018)
Sp1	whole cell (*Shigella sonnei* ATCC 51334)	LB, 37 °C, overnight	**NR**	*S. dysenteriae, S. flexneri, S. boydii, S. typhimurium, E. coli*	Optical (fluorescence)	30CFU/ml	(Gong, Duan et al. 2015)
				NA	Optical (SERS)	10 CFU/ml	(Wu, Duan et al. 2020)
Sp20	whole cell (*Shigella sonnei* ATCC 51334)	LB, 37 °C, overnight	**NR**	*S. dysenteriae, S. flexneri, S. boydii, S. typhimurium and E. coli*	Optical (Fluorescence)	30 CFU/ml	(Gong, Duan et al. 2015)

S.flexneri aptamer1	whole cell (*Shigella flexneri*)		**NR**	**NR**	Optical (fluorescence)	100 CFU/ml	(Zhu, Li et al. 2015)
SS-3,SS-4	whole cell (*Shigella sonnei*)	NB,37 °C	**NR**	*E. coli*	Optical (Fluoresence)	1000 CFU/ml	(Song, Sekhon et al. 2017)
S.flexneri aptamer`	whole cell (*Shigella flexneri* ATCC 12022)	NB,37 °C, 12 hrs	**NR**	**NR**	Optical (colorimetric)	80 CFU/ml	(Feng, Shen et al. 2019)
Vibrio cholerae							
CT916	Cholerae toxin	*NA*	*NA*	ethanolamine-blocked magnetic beads	Optical (colorimetric)	2.1 ng/ml	(Frohnmeyer, Frisch et al. 2018)
				NA	Optical (colorimetric)	1-100ng/ml	(Frohnmeyer, Tuschel et al. 2019)
	whole cell (*V. cholerae* O1 - Inaba, ATCC 39315 and Ogawa)	LB broth, 37 °C	0.4/E	*E. coli O157:H7, S.a dysenteriae, S. enteritidis, S. Typhimurium, Yersi nia spp., S.flexneri.*	Optical (colorimetric)	10^4 CFU/ml	(Mojarad and Gargaria 2020)
Yersinia							
N30yc5, N71yc2	recombinant Yop51	**NR**	**NR**	**NR**	*NA*	*NA*	(Bell, Denu et al. 1998)
M1, M5, M7	whole cell (*Yersinia entercolitica*)	Specific media (NaCl, beef extract, peptone, pH 7.2-7.4), 26 °C	0.3 (L), 0.6 (E), 0.9 (PE)	*B. cereus, S. dysenteriae, L. monocytogenes, S. typhimurium, S. aureus, and E. coli*	*NA*	*NA*	(Shoaib, Shehzad et al. 2020)

NR : Not Reported, NA: Not applicable, *: Extrapolated from culture conditions

1) Microbial culture conditions and growth conditions are listed for aptamer development only. The
   Microbial culture and growth conditions used for aptasensors development are listed in TableS1

2) State: L, lag phase; E, exponential; PE, post-exponential.

3) If number of strains used for counter selection is higher than to 5, they are listed in Table S1.

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CHAPTER 3: CONNECTING TEXT

Taking into consideration the source environment and certain physiological traits of the microbial target, *Legionella pneumophila*, we performed Cell-SELEX to identify aptamers binding to the bacterium.

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Identification of two aptamers binding to *Legionella pneumophila* with high affinity and specificity

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3.1 ABSTRACT

Legionella pneumophila (*Lp*) is a water borne bacterium causing Legionnaires' Disease (LD) in humans. Rapid detection of *Lp* in water system is essential to reduce the risk of LD outbreaks. The methods currently available require expert skills and are time intensive, thus delaying intervention. In situ detection of *Lp* by biosensor would allow rapid implementation of control strategies. To this end, a biorecognition element is required. Aptamers are considered promising biorecognition molecules for biosensing. Aptamers are short oligonucleotide sequence folding into a specific structure and are able to bind to specific molecules. Currently, no aptamer and thus no aptamer-based technology exists for the detection of *Lp*. In this study, Systemic Evolution of Ligands through EXponential enrichment (SELEX) was used to identify aptamers binding specifically to *Lp*. Ten rounds of positive selection and two rounds of counter-selection against two *Pseudomonas* species were performed. Two aptamers binding strongly to *Lp* were identified with K_D of 116 and 135 nM. Binding specificity of these two aptamers to *Lp* was confirmed by flow cytometry and fluorescence microscopy. Therefore, these two aptamers are promising biorecognition molecules for the detection of *Lp* in water systems.

Key words: *Legionella pneumophila*, aptamers, SELEX, flow cytometry, fluorescence microscopy, *Pseudomonas*

3.2 INTRODUCTION

Legionella pneumophila (Lp) is a pathogenic Gram-negative bacterium responsible for two types of respiratory diseases, namely the severe pneumonia Legionnaires' Disease (LD) and the milder flu-like Pontiac fever [34]. *Lp* occurs in both natural and engineered water systems and is one of the most prevalent pathogens in man-made, engineered water systems [26]. Infections occur when the bacteria are aerosolized, and the contaminated aerosols are inhaled, at which point *Lp* can then infect and replicate inside alveolar macrophages[3]. Modern water systems provide optimal transmission conditions for *Lp* by generating aerosols [40]. Leading sources of infection are cooling towers, hot water distribution systems, humidifiers, misters, showers, fountains, spa pools and evaporative condensers [51].

Outbreaks of LD occur consistently globally and have increased in recent years. The average incidence rate is about 10-15 cases per million people[32]. According to the Centre for Disease Control, incidences of legionellosis have increased by four and a half times between 2000 and 2016 [39]. The Public Health Agency of Canada reports a 485% increase in the rate per 100,000 Legionellosis cases between the years 2000 to 2017 [6]. The rise in LD outbreaks can be attributed to several factors such as aging infrastructures and an aging population who is more vulnerable to such infections, as well as an increase in diagnosis and reporting of LD [40,13]. Most LD outbreaks, however, are the result of mismanagement of man-made water systems[41]. Examples of mismanagement of water distribution systems include keeping the temperature of the water below 50°C and allowing water to stagnate[41]. In the case of cooling towers, a lack of regular cleaning and disinfection is associated with an increased risk of *Lp* spread[41]. In both cases, routine monitoring of *Lp* is critical to evaluate risk, initiate treatment of water systems, and prevent outbreaks[41]. The European Center of Disease Control (ECDC) specifies that immediate corrective measures must be taken when *Lp* levels reach a value of 10,000 CFU/L [19].

Currently, there are two ISO-certified strategies to detect *Lp* from water systems: the standard plate count method (AFNOR NF T90-431, ISO 11731) and qPCR (AFNOR NF T90-471, ISO/TS 12869). The plate count method is the gold standard for detecting *Lp* and involves its cultivation on selective media and the enumeration of bacterial colonies showing *Lp*-specific morphology [5,8,22]. The whole procedure takes up to 14 days which delays the application of corrective measures and increases the chances of an outbreak[49]. A pilot study performed in 2011 evaluated the

consistency of the results obtained by this method between several different laboratories. Qualitative results did not differ drastically between laboratories, but quantitative results showed large variation, within and between laboratories[31]. Therefore, the culture method should be used with caution to precisely enumerate *Lp*. A second major limitation is the presence of viable but non-culturable (VBNC) *Lp* cells which leads to an underestimation of the true amount of infectious *Lp* in a system [29,18]. The qPCR method relies on the quantification of *Legionella* DNA. Its major advantages in comparison with conventional culture method is the rapid turn-around time, high sensitivity and specificity, low limit of detection, as well as the ability to detect VBNC cells. When used in conjunction with the culture method, qPCR can serve as a powerful tool. There are, however, several drawbacks: qPCR typically overestimates *Lp* burden because it detects dead cells and the presence of PCR inhibitors may limit the use of this method [12,48]. In addition, multiple processing steps are required which increases the overall cost of the qPCR method[52]. Unfortunately, it is impossible to develop these two methods into rapid, cost-effective, sensitive tests that would detect *Lp* in real-time, on-site, without any additional processing steps[27,11].

Biosensors are attractive detection technology that could address the problems associated with culture-based bacterial detection methods. These analytical devices are commonly used to assess and quantify in real-time, with high sensitivity, the presence of an analyte such as a protein, peptide or cell in a fluid[1]. However, a biosensing approach to *Lp* detection would require a specific biorecognition element, which, when coupled with a transducer, translates its interaction with *Lp* cells into a meaningful readout[1].

Various biorecognition elements, such as antibodies, lectins or aptamers, can be used. The latter are becoming the primary choice for biosensing strategies due to their easily modifiable nature and versatility[54,10]. Aptamers are antibody analogues. They are short single stranded DNA or RNA oligonucleotides that can be cost effectively synthesized in a high throughput manner. The aptamer folds into a specific, stable structure and can interact with its targets via shape complementarity, hydrogen bonding, electrostatic interactions and stacking interactions[20]. This allows aptamers to bind with high affinity and specificity to a wide variety of targets ranging from small molecules, peptides, proteins to whole cells[20]. A key characteristic of aptamers is the possibility to generate them *in vitro* in the same condition as those used for detecting the analyte. This is a clear advantage over antibodies which are produced under strict physiological conditions [25]. In addition, aptamers

can be easily modified and therefore be optimized for various sensing platforms such as lateral flow assays, surface plasmon resonance sensors, flow cytometry or fluorescence microscopy [42].

The procedure by which an aptamer is created is known as Systemic Evolution of Ligands through EXponential enrichment (SELEX). Developed in 1990 by the teams of Gold and Szostak[50,16], SELEX is an iterative process which involves incubating a target with a large library of oligonucleotides, separating the target bound and unbound oligonucleotides and then amplifying the target bound sequences via PCR for the next round of selection. The selection rounds are repeated until the oligonucleotide pool is enriched with sequences that bind specifically and with high affinity to the target[16]. Over the years, many variations of the original SELEX methods were published. Among those, one is particularly useful for the present study. Cell-SELEX can be used to select aptamers binding to whole living cells and, thus, eliminate the need for prior knowledge of a target molecule [9,23]. Rounds of counter-selection are typically used to reduce aptamer cross-reactivity across targets by eliminating non-specific aptamers[42]. Cell-SELEX has been successfully employed to isolate aptamers against various bacterial species such as *E. coli*, *Salmonella typhimurium, Campylobacter jejuni, Listeria monocytogenes, Staphylococcus aureus* and *Vibrio parahaemolyticus* [25,14,33,15,37,23,2]. Several of these aptamers are used in conjunction with optical, mechanical or electrical/electrochemical biosensors to mitigate the problems associated with traditional bacterial detection methods. Although numerous works have been done to detect *Lp* with the use of biosensors [53,35,17] no aptamers binding to *Lp* have been reported yet. Consequently, no aptamer and thus no aptamer-based technology currently exists for the detection of *Lp*.

In this work, the cell-SELEX procedure was employed to generate aptamers binding to *Lp*. Two *Pseudomonas* species were used for counter-selection to improve the specificity of the aptamers. These species were chosen because they are γ-proteobacteria like *Lp* and are routinely found in premise plumbing and water systems where *Lp* is prevalent [46,24]. Two aptamers were identified and their binding affinity and specificity for *Lp* were evaluated by flow cytometry and fluorescence microscopy.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial Strains and Culture Conditions

The environmental *Lp* strain *lp120292*, isolated from a cooling tower implicated in the 2012 outbreak in Quebec City, was used as the target strain for aptamer generation[28]. The strain Lp*GFP is *lp120292* transformed with plasmid pXDC31 expressing the green fluorescent protein (GFP) under the *Ptac* promoter[21]. The thymidine auxotroph *Lp* strain Lp02, derived from *Lp* Philadelphia-1 was used to confirm binding of the aptamers[4]. *Lp* was cultured on CYE (ACES-buffered charcoal yeast extract) agar plates supplemented with 0.25 mg/ml L-cysteine and 0.4 mg/ml ferric pyrophosphate, at 37 °C for 3 days. Lp*GFP strain was grown on CYE media supplemented with 5 µg/ml chloramphenicol and 1 mM isopropyl β-D-1-thiogalactopyranoside. For liquid culture, *Lp* was suspended in AYE (ACES-buffered yeast extract) broth supplemented with 0.25 mg/ml L-cysteine and 0.4 mg/ml ferric pyrophosphate until post-exponential phase (OD_{600} of 2.5). *Pseudomonas putida* KT2440 and *Pseudomonas fluorescens* LMG1794, were first cultured on LB agar plates at 30 °C for 24 hours and then grown in LB medium (Difco) until the cultures reached post-exponential phase (OD_{600} of 2.0). *Pseudomonas sp., Brevundiomonas sp., Bacillus sp., Staphylococcus sp. Sphingomonas sp., Stenotrophomonas sp. and Cupriavidus sp.* were isolated from cooling towers as part of a different study (Paranjape, in preparation). Briefly, the bacteria were isolated on nutrient agar incubated at 30 °C for 24h. Isolates were then classified using 16S rDNA sequencing. These strains were first cultured on nutrient agar plates (Difco) at 30 °C for 24 hours and then grown in nutrient broth medium (Difco) overnight until the cultures reached post-exponential phase (OD_{600} of 2.0-2.5).

3.3.2 Oligonucleotides Library and Primers

A random ssDNA library of 10^{15} sequences was chemically synthesized and purified by HPLC (Integrated DNA Technologies). The library consisted of a central random region of 45 nucleotides flanked by two different primer binding regions at the 5' and 3' ends: 5'-GCAATGGTACGGTACTTCC-45N-CAAAAGTGCACGCTACTTTGCTAA-3'. The forward and reverse primers were conjugated with fluorescein (FITC) and biotin, respectively. The forward primer (FP) sequence is 5'-fluorescein-GCAATGGTACGGTACTTCC-3'. The reverse primer (RP) sequence is 5'-biotin-TTAGCAAAGTAGCGTGCACTTTTG-3'[25]. FITC was used to quantify ssDNA and monitor the SELEX procedure via flow cytometry. The biotin was used in

conjunction with streptavidin coated magnetic beads (Promega) to generate ssDNA from the amplified double-stranded aptamer pool following PCR. For cloning and sequencing, PCR was performed with unmodified versions of the primers.

3.3.3 Bacterial Cell-SELEX procedure

Cell-SELEX was performed as previously described [25]. Each round of SELEX consisted of three steps: Binding and elution, amplification, and recovery of ssDNA. Ten rounds of positive selection and two rounds of counter selection were performed (Figure 1). The first round of counter selection was performed with *P. putida* KT2440; the second one with *P. fluorescens* LMG1794.

Binding and Elution: Cell-SELEX was performed with cells suspended in an artificial freshwater medium (Fraquil) to replicate the physiological state of nutrient-limited environmental conditions[36, 30]. Fraquil was prepared as described previously [38] with a final iron concentration of 10 nM and filter-sterilized using a 0.2 µm filter (Sarstedt). Post-exponential phase cultures were rinsed twice with Fraquil (6000 g, 15 minutes) and suspended in Fraquil at an OD_{600} of 1 corresponding to a concentration of 10^9 CFU/ml. The concentration of cells was confirmed by CFU counts for each round. The suspension was incubated at room temperature for 24 h. Fraquil exposed cells were washed three times in 1X binding buffer (phosphate buffered saline with 0.1 mg/ml salmon sperm DNA, 1% bovine serum albumin, and 0.05% Tween 20) at room temperature (25°C) using 6,000 g for ten minutes. The cell pellets were then suspended in 330 µl of 1X binding buffer. The aptamer pool was denatured by heating at 95 °C for 10 minutes, cooled immediately on ice for 10 minutes, and added to the cell suspension. Finally, 1X binding buffer was added to a total volume of 1 ml. For the first round, 32 µg of the initial library was used. For the subsequent rounds, approximately 400 ng of aptamer pool was used. The final mixture was incubated at 25 °C for 1 hour with mild shaking using a tube rotator at 150 rpm. Following incubation, the mixture was centrifuged at 6000 g for 10 minutes and washed twice with wash buffer (phosphate buffered saline containing 0.05% Tween 20) to remove unbound sequences. To elute the bound sequences from the cells, the final cell pellet was resuspended in 100 µl nuclease free water (Ambion) and heated at 95 °C for 10 minutes and immediately placed on ice for 10 minutes. After centrifuging at 6,000 g for 10 minutes at 25°C, the supernatant was collected and purified using overnight ethanol precipitation at -20 °C with 5 µg of glycogen as a carrier to recover the eluted ssDNA. The pellet was recovered, dried and suspended in nuclease free water (Ambion). The concentration and

quality of the ssDNA was determined using a Nanodrop spectrophotometer (Thermofisher). For counter-selection the supernatant containing the unbound sequences was collected and purified via ethanol precipitation, as described above. To ensure there was no amplification or collection of unwanted bacterial DNA (instead of the desired amplification and collection of ssDNA oligonucleotides), a control sample consisting of bacterial cells without aptamer was included in each round.

PCR amplification: The purified aptamer pool was then amplified by PCR with One Taq DNA polymerase (NEB), according to the manufacturer's protocol. All primers were used at a final concentration of 0.5 µM. PCR conditions were as follows: initial heat activation at 95 °C for 5 min and 25 cycles of 95 °C for 30 s, 56.3 °C for 30 s, 72 °C for 10 s, and a final extension step of 10 min at 72 °C. After amplification, the concentration and size of the PCR product were confirmed by gel electrophoresis using a 2.0% agarose gel. PCR products were then purified using a MinElute PCR Purification Kit (Qiagen). As expected, no amplification was observed for the control samples, lacking aptamer template.

Recovery of ssDNA: Streptavidin coated magnetic beads (Promega Technology) were used, according to the manufacturer's recommendation. Briefly, 600 µg of magnetic beads were washed twice and then resuspended in 900 µl of washing buffer (phosphate buffered saline with 0.05% Tween 20). Next, approximately1 µg of PCR product was incubated with the magnetic beads for 10 min, mixing gently by inversion after every few minutes. The mixture was then washed in 1 ml of washing buffer. Finally, the beads were incubated with 500 µl of 200 mM NaOH for 5 minutes. The supernatant was then collected, and the FITC-labelled ssDNA was purified using ethanol precipitation as mentioned previously and quantified with a Nanodrop spectrophotometer (Thermofisher).

Figure 1: Schematic illustration of bacterial cell-SELEX procedure used in this study. A random library of oligonucleotides is incubated with *Lp lp120292* at room temperature for 1h. Sequences that do not bind are washed off and the cell-bound sequences are then released and amplified via PCR. The resulting sequences are then submitted to another round of positive selection. *P. fluorescens* and *P. putida* were used to perform counter-selection rounds to eliminate non *Lp*-specific sequences.

3.3.4 Monitoring of SELEX by flow cytometry

The binding of the FITC-labelled aptamer pools from rounds 1 (R1), 6 (R6), 7 (R7), 8 (R8) and 10 (R10) to *Lp* was assessed using flow cytometry. Briefly, 35 nM of aptamer pools from each of these rounds was incubated with 10^6 CFU/ml of *Lp* cells at 25 °C for 1 hour. Analysis was performed on a Guava easyCyte (Millipore) using the green fluorescence channel. A total of 5,000 events were recorded. Unlabeled cells were used as a control to measure autofluorescence. The Lp*GFP strain, producing strong green fluorescence from GFP, was used to adjust the gain of the green fluorescence channel. For analysis, a gate was first defined based on the forward and side scatters that included most of the cells. Then, a histogram of the number of cells vs the fluorescence intensity was used to define a region named Green_Lp where cells were considered positive for

green fluorescence and therefore stained with aptamers. This region was setup to include very few cells of the unstained control and therefore represent fluorescence above the autofluorescence. Aptamer pools from R10 alone, without cells, was also analyzed to ensure that the aptamer alone was not forming aggregates that would be confused with cells.

3.3.5 Cloning and Sequencing

To identify sequences binding to *Lp*, the aptamer pool from the 10th round of SELEX was cloned with the pGEMT-easy Cloning and Ligation Kit (Promega). To investigate the effect of counter-selection on the aptamer pools, we also cloned and sequenced aptamers from the 6th round of SELEX. Positive colonies, containing aptamer inserts, were determined via blue-white screening and confirmed by PCR. Plasmids were extracted and purified using a Miniprep Kit (Qiagen) and sequenced by Sanger Sequencing at the Plate-forme d'Analyse Génomique of Laval University. Secondary structures of the aptamer sequences were determined using the Mfold web server using default parameters. [56]

3.3.6 Characterization of aptamers R10C5 and R10C1

The binding of the aptamers R10C5 and R10C1 to *Lp* and to the species used for counter-selection was further characterized. R10C5 and R10C1 were individually synthesized with FITC at the 5' end (Integrated DNA Technology).

Determination of the Disassociation constant (K_D): To determine the K_D of R10C5 and R10C1, varying concentrations of FITC-tagged aptamers (1000 nM, 100 nM, 10 nM, and 1 nM) were incubated with 10^6 CFU/ml of *Lp* cells suspended in Fraquil and the fluorescence obtained at each concentration was measured using flow cytometry, as described above, in triplicate. The number of bound cells (FITC-positive) were recorded and used to determine the K_D by interpolating the logarithmic curve using GraphPad Prism 7.03.

Specificity assay: To determine the specificity of R10C5 and R10C1 for *Lp* cells, the binding to counter-SELEX *Pseudomonas* strains as well as cooling tower isolates was tested using flow cytometry. All cells were suspended in Fraquil and prepared as described above for cell-SELEX. Briefly, 100 nM of R10C5 and R10C1 was incubated with 10^7 CFU/ml of the strain used for SELEX (*lp120292*), another *Lp* strain (Lp02), the strains used for counter-selection (*P. putida KT2440* and *P. fluorescens LMG1794*), and the isolates from cooling towers (*Pseudomonas sp.,*

Brevundimonas sp., Bacillus sp., Staphlyococcus sp., *Sphingomonas sp., Stenotrophomonas sp. and Cupriavidus sp.*) for 1 hour at 25 °C with mild shaking. *lp120292* was also incubated with 100 nM of a FITC-labeled scrambled sequence of aptamer R10C5 (5'-fluorescein-ACAGAATCAGTTCGAGTACATACGCGCGAAGACTCCTAAGGCCGTAGCGTTCTTCCC GGTAATACCATG) and R10C1 (5'-fluorescein-TGTACTCCCGCGTCCCACCTGCTACCCGAAATAGAGTTTCCCTAGAAAGGCTTGCCC AAC). The suspension was centrifuged for 10 minutes at 6000 *g* to eliminate excess aptamer and resuspended in Fraquil. These suspensions were then analyzed using flow cytometry as described above. This experiment was done in triplicate. Cells suspended in Fraquil without any aptamer added were used as controls. The percentage of bound cells was determined as described above. Statistical differences were assessed using a one-way ANOVA and Dunnett correction for multiple comparison using GraphPad Prism 7.03.

3.3.7 Confocal Fluorescence microscopy assay

FITC-labelled R10C5 and R10C1 aptamer (100 nM) were incubated with 10^8 CFU/ml of target cell *lp120292* or counter-selection strain *P. fluorescens LMG1794* for 1 hour at 25 °C on a tube rotator at 150 rpm. Cells were suspended in Fraquil as mentioned previously. Negative controls included cells suspended in Fraquil without any aptamers. The suspensions (10 µl) were dropped on a glass slide (Fisherbrand), and a #1.5 cover slip (VWR) was used to make a thin layer. The slides with suspensions were then transferred onto a microscope chamber and imaged using a Zeiss LSM 710 confocal microscope (Carl Zeiss, Oberkochen, Germany), a 100 X oil objective (Plan-Apochromat 100x/1.40 Oil DIC M27a) and a 488 nm argon laser (25 mW,). A pre-set FITC filter was used with excitation and emission wavelengths of 488 and 564 nm respectively. Images were analyzed using Fiji (Schindelin et al. 2012).

3.4 RESULTS AND DISCUSSION

3.4.1 Selection of Aptamers binding to *Lp*

Cell-SELEX was used to select aptamers binding specifically to *lp120292*. This strain was selected because it was involved in the Quebec City Outbreak in 2012 [28]. To mimic the physiological state of *Lp* in a water system, *Lp* cells were grown to post-exponential phase and suspended in Fraquil

for 24 h at 25 °C to induce starvation and the associated morphological and physiological changes [36,30]. Seven rounds of positive selection were performed, followed by one round of counter-selection, two rounds of positive selection, an additional round of counter-selection and a final round of positive selection (Figure 1). Two *Pseudomonas* strains were used for counter-selection because they are also Gram-negative γ-proteobacteria frequently isolated from water systems where *Lp* is found as mentioned previously.

To monitor the progress of the SELEX procedure and ensure that the proportion of sequences binding to *Lp* was increasing, the binding of the FITC-labelled aptamer pools from rounds 1 (R1), 6 (R6), 7 (R7), 8 (R8) and 10 (R10) to *Lp* was examined using flow cytometry. Cells incubated with the initial aptamer library showed minimal fluorescence compared to the negative controls (Figure 2d, Lp Lib). Cells incubated with aptamer from the first positive selection round showed a drastic increase in fluorescence (Figure 2e, Lp R1). The saturation in the fluorescence intensity and the percentage of bound cells starting at R6 suggests that the pool is dominated by sequences binding to *Lp* (Figure 2f-i, R6, R7, R8, R10). A small decrease in fluorescence and percentage of bound cells at R8 suggests that the first counter-selection step removed a few sequences. The fluorescence intensity remains similar between round 8 and 10 indicating that the second round of counter-selection did not remove *Lp*-specific sequences and that our strategy was successful in retrieving aptamers binding to *Lp*.
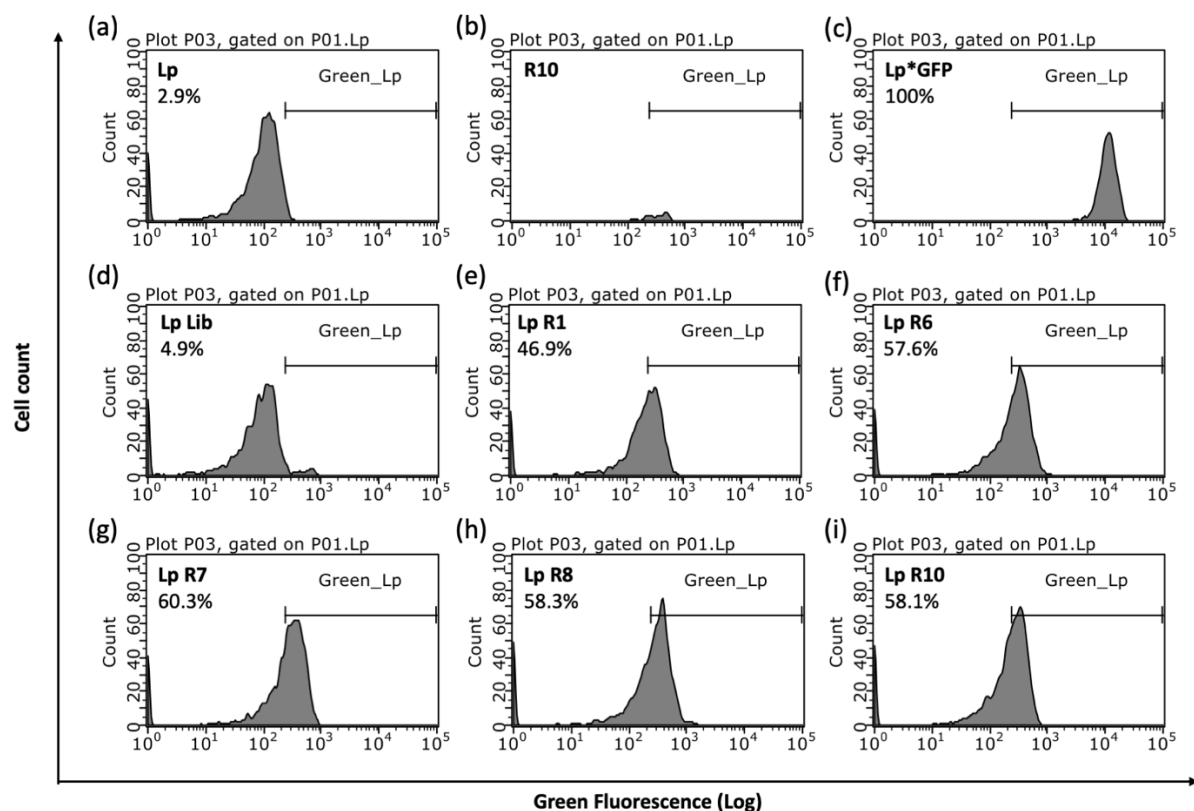
Figure 2: Fluorescent labeling of *Lp* with FITC-labeled aptamers pools obtained after selected round of SELEX. *Lp* strain *lp120292* was incubated without aptamers (Lp, a), with 35 nM of the aptamer library (Lp-lib, d) and with the aptamer pools obtained after round 1 (Lp-R1, e), 6 (Lp-R6, f), 7 (Lp-R7, g), 8 (Lp-R8, h) and round 10 (Lp-R10, i). The fluorescence obtained with aptamer from round 10 alone (R10, b), without cells, was also evaluated. Lp*GFP is a GFP producing version of *lp120292* and is used as a positive control (c). The percentages refer to the proportion of cells with fluorescence above the autofluorescence, falling in the Green_Lp region.

3.4.2 Cloning and Sequencing

Analyzing the sequences obtained from the 10th round of positive selection allowed for identifying two different ssDNA aptamers, named R10C5 and R10C1 (Table 1 and Figure 3). Of the 13 sequences that were retrieved, 12 of them were R10C5 whereas 1 was R10C1. In contrast, the survey of a non-exhaustive list of the sequences present in the R6 aptamer pool revealed eight different sequences out of 9 clones, but none similar to R10C1 and R10C5. This illustrates the

directional evolution of the pool as a result of the additional positive selection rounds and counter-selection steps [44,47]. A strong bottleneck effect was likely caused by the last four positive selection rounds and the apparently stringent counter-selection rounds, which most likely led to the removal of several aptamers.

R10C5   **R10C1**
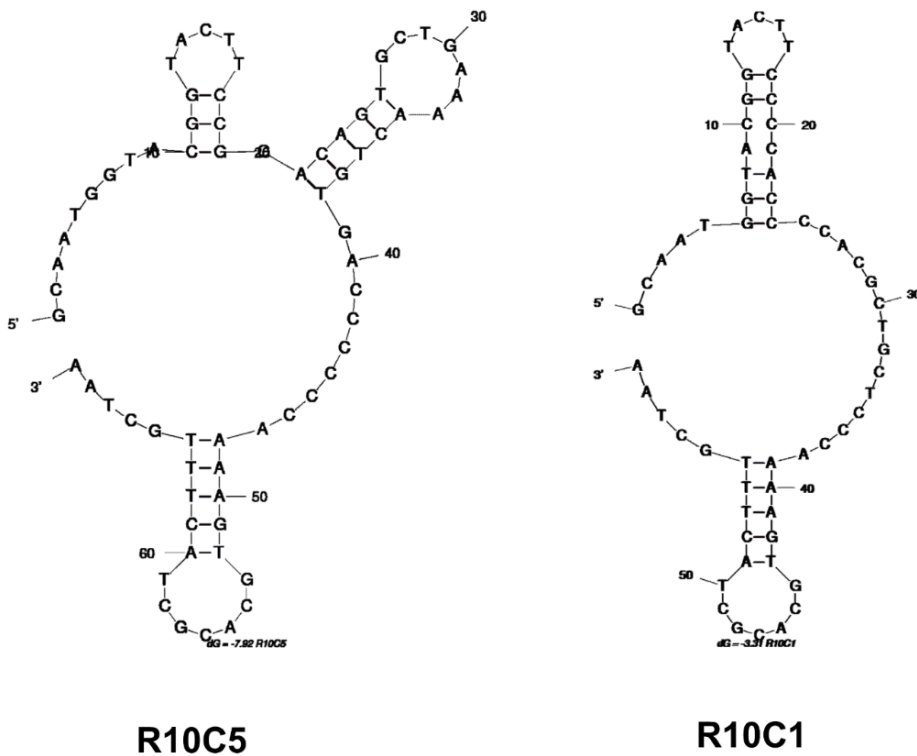
Figure 3: The structure of the aptamers R10C5 and R10C1 were determined using Mfold.

Table 1: Aptamer's sequences from round 6 and round 10.

APTAMER ID	SEQUENCE	FREQUENCY (%)
Before counter-SELEX (Round 6)		
R6C1	GCAATGGTACGGTACTTCCCCACTAACGCG CCCACGCACCCCTCGGCTACATCCAGCACC CGCCCAAAAGTGCACGCTACTTTGCTAA	2/9 (22.2%)
R6C3	GCAATGGTACGGTACTTCCCCACTCCACGCA TCACAGCCTTTCACTGCCCACGCCTCAAAAG TGCACGCTACTTTGCTAA	1/9 (11.1%)
R6C7	GCAATGGTACGGTACTTCCACCACCGGAGT GTGCTTCAGCCGTGGTACAATACTGCCGTGT ATCCAAAAGTGCACGCTACTTTGCTAA	1/9 (11.1%)
R6C11	GCAATGGTACGGTACTTCCCCCACTGCACAC ACAAAGGGCCAGCATCAACACACGCGCCGT TCCAAAAGTGCACGCTACTTTGCTAA	1/9 (11.1%)
R6C12	GCAATGGTACGGTACTTCCCACCCCGCCAC GCCGATAGCCTCCCATACTCCCCCCGCANGT CCAAAAGTGCACGCTACTTTGCTAA	1/9 (11.1%)
R6C15	GCAATGGTACGGTACTTCCCGCGCACCCCA CACCTCCGCACACCGCATGCCTCCCCTTAGG CCCCAAAAGTGCACGCTACTTTGCTAA	1/9 (11.1%)

R6C16	GCAATGGTACGGTACTTCCCACTGCCGAAC	
	GCGCCCTCTCCTGCTGCCTCCACACATGGTC	1/9 (11.1%)
	GCCAAAAGTGCACGCTACTTTGCTAA	
R6C18	GCAATGGTACGGTACTTCCCCCACCAAGCC	
	CATACACGTACAGCCTACCACAATCCACAT	1/9 (11.1%)
	CGGGCCAAAAGTGCACGCTACTTTGCTAA	

Post Counter-SELEX (Round 10)

R10C5	GCAATGGTACGGTACTTCCGGACAGTGCTG	
	AAAACTGTGACCCCCAAAAGTGCACGCTA	12/13 (92.3%)
	CTTTGCTAA	
R10C1	GCAATGGTACGGTACTTCCCCACCCCACGCT	
	GCTCCCAAAAGTGCACGCTACTTTGCTAA	1/13 (7.7%)

3.4.3 Determination of K_D

The calculated K_D is 116 nM for R10C5 and 135 nM for R10C1 (Figure 4). These values are comparable to high affinity antibodies that typically show nanomolar ranges of K_D for small protein targets[55]. These values are also comparable to values of published aptamers created against whole bacterial pathogens. For example, aptamers isolated against *Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae, Citrobacter freundii, Bacillus subtilis*, and *Staphylococcus epidermidis* showed K_D ranging from 9.22–38.5 nM [45]. Additionally two 62 nucleotide aptamers were isolated that bind to *Staphylococcus aureus* with K_D of 35 nM and 129 nM [7].
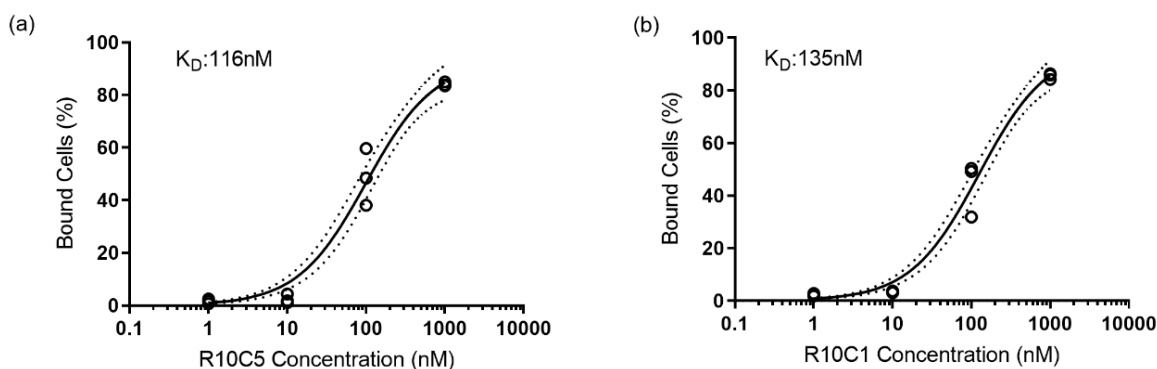
Figure 4: Determination of the K_D of the aptamers R10C5 (a) and R10C1 (b). *Lp (lp120292)* was incubated with 10-fold dilutions of the FITC-tagged aptamers and the fluorescence was measured by flow cytometry. The number of cells displaying fluorescence above the autofluorescence were counted as bound cells, as described in Figure 2. The graphs show individual values of three experiments. The equilibrium dissociation constant, K_D was calculated using GraphPad Prism 7.03. The non-linear regression (solid line) with 95% confidence interval error envelopes (dotted lines) is shown.

3.4.4 Specificity of R10C5 and R10C1

Figure 5A shows the binding of R10C5 and R10C1 to the strains used for counter-selection. Around 60% of *lp120292* cells are stained by R10C5, consistent with previous results shown in Figure 2, but only 20% of *Pseudomonas* strains are labelled. Similarly, R10C1 shows significantly more binding to *Lp* than to *Pseudomonas* (Figure 5b). Of note, the scrambled sequences of aptamer R10C5 and R10C1 bind minimally to *lp120292* (Figure 5a and b). The aptamer R10C5 stained Lp02 similarly to *lp120292*, suggesting that the binding of this aptamer is not restricted to a particular strain of *Lp* (Figure 5a). Moreover, both aptamers show very low binding to environmental isolates from cooling tower water. For the majority of the isolates, less than 10% of cells were labeled by the aptamers (Figure 5c and 5d). The specificity of these aptamers for *Lp* was further analyzed by confocal fluorescence microscopy. Both aptamers strongly stained *Lp* (Figure 6) but not *P. fluorescens LMG1794*, one of the strains used for counter-selection. These results support the notion that the aptamers R10C5 and R10C1 are highly specific to *Lp*.
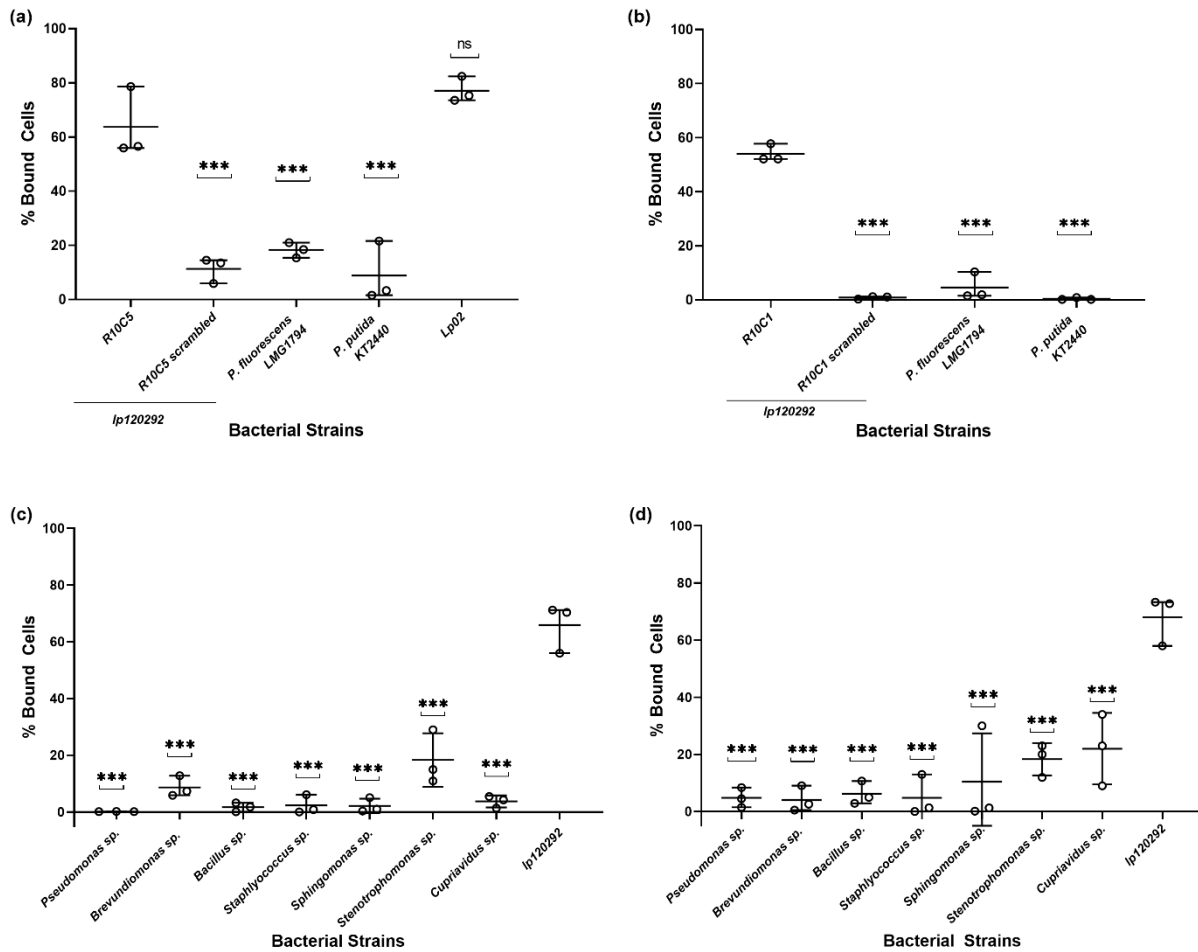
Figure 5: The specificity of FITC-labelled R10C5 and R10C1 aptamers binding to *Lp* strain *lp120292* (positive control), to counter SELEX *Pseudomonas* strains (a and b) as well as to environmental isolates (c and d) was analyzed by flow cytometry. The binding of R10C5 to *Lp* strain Lp02 was also analyzed. The percentage of cells bound by R10C5 (a and c) and R10C1 (b and d) to counter-SELEX strains (a and b) and environmental isolates (c and d) are presented. The binding of a scrambled sequence of aptamer R10C5 and a scrambled sequence of aptamer R10C1 to *Lp* strain *lp120292* was also investigated (scrambled). The values of three experiments are shown with the mean and standard deviation. A one-way ANOVA with a Dunnett correction for multiple comparisons was used to infer statistical significance compared to *Lp* strain *lp120292*: *** $P < 0.001$; ns, not significant.
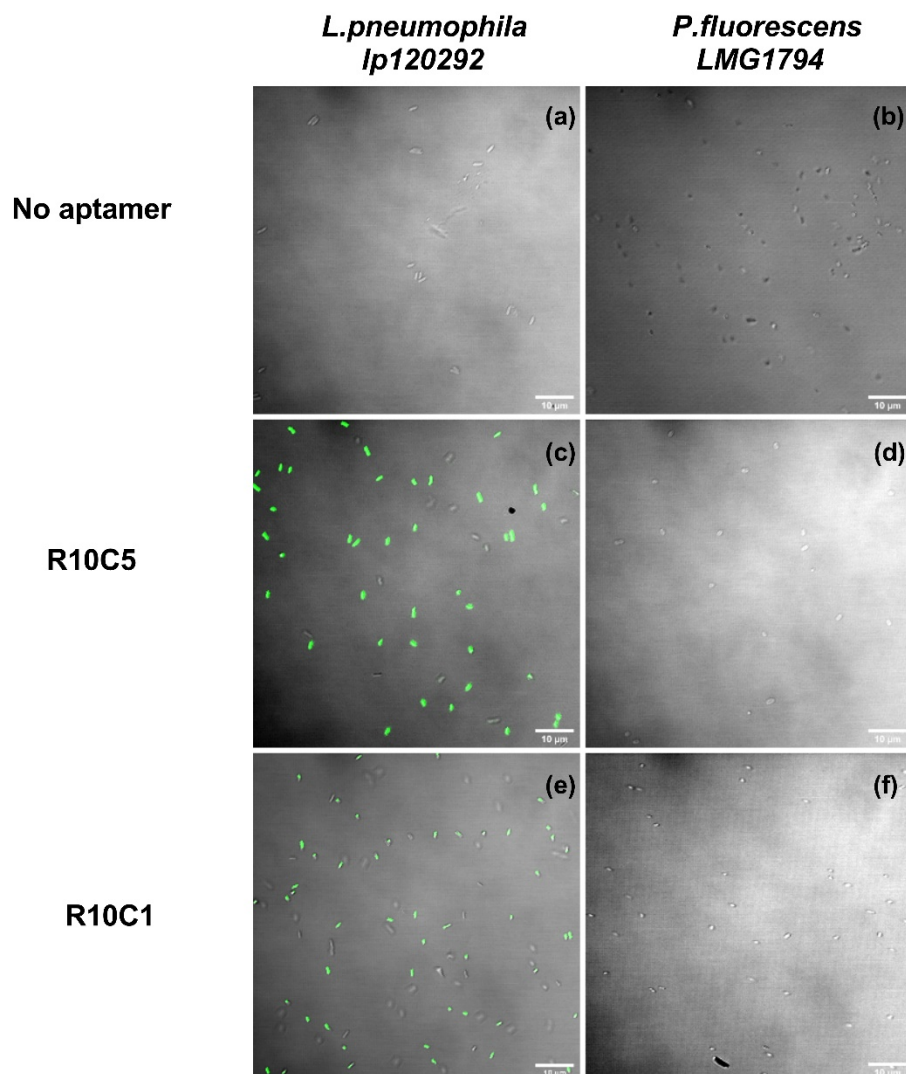
Figure 6: The specificity of FITC-labelled R10C5 and R10C1 aptamer was tested by measuring their ability to bind to *Lp* strains *lp120292* (a, c and e) and to *P. fluorescens* LMG1794 (b, d and f) by confocal fluorescence microscopy. The No aptamer controls consists of cells alone (a,b). Cells were incubated with aptamer R10C5 (c,d) and aptamer R10C1 (e,f).

In conclusion, our cell-SELEX strategy was successful in identifying two aptamers binding to *Lp* with high affinity (K_D = 116 nM for R10C5 and 135 nM for R10C1). Whereas R10C5 seems to stain *Lp* more strongly then R10C1, the latter seems more specific to *Lp*, showing minimal binding to the counter SELEX *Pseudomonas* strain. Both aptamers showed minimal binding to cooling towers isolates, indicating that the aptamers are suitable to detect *Lp* in complex water samples.

Modification of these aptamers could be attempted to further increase their affinity and specificity to *Lp*. Based on the results presented here, these aptamers are promising candidates as biorecognition elements to develop a biosensor to detect *Lp* in real time and *in situ.*

3.5 ACKNOWLEDGEMENTS

3.6 AUTHOR CONTRIBUTIONS STATEMENT

MS, MT and SPF designed the study. MS and SPF planned the experiments. MS and DC performed the experiments. MS wrote the first draft of the manuscript. MS, MT and SPF edited the manuscript. All authors approved the submitted version of the manuscript.

3.7 COMPETING INTERESTS STATEMENT

All the work described in this manuscript is the subject of patent applications filed in USA, patent application no US 16/850,355; and in Canada – patent application number pending at the time of revised manuscript submission. The inventors are Mariam Saad, Maryam Tabrizian and Sebastien P. Faucher. At the time of submission of the manuscript, the applications were under review. The authors declare no other competing interest.

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CHAPTER 4: CONNECTING TEXT

The aptamer R10C5, identified in the previous chapter, was then integrated with the SPRi sensing platform to detect *Legionella pneumophila* (*Lp*).

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Introducing an SPRi-based titration assay using aptamers for the detection of *Legionella pneumophila*

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4.1 ABSTRACT

Legionella are waterborne bacteria and the causative agents behind a severe respiratory disease known as Legionnaires Disease (LD). Standard ISO-certified methods to detect *Legionella* (plate count and qPCR) are time-consuming and tedious necessitating the development of novel detection strategies. This study introduces an SPRi-based titration assay that uses *L. pneumophila (Lp)* aptamers. Multiple parameters including buffer conditions, aptamer concentrations, complementary aptamer probe (cApt) concentrations and incubation times were investigated and evaluated for optimal signal response. For maximum hybridization efficiency on the sensor surface, high salt buffer (SSC) as well 500nM of immobilized cApt resulted in a maximum signal response. Additionally, it was observed that of the three buffers (PBS, Fraquil, SSC) tested in this assay only SSC facilitated incremental binding of aptamers to *Lp* such that varying concentrations of *Lp* could be determined. We were able to detect varying concentrations of *Lp* down to a limit of 10^4 cells/ml without any sample processing or signal amplification steps, highlighting the potential of this system for SPRi-based *Lp* detection. The assay is also specific to *Lp* and shows negligible detection of *Pseudomonas*, a common inhabitant of water systems.

Keywords: aptamer, *Legionella*, SPR, biosensor, waterborne pathogen

4.2 INTRODUCTION

Legionellosis is a severe form of pneumonia caused by the opportunistic pathogen known as *Legionella*. These bacteria are found primarily in water. Inhalation of microdroplets and aerosols from water systems containing *Legionella* results in an infection of the lungs. Though widely prevalent in natural water systems, it is the elevated numbers of these bacteria in engineered water systems that cause the disease as the latter are prone to producing aerosols which harbour the bacteria. Examples of aerosol producing engineered water systems include cooling towers, misters, showers and premise plumbing.

The National Academies of Sciences, Engineering, and Medicine (NASEM) in the United States reports that incidences of legionellosis in 2017 are 5 times higher than they were 20 years ago (National Academies of Sciences and Medicine 2020). Similar trends are observed in Canada according to the Public Health Agency of Canada (PHAC 2018). In both the USA and Canada, the actual number of cases is considered to be much higher, (PHAC 2019, CDC 2021). The situation is particularly pressing in recent times, in the context of the SaRs-CoV2 pandemic, as the unprecedented worldwide "stay-at-home" orders has created favorable environment for *Legionella* growth and proliferation (CWWA-ACEPU 2020, Proctor, Rhoads et al. 2020). The orders have led to the closures of buildings, institutions, and offices in response to the novel coronavirus pandemic. These widespread closures and "shut-downs" have reduced water usage and flow, consequently leading to a stagnation in water. Stagnation of water leads to a decrease in disinfectant residuals, degradation in water quality, temperature fluctuations that promote microbial growth conditions, as well as the formation of biofilm, all of which favor *Legionella* growth (CWWA-ACEPU 2020, Proctor, Rhoads et al. 2020). Routine surveillance and detection are therefore critical for monitoring the spread of the *Legionella* and limiting the risk of infection.

Standard *Legionella* detection and testing procedures consist of the ISO 2017 culture methods (AFNOR NF T90-431, ISO 11731) and the qPCR method (AFNOR NF T90-471, ISO/TS 12869). The culture method is very time-consuming, highly affected by variation in sampling strategies (Lucas, Taylor et al. 2011, Wang, Bédard et al. 2017, National Academies of Sciences and Medicine 2020), and is plagued by false negative results due to the presence of interfering flora and VBNC cells thereby underestimating true *Legionella* load (Whiley and Taylor 2016, Wang, Bédard et al. 2017). The qPCR method is a more sensitive and quick method, but it has the added

caveat of overestimating Lp amounts because it cannot distinguish between viable and non-viable bacteria (Taylor, Bentham et al. 2014, Whiley and Taylor 2016, Wang, Bedard et al. 2017) It also requires additional steps to process and eliminate compounds from complex matrices that would have adverse effects on the qPCR reactions (Gentry-Shields, Wang et al. 2013, Taylor, Bentham et al. 2014). Several of the problems incurred by these methods can be overcome with the use of SPRi biosensors. Unlike the currently used methods, SPRi technology has the potential to offer real-time detection, allow the use of complex samples, multiplexing capabilities as well as regeneration of detection surfaces, all of which would eliminate multiple processing steps and ensure timely operation of the system at a low cost (Nguyen, Park et al. 2015, Wang, Loo et al. 2019). For bacterial detection, SPRi systems can be adapted to be highly sensitive, providing low limits of detection, as well as highly specific, with the aid of a selective biorecognition element (Ahmed, Rushworth et al. 2014, Nguyen, Park et al. 2015, Kumar, Hu et al. 2018).

Aptamers are an increasingly promising choice as biorecognition elements. They are antibody analogues, made up of short single-stranded DNA or RNA oligonucleotides (McKeague, De Girolamo et al. 2015). Unlike antibodies which require strict physiological conditions, aptamers can be generated *in vitro* in tailored conditions. Their physicochemical properties make them versatile and cost-effective to synthesize with high fidelity as compared to antibodies. They also have high affinities to a wide array of targets including non-immunogenic targets (McKeague, De Girolamo et al. 2015). Given their chemical make-up, aptamers are also easily modifiable which allow for ease of functionalization and use in bio-sensing applications (Morales and Halpern 2018, McConnell, Nguyen et al. 2020) Several aptamers have already been made against various bacteria and used on multiple platforms ranging from lateral flow assays (Bruno 2014, Saad and Faucher 2021) to electrochemical impedance spectroscopy (Brosel-Oliu, Ferreira et al. 2018, Saad and Faucher 2021) to SPR-based systems (Yoo, Kim et al. 2015, Oh, Heo et al. 2017, Saad and Faucher 2021). In previous work, we reported on the development of two aptamers specific to *Legionella pneumophila (Lp)*. Following ten rounds of positive selection and two rounds of counter-selection against two *Pseudomonas* species, two aptamers (R10C5 and R10C1) binding strongly to Lp were identified with K_D in 100nM range. The binding specificity of these two aptamers to Lp was then confirmed by flow cytometry and fluorescence microscopy (Saad, Chinerman et al. 2020). This study aimed to use the aptamer R10C5 to develop an SPRi-based assay for the detection of Lp in water systems.

Generally, the use of SPR for whole-cell bacterial detection suffers from inherent drawbacks such as the limited range of the evanescent electromagnetic field wave (~ 300nm) produced by the instrument, the similarity of the refractive index (RI) of the bacterial cytoplasm, and aqueous medium, and the diffusion-limited mass transport of the bacteria to the metal-dielectric surface (Torun, Boyacı et al. 2012, Galvan, Parekh et al. 2018). Although much research is dedicated to circumventing the aforementioned limitations, such as the use of nanoparticles, nanorods, Long Range-SPR (LSPR), and transducer surface modifications (Gasparyan and Bazukyan 2013, Boulade, Morlay et al. 2019, Castiello and Tabrizian 2019), several researchers also make use of specific molecules such as 16 sRNA gene or biomarkers such as outer membrane protein antigens to mitigate these problems and assess bacterial cell concentrations indirectly (Wang, Ye et al. 2011, Taheri, Rezayan et al. 2016, Melaine, Saad et al. 2017, Sikarwar, Singh et al. 2017, Masdor, Altintas et al. 2019) The use of specific molecules and biomarkers, however, requires cumbersome extraction, purification and enrichment steps as in the case of RNA or DNA isolation. To overcome drawbacks from sample processing, in this work an SPRi-based titration assay is proposed for whole *Lp* cell detection. The titration assay format was previously used with antibodies to enable SPR-based detection of *Campylobacter jejuni* to a LOD of 131 CFU/ml (Masdor, Altintas et al. 2019). However, the present work shows for the first time that aptamers can be adapted in this format for SPRi-based detection of bacteria providing enhanced specificity and simplicity. The use of aptamers improves this method by minimizing complex handling steps as well as costs since antibodies are less stable and more expensive to produce.

The present assay determines the number of *Lp* by measuring the amount of unbound aptamers hybridizing to a complementary sequence (cApt) immobilized on the SPRi chip. Consequently, increasing *Lp* concentrations results in reduced signal responses, enabling detection of *Lp* without the use of labeling or signal amplification strategies. This type of assay is advantageous as it eliminates the need to inject whole bacterial cells, thus circumventing the SPR limitations with field range and similar RI while minimizing sample processing. To develop the titration assay format for SPRi-based detection of *Lp* using aptamer R10C5, we analyzed conditions for optimal hybridization of the aptamer to the cApt probe, as well as aptamer binding to the *Lp* cell. Using these optimal conditions, we determined both sensitivity and specificity of our titration assay for SPRi-based detection of *Lp*. To our knowledge, this work presents the first use of an SPRi-based titration assay using *Lp*-specific aptamers.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial Culture Strains and Conditions

The thymidine auxotroph *Lp* strain Lp02, derived from *Lp* Philadelphia-1 was used to perform the titration assay (Berger et al.1993). *Lp* was cultured on CYE (ACES-buffered charcoal yeast extract) agar plates supplemented with 10 ug/ml thymidine, 0.25 mg/ml L-cysteine, and 0.4 mg/ml ferric pyrophosphate, at 37 °C for 3 days. For liquid culture, *Lp* was suspended in AYE (ACES-buffered yeast extract) broth supplemented with 10 ug/ml thymidine, 0.25 mg/ml L-cysteine, and 0.4 mg/ml ferric pyrophosphate until post-exponential phase (OD_{600} of approximately 2.5). Following growth in liquid culture, the cells were suspended in an artificial freshwater medium (Fraquil) to replicate the physiological state of nutrient-limited environmental conditions (Li, Mendis et al. 2015, Mendis, McBride et al. 2015). Fraquil was prepared as described previously (Morel, Westall et al. 1975) with a final iron concentration of 10 nM and filter-sterilized using a 0.2 µm filter (Corning). Post-exponential phase cultures were rinsed twice with Fraquil (6000 ×*g* 15 minutes) and suspended in Fraquil at an OD_{600} of 1 corresponding to a concentration of 10^9 CFU/ml. This final suspension was left to incubate at room temperature for 24 hours.

For the specificity assay experiment *Pseudomonas fluorescens* LMG1794, was first cultured on LB agar plates at 30 °C for 24 hours and then grown in LB medium (Difco) until the cultures reached post-exponential phase (OD_{600} of 2.0). Following growth in liquid culture, the cells were resuspended in Fraquil and left to incubate at room temperature for 24 hours as described for *Lp*.

4.3.2 *Lp* Aptamer Development

Aptamers were previously created against *Lp* using Cell-SELEX (Saad, Chinerman et al. 2020). In total, ten rounds of positive selection and two rounds of negative selection were performed. Briefly, a random ssDNA library of 10^{15} sequences was chemically synthesized and flanked by primer binding regions at the 5' and 3' ends (IDT). This library served as the input pool for Cell-SELEX. Each round of SELEX consisted of three steps: Binding and elution, PCR amplification, and recovery of ssDNA. For binding and elution, 24 hours Fraquil exposed cells were incubated with denatured aptamer pools in 1X binding buffer (phosphate buffered saline with 0.1 mg/ml salmon sperm DNA, 1% bovine serum albumin, and 0.05% Tween 20) at 25 °C for 1 hour with mild shaking (150 rpm). Following incubation, the mixture was pelleted and washed twice with

wash buffer (phosphate buffered saline containing 0.05% Tween 20) to remove unbound sequences. To elute the bound sequences from the cells, the final cell pellet was resuspended in 100 µl nuclease-free water (Ambion) and heated at 95 °C for 10 minutes. After centrifugation, the supernatant was collected and purified using overnight ethanol precipitation at -20 °C. The pellet was recovered, dried and suspended in nuclease-free water (Ambion). The purified aptamer pool was then amplified by PCR with One Taq DNA polymerase (NEB), according to the manufacturer's protocol. All primers were used at a final concentration of 0.5 µM. To recover ssDNA for subsequent rounds of SELEX, streptavidin-coated magnetic beads (Promega Technology) were used, according to the manufacturer's recommendation. Aptamer R10C5, was identified as the dominant sequence following cloning and sequencing, (pGEMT-easy Cloning and Ligation Kit, Promega) of the aptamer pool from the 10th round of SELEX (Figure 1).
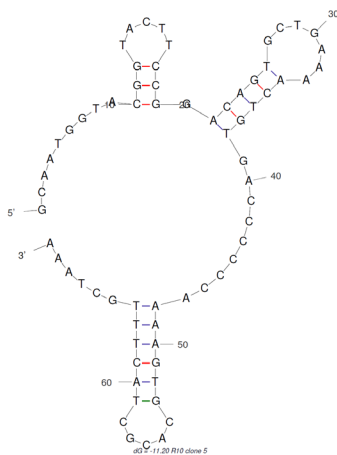
Figure 1. Predicted secondary structure of R10C5 using Mfold (Zuker 2003)

4.3.3 Buffer Test on *Lp* Growth

Lp was cultured on thymidine supplemented CYE plates followed by culture in AYE broth as mentioned above. The culture was aliquoted into three tubes, and each tube was then resuspended in either 1X PBS, 1X Fraquil, or 5X SSC respectively. The cultures were rinsed twice with the corresponding buffer (1X PBS, 1X Fraquil, 5X SSC) each time at 6000 ×g for 15 minutes and left to incubate for 1 hour at room temperature. Serial dilutions were made in triplicate at the beginning of incubation, T=0, and at one hour following incubation, T=1 hr to determine buffer effects on

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Lp survival. The dilutions were plated on thymidine supplemented CYE plates and incubated at 37 °C for 3 days. Colony counts were then taken, and the CFU/ml was calculated.

4.3.4 Substrate Preparation for SPRi Sensing

Cleaned microscope glass slide (12 mm × 25 mm × 1 mm, *n* = 1.518) substrates were coated with 2 nm Cr as an adhesion layer, followed by the deposition of a thin Au layer of 48 nm using E-beam vapor deposition under ultra-high vacuum. Au coated slides were cleaned using acetone, isopropanol and nuclease-free water before being dried under a nitrogen stream. Post surface modification, the slides were then coupled to an SF11 equilateral triangular prism (*n*SF-11 = 1.765) using a refractive index matching liquid (Horiba Scientific). For experiments determining the optimal concentration of cApt (sequence complementary to the R10C5 aptamer), gold-coated prisms were used. Gold-coated prisms (*n* = 1.765) were purchased from Horiba Scientific, NJ, USA, and used as received.

4.3.5 Surface Immobilization of Nucleic Acids

Cleaned Au-coated slides were immersed and incubated in a solution of 5X SSC and 500nM of thiolated cApt (IDT DNA) for 2 hours (Table 1). After incubation, the slides were rinsed with nuclease-free water and dried under a nitrogen stream. To minimize nonspecific adsorption, the slide was further blocked for 16 hours with a solution of 1mM Triethylene glycol mono-11-mercaptoundecyl ether (Sigma-Aldrich) dissolved in 200 proof EtOH. This was followed by an additional rinsing step and drying under nitrogen stream. The slides were used no longer than 48 hours after functionalization and were stored at 4°C.

Table 1: List of DNA sequences used in this study

ID	Sequence
Aptamer R10C5/ +ve control	GCAATGGTACGGTACTTCCGGACAGTGCTGAAAACTGTGACCCCCC AAAAGTGCACGCTACTTTGCTAA
Scrambled R10C5/-ve control	ACAGAATCAGTTCGAGTACATACGCGCGAAGACTCCTAAGGCCGTA GCGTTCTTCCCGGTAATACCATG

cApt (complementary sequence)	TTTT- TTAGCAAAGTAGCGTGCACTTTTGGGGGGTCACAGTTTTCAGCACT GTCCGGAAGTACCGTACCATTGC

4.3.6 SPRi Measurements

SPRi detection was performed using a scanning-angle SPRi instrument (model SPRi Lab+, Horiba, France). The SPRi apparatus is equipped with an 800 nm LED source, a CCD camera, and a microfluidic flow cell. All experiments were performed at 25 °C by keeping the instrument inside an incubator (Memmert Peltier, Rose Scientific, Canada).

To select the working angle for kinetic analysis, the slope of the plasmon curves was computed automatically using the instrument's software. The selected angle corresponds to the point of the plasmon curve at which the slope was maximum. For all measurements, reflectivity shift (ΔR (%)) was recorded upon stabilization of the baseline. Prior to injections, the sensor surface was pre-conditioned by exposing it to 200 mM NaOH (regeneration solution) to eliminate loosely bound substances, and to establish a stable baseline. After each analyte/supernatant injection, the surface was rinsed with running buffer 5X SSC (sodium saline citrate buffer, pH 7.6) (3M NaCl, 300mM NaCit, pH adjusted with 1M HCl). ΔR was calculated by the difference between the signals before and after the analyte injection. The signal was recorded at minimum on three spots for each analyte and control to determine the average ΔR values. Final ΔR values reported are the average of 3 independent experiments (using 3 different glass slices of prims). All experiments were performed using an injection loop with a fixed volume of 200 μL and a constant flow rate of 20 μL min^{-1}.

4.3.7 Selection of Hybridization Buffer

50nM of R10C5 aptamer buffer was dissolved in either 5X SSC, 1X PBS or 1X Fraquil and was injected onto the SPRi sensing surface. The running buffer of the SPR was changed each time to match the buffer of the analyte solution and rinsed with the specified buffer for 30 minutes before subsequent analyte injections.

4.3.8 Titration Assay

The working principle of the assay is the capture of biorecognition probes (aptamers) directly by the target cells (*Lp*) and measurement of the free unbound aptamers by SPRi (Figure 2). The free aptamers are detected following hybridization to complementary sequences (cApt) functionalized on the Au-coated sensor chip. A higher concentration of cells would result in an increased capture of the aptamer and thus reduce concentration of free aptamers.

All handling and incubation steps were performed at room temperature. Serial dilutions were made of 24hr Fraquil-exposed bacterial cultures in 5X SSC (sodium saline citrate buffer, pH 7.6) to obtain bacterial concentrations from 10^8 to 10^2 cells/ml. For experiments involving the effect of buffers on bacteria-aptamer binding, serial dilutions were also made in 1X Fraquil and 1X PBS. Each bacterial concentration was then incubated with 100 nM of R10C5 aptamer under gentle agitation for 45 minutes. The mixtures were then centrifuged for 15 minutes at 6000 $\times g$ after which the supernatant containing the free aptamer was collected and injected into the SPRi system. For supernatants collected from the dilution series made in PBS and Fraquil, the final concentration was adjusted to 5X SSC, by the addition of 20X SSC. Supernatants from each bacterial concentration (highest to lowest) were then serially injected over the functionalized sensor chip (Figure 2).
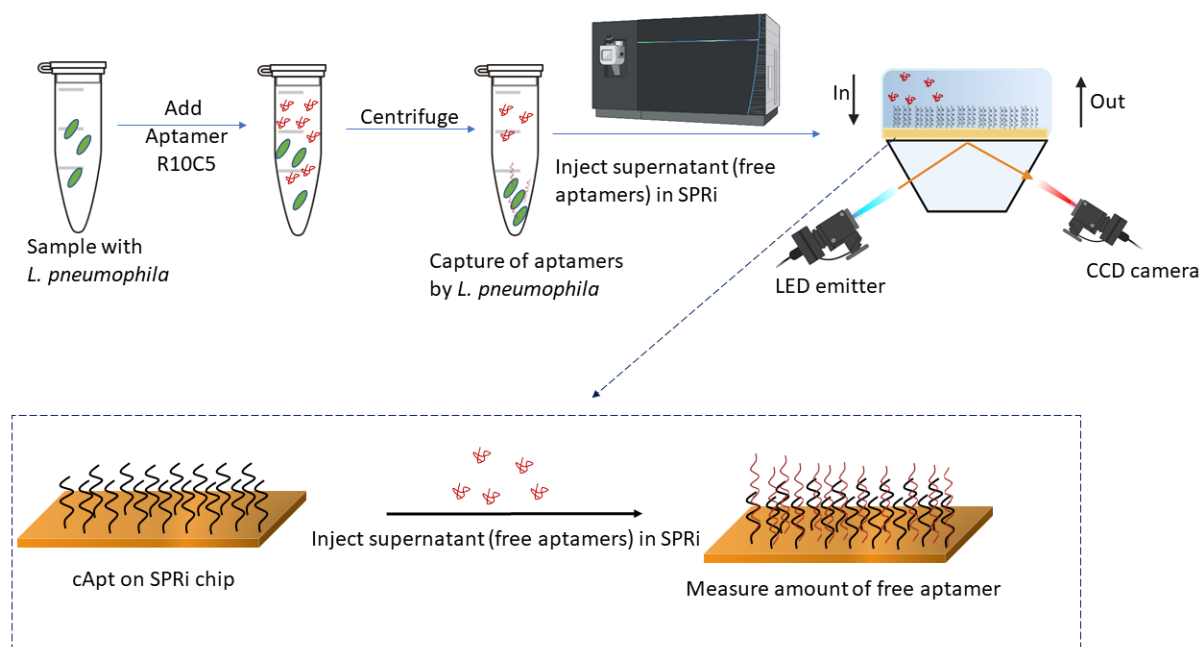
Figure 2. Schematic illustration of the titration assay. Aptamers are added to the water samples. Aptamers are captured by the bacteria. After centrifugation, the free aptamers are collected in the supernatant and detected following hybridization to a complementary probe (cApt) immobilized on the surface of the sensor. Created with BioRender.com.

Prior to injections, the sensor surface was pre-conditioned by exposing to 200 mM NaOH (regeneration solution) to eliminate loosely bound substances, and to establish a stable baseline. As a positive control, a solution containing a fixed concentration (100nM) of R10C5 aptamer dissolved in 5X SSC was injected over the sensor chip (Table 1). As negative controls, a scrambled sequence of R10C5 (Table 1) dissolved in 5X SSC and supernatants of bacterial suspensions without any aptamer were injected over the sensor chip. The sensor surface was regenerated with 200 mM NaOH following each injection. Consequently, each sensing cycle consisted of supernatant injection with a contact time of 13 minutes, buffer rinsing, and regeneration injections with a contact time of 30 seconds.

4.3.9 Determination of Incubation Time

To determine the time required for the bacteria-aptamer complex formation, serial dilutions were made of bacterial cultures to obtain a concentration of 10^6 cells/ml in 5X SSC. This concentration

of 10^6 cells/ml was then incubated with 100nM of R10C5 aptamer under gentle agitation for 5, 15, 30, and 45 minutes. At the specified time points the mixtures were then centrifuged for 10 minutes at 6000 $\times g$ after which the supernatant was collected. Following the pre-conditioning steps as outlined in the titration assay, the supernatants from each specified time point were serially injected over the functionalized sensor chip.

4.3.10 Statistics

The difference in cell concentrations because of buffer effects (Figure 3a) was calculated using a One-Way ANOVA with a Tukey multiple comparison test in GraphPad Prism 8.3.0. The difference in signal response as a result of varying bacterial concentrations (Figure 5C) was calculated using a One-Way ANOVA with Dunnetts Multiple Comparison Test in Graphpad Prism 8.3.0 The change in signal response (ΔR) was determined by subtracting the baseline following analyte injection from the previous baseline. The calibration curves were fitted using a non-linear 4 parameter logistic (4PL) model using GraphPad Prism 8.3.0. The signal change of the positive control solution, containing only a fixed concentration of aptamer, is (C_0). The LOD for the assays was calculated from the calibration curve as the maximum signal from the positive control (C_0) subtracted from the average (C_0), minus three times its standard deviation. The dynamic range for the titration assay was established between $0.2C_0$ and $0.8C_0$ and was determined from fitting the data to a non-linear 4PL regression model shown in the calibration curve in Figure 5(d). All data are expressed as the average of and with the standard deviation of 3 experiments.

4.4 RESULTS

To test the feasibility of the titration assay, two different parameters needed to be evaluated, namely (i) conditions that would allow efficient hybridization of the aptamer to cApt sequence and result in an optimal signal response (ii) conditions that would allow proportional binding of the aptamer to viable whole *Lp* cells.

(i)   Effect of Experimental Conditions on Hybridization Efficiency

First, to visually evaluate the specific hybridization of the complementary aptamer sequences to the SPR chips, FITC-labelled R10C5 aptamer was flooded over the sensor chip spotted with

unlabeled cApt and then visualized using a fluorescence microscope. Figure 3a shows that hybridization occurs between the R10C5 aptamer and only the cApt spots with no binding elsewhere on the sensor surface.

Next, to determine whether buffer composition affects nucleic acid hybridization, three buffers were selected to determine the optimal media for hybridization. 1X PBS, 1X Fraquil and 5X SSC. SSC is a high salt media common in hybridization buffers used for molecular biology applications whereas Fraquil is a low salt media, modeled after tap water. Figure 3b shows that maximum signal was obtained in 5X SSC, whereas hybridization of the R10C5 aptamer to cApt seems to be minimal in 1X PBS or 1X Fraquil. Consequently, 5X SSC was used for all hybridization steps listed in this study.

Next, the amount of cApt immobilized on the sensor surface was studied to observe its effect on hybridization efficiencies. Figure 3c demonstrates the change in signal upon injection of 100 nM of R10C5, following hybridization to varying concentrations of cApt immobilized on the sensor surface. The negative (-ve) control (Table 1) is a sequence that is not complementary to the R10C5 aptamer and thus shows a negligible change in signal. The highest concentration of the cApt (10 µM) shows a lower signal as do concentrations below 500 nM. When using 1 nM the signal response becomes too low and close to the limit of the instrument signal detection range. cApt concentrations of 1 µM and 500 nM provide maximal signal responses. Therefore, a cApt concentration of 500nM was chosen for further characterization of this assay.

Finally, varying concentrations of aptamer R10C5 were investigated to observe its effect on the SPR signal. The goal was to achieve a detectable signal following hybridization in order to optimize the use of reagents while still being reliable and sensitive similar to its SPR immunoassay counterparts (Estevez, Belenguer et al. 2012). Figure 3d indicates the signal range obtained following hybridization of R10C5 aptamer of different concentrations. Decreasing aptamer concentration results in a proportional signal decrease. Below 100nM the signal range becomes barely detectable, limiting the performance of a titration assay. For this reason, 100nM was selected as the optimal concentration to perform all titration assays.
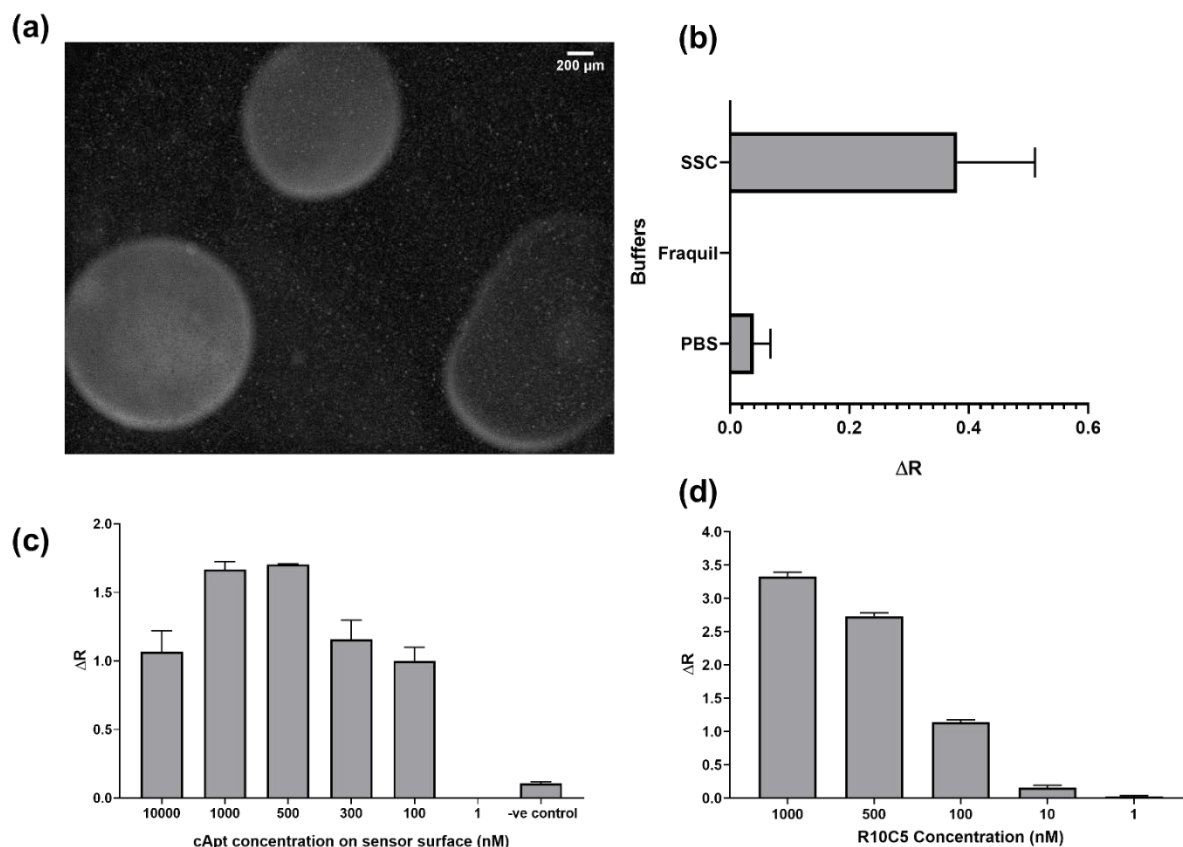
Figure 3. Conditions for optimal hybridization efficiency: a) Fluorescence microscopy image of FITC-labelled R10C5 aptamer hybridized to non-labeled cApt spots immobilized on an Au coated glass slide. b) ΔR from hybridization of 50nM R10C5 aptamer dissolved in 1X PBS, 1X Fraquil or 5X SSC. c) ΔR from hybridization of 100nM aptamer with different concentrations of cApt functionalized on the sensor surface. The negative control refers to the scrambled sequence which is not complementary to R10C5. d) ΔR in response to different aptamer R10C5 concentration hybridizing with 500nM cApt.

(ii) Effect of Experimental Conditions on Aptamer-Lp Binding and efficiency of the SPRi Titration Assay

Figure 4 illustrates the effects of PBS, Fraquil, and 5X SSC buffers on the capture of the R10C5 aptamer by *Lp* cells. The positive control consisted of pure R10C5 aptamer solutions (100nM) to show the range and maximum response of the SPR aptamer sensing system. The negative control (scrambled sequence) shows that the hybridization of the aptamer to cApt is specific. As additional controls, supernatants were taken from solutions containing 10^8 cells/ml in either PBS, Fraquil or

5X SSC with no aptamer. This step eliminates any possible non-specific binding coming from the cell culture supernatant to the sensor surface.

Next, the titration assay was performed, and supernatants were taken from solutions in the various buffers- PBS, Fraquil or SSC- containing either 10^8 cells/ml or 10^4 cells/ml of bacterial cells incubated with 100 nM R10C5 aptamer added. For aptamer-bacteria solutions made in PBS and Fraquil, the supernatants show negligible changes in signal between high (10^8 cells/ml) and low (10^4 cells/ml) bacterial concentrations (Figure 4a and 4b). However, when using 5X SSC, a lower signal is obtained for the cell suspension containing 10^8 *Lp* cells (Figure 4c). In this condition, higher *Lp* concentration corresponds to lower levels of free aptamer proving the working principle of the titration assay. 5X SSC buffer was, thus, selected as the optimal media for binding the aptamer to the bacteria in the titration assays.

Figure 4d illustrates that a one-hour exposure of Post Exponential phase *Lp* to either PBS, Fraquil, or 5X SSC buffer results in no adverse effects on cell cultivability. This indicates that 5X SSC is safe for use with this assay and does not alter cell counts, at least in the time frame necessary to perform the assay.

After this, to determine the effect of incubation time on the capture of aptamers by *Lp*, 100nM of R10C5 aptamer was incubated in solutions containing 10^6 cells/ml diluted in 5X SSC and were gently mixed for either 5, 15, 30, or 45 minutes. After specified time points the supernatants were collected and the signal response was measured. Figure 4e shows that the signal response for 10^6 *Lp* cells/ml incubated with R10C5 aptamer at varying time lengths remains unchanged suggesting that levels of free aptamer are similar at all time points. This means that the binding occurs within 5 minutes and a longer incubation time does not lead to more binding of the aptamer to *Lp*. We therefore selected a binding time of 5 minutes to perform the assay.

Finally, the specificity of the assay was evaluated using *P. fluorescens*. Figure 4f shows the signal change in response to R10C5 aptamer incubated with *P. fluorescens*. For high concentrations of bacteria, there are high levels of free aptamer suggesting that very little aptamer binds to the bacterium *P. fluorescens.* This illustrates the specificity and selectivity of the aptamer R10C5 for *Lp* as previously reported and the specificity of the titration assay (Saad, Chinerman et al. 2020) . The 5X SSC buffer also does not cause high cross-reactivity of the aptamers to the *P. fluorescens* strain given that a similar signal was obtained with aptamer alone.
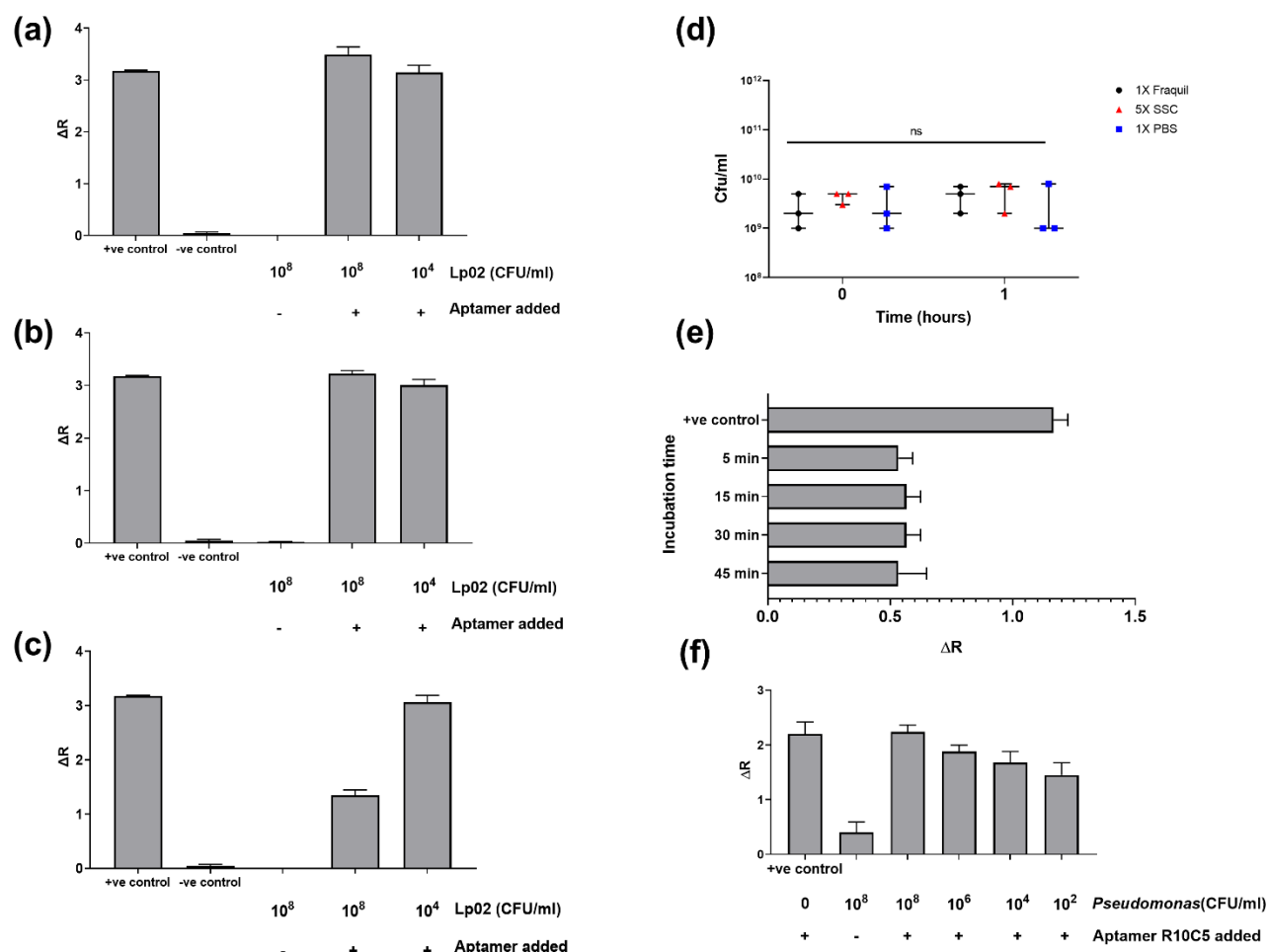
Figure 4. Parameters affecting aptamer-bacterial binding and titration assay efficiency. -ve control is the scrambled sequence. +ve refers to 100nM of R10C5. a) Signal change in response to aptamers incubated with Lp in 1X PBS. b) Signal change in response to aptamers incubated with *Lp* in Fraquil. c) Signal change in response to aptamers incubated with Lp in 5X SSC. d) Viability test showing *Lp* concentrations following 1-hour exposure 1X Fraquil, 5X SSC, and 1X PBS. e) Signal change in response to incubation of 100 nM R10C5 aptamer mixed with 10^6 *Lp* cells/ml for 5, 15, 30, and 45 minutes. f) Specificity test using *Pseudomonas fluorescens* LMG1794 in the titration assay which shows the signal response is not inversely proportional to bacterial concentrations.

(iii) Titration Assay and Calibration Curve

Next, we evaluated the response of the system to a dilution series of *Lp* in Fraquil with the optimal experimental parameters determined in previous sections (100 nM R10C5, 500 nM cApt, 5X SSC for hybridization, 5X SSC for binding, 5 minutes incubation time). Figure 5a and 5b show sensograms whereas Figure 5c show a bar graph of the titration assay experiments. The figures indicate that decreasing bacterial cell concentrations from 10^8 cells/ml to 10^4 cells/ml causes a direct increase in the SPR reflectivity signal. This signifies that the SPR sensor response is inversely proportional to the concentration of *Lp*, as expected. A higher concentration of cells results in an increased capture aptamer, thereby reducing the concentration of unbound aptamer detected. The specificity of the detection is reported in Figure 5b and 5c, which show the signal change in response to positive and negative controls. The limit of detection for this system was found to be $10^{4.4}$ cells/ml with a linear dynamic range of $10^{4.3}$ cells/ml – $10^{7.7}$ cells/ml as determined from fitting the data to a non-linear 4 parameter logistic regression model shown in the calibration curve in Figure 5(d). The R^2 for this region was calculated as 0.9544 indicating a strong goodness of fit.

Figure 5. The titration assay shows a proportional inverse relationship between the number of cells and signal response. a) Sensograms showing increasing signals in response to decreasing bacterial concentrations. b) Sensograms showing signal response for the positive control (100nM R10C5 without cells) and negative controls (supernatant without aptamer and 100 nM scrambled sequence without cells). c) Average change in signal in response to the concentration of free aptamers which is inversely proportional to bacterial concentrations. Statistical significance compared to the positive control was determined using a one-way ANOVA with Dunnetts Multiple Comparison Test was done using Graphpad Prism 8.3.0. d) Calibration curve from three independent titration assay experiments. Curves were fitted using a non-linear 4 parameter logistic regression model using Graphpad Prism 8.3.0.

4.5 DISCUSSION

To date, only two studies using an SPR-based immunosensor have detected whole *Lp* cells both with detection limits of 10^5 cells/ml (Oh, Kim et al. 2003, Oh, Lee et al. 2005). Previously reported

methods using SPR to detect *Lp* involved antibodies-protein complexes (Enrico, Manera et al. 2013) and detecting 16S rRNA of *Lp* (Foudeh, Daoud et al. 2014, Foudeh, Trigui et al. 2015, Melaine, Saad et al. 2017). None of these studies used aptamers as biorecognition elements or as part of a titration assay.

In comparison, this work presents the first report of an SPRi, aptamer-based *Lp* biosensor using a titration assay to establish a novel *Lp* detection strategy. Several parameters were studied in concert for the first time, including the use of an *Lp*-associated aptamer, conditions on the sensor surface (hybridization) as well as the parameters of bacterial-aptamer binding that affect SPR signal efficiency. The result is a simple method that requires no additional labeling, no extra amplification steps, and allows rapid detection of *Lp* (roughly 30 minutes). The use of aptamers in the titration assay also reduces the overall cost and tedious handling steps associated with this detection method. This is because aptamers are cheaper to synthesize, easier to functionalize, and more stable across a range of conditions as compared to their antibody counterparts (Morales and Halpern 2018).

SPR-based biosensors are an attractive technology for bacterial detection due to their intrinsic sensitivity to changes in refractive index (RI),produced during analyte-bioreceptor binding, which means they require no labelling or sample processing steps (Wang, Loo et al. 2019). Drawbacks for whole-cell microbial detection using SPR however are the low sensitivities and high LODs, as a result of the limited range of the electromagnetic field (~ 300 nm), the similarity of the refractive index (RI) of the microbial cytoplasm and aqueous medium, and the diffusion-limited mass transport of the microbe to the metal-dielectric surface (Torun, Boyacı et al. 2012, Galvan, Parekh et al. 2018). Consequently, microbial surrogate biomarkers and molecules are used as alternative detection strategies, but the main limitations of these methods are the requirement of multiple sample processing steps as well as an inability to discern whole, membrane-intact, viable cells. A titration assay can thus prove beneficial by eliminating the need to inject whole bacterial cells, thus circumventing the SPR problems with field range and similar RI while detecting membrane-intact and potentially viable microbial cells.

In the present work, using a titration assay, we have determined the concentration of *Lp* cells by measuring the reflectivity change in response to the amount of available free aptamer left in the supernatant after exposure to *Lp* cells. The free aptamer in the supernatant was allowed to bind to

its complementary sequence (cApt) previously functionalized to the SPRi sensor surface. Thus, the response of the SPRi sensor was inversely proportional to the concentration of the bacteria, in that, a higher concentration of cells would result in increased binding of aptamer, reducing the concentration of unbound/free aptamer detected. To test the feasibility of the assay, two different conditions needed to be evaluated namely, (i) conditions that would allow efficient hybridization of the aptamer to the cApt cognate sequence and (ii) conditions that would allow proportional binding of the aptamer to *Lp*. Figure 3 outlines optimal conditions for hybridization. The aptamer does not interact with non cApt regions on the sensor chip (Figure 3a) signifying that any signal observed is the result of specific aptamer-cApt hybridization. Since the sensor signal response is dependent on hybridization, it was critical to select a high ionic strength buffer as well as to determine the optimal spacing of the cApt (Zhang, Chen et al. 2012, Giamblanco, Petralia et al. 2017). Optimal cApt spacing is necessary to reduce high surface coverage density which can inhibit binding or analyte access due to steric or electrostatic hindrances (Macedo, Miller et al. 2017). SSC is a high ionic strength buffer widely used in DNA microarrays and hybridization studies (Figure 3b). For our assay, the combination of 5X SSC's (high ionic buffer strength) with a low cApt surface density achieved through spacing by using lower concentrations of cApt resulted in the optimal hybridization efficiencies, and thus the best signal intensity (Figure 3b and 3c).

Given that this titration assay is contingent on the amount of free aptamer, it was critical for the aptamer to be the limiting factor for the assay to be sensitive. If aptamers are in excess, then the concentration of free unbound aptamers will not differ for different bacterial concentrations. As such, a trade-off was made in selecting the minimum amount of aptamer possible i.e., 100nM, while still yielding a sufficient SPR signal response range (Figure 3d). Additionally, in previous work, 100 nM of R10C5 aptamer was deemed optimal to label at least 50% of *Lp* cells (Saad, Chinerman et al. 2020). Consequently, all titration assays mentioned in this study were conducted with 100 nM of R10C5 aptamer.

When determining optimal conditions for binding efficiency, 5X SSC buffer results in more sequestering of the aptamer by *Lp* cells at higher concentrations signifying that the buffer affects the formation of aptamer-target complex (Figure 4c). Previous studies using thrombin and cocaine aptamers have shown that electrostatic interactions between an aptamer and its target are affected

by salt concentrations. (Lin, Chen et al. 2011, Sachan, Ilgu et al. 2016). Additionally, low salt buffers have been shown to promote the formation of complexes which enhance aptamer stability (Wiedman, Zhao et al. 2017, Wildner, Huber et al. 2019). If an aptamer or aptamer complexes are in a highly stable configuration, then they would be too rigid to bind to cognate targets in an induced-fit manner suggesting that the use of PBS and Fraquil would result in stable aptamer complexes that do not bind to *Lp* (Grytz, Marko et al. 2016, Munzar, Ng et al. 2018). Following the results presented in (Figure 4c), it was crucial to assess the buffers' effects on *Lp* viability to ensure that any changes in free aptamer levels were not confounded by death or lysis of *Lp* (Figure 4d). Several studies show that *Lp* can tolerate high salt conditions at lower temperatures (Heller, Höller et al. 1998, Ohno, Kato et al. 2003, Vatansever and Türetgen 2015), confirming that the short exposure time to 5X SSC would not be harsh enough to affect *Lp* cultivability (Figure 4d). Furthermore, the 5X SSC buffer also does not cause high cross-reactivity of the aptamers to the *P. fluorescens* strain given that similar signals were obtained with a pure aptamer solution alone (Figure 4f). This is an important validation of the assay, since environmental water samples are often complex matrices ranging from tap water to cooling tower water (Wang, Bédard et al. 2017). The complex microbial flora of a real environmental sample may have microbial strains whose membranes or surface structures are susceptible to the effects of 5X SSC, resulting in loose binding of free aptamers (Helander and Mattila-Sandholm 2000, Nagaoka, Murata et al. 2010, Gandhi and Shah 2016, Santos, Chaumette et al. 2019, Kundukad, Udayakumar et al. 2020). This effect of non-specific binding however was not observed with a pure culture of *P. fluorescens* (Figure 4f). This confirms the specificity and selectivity of the aptamer R10C5 for *Lp* as previously reported (Saad, Chinerman et al. 2020).

Incubation temperature as well as target properties and characteristics can influence aptamer binding capabilities, as discussed previously (Saad and Faucher 2021). Our assay was validated at a temperature of 25 °C to facilitate environmental testing. It is important to note that samples should be allowed to come to room temperature before doing the assay to ensure optimal binding of the aptamer with *Lp*. Others have shown that aptamers may differentiate a bacterium's physiological state. An example is the *Campylobacter jejuni* aptamer ONS-23 which can discern live cells from non-viable cells (Dwivedi, Smiley et al. 2010, Kim, Kim et al. 2018). In our case, we used Fraquil-exposed bacteria to mimic the physiological state of *Lp* in water systems, which is the target application.

Given that the incubation time for aptamer-bacterial binding in this assay is just 5 minutes (Figure 4e), the titration assay is very quick to perform with minimal labour required. A result can be obtained in just 30 minutes which is more rapid when compared to current *Legionella* detection standards which take 7-15 days for conventional culture methods and 60-90 minutes for qPCR methods.

Several SPR techniques have been coupled with signal amplification strategies for the detection of waterborne bacterial contaminants (Saad and Faucher 2021). The current SPRi-based aptamer titration assay was able to reach a detection limit of $10^{4.3}$ cells/ml (Figure 5) which is comparable to various SPR-based sensors for whole-cell bacterial detection having either limited or no amplification strategies (Usachev, Usacheva et al. 2014, Yamasaki, Miyake et al. 2016, Oh, Heo et al. 2017, Nakano, Nagao et al. 2018, Saad and Faucher 2021). For example, SPRi was used by two groups to identify various serogroups of Shiga-Toxin producing *Esherichia coli* (STEC) cells via antibodies with limits of detection that were roughly 10^6 cells/mL and 10^4 cells/ml respectively (Yamasaki, Miyake et al. 2016, Nakano, Nagao et al. 2018). An aptamer labeled- gold nanoparticle (AuNP) coated sensor surface coupled with localized SPR detected whole *Salmonella Typhimurium* cells to an LOD of 1.0×10^4 cells/ml (Oh, Heo et al. 2017). The current assays LOD was also consistent with detection limits obtained using a similar titration assay but with antibodies where the LOD was 10^5 *Listeria monocytogenes* cells/ml and 10^4 *E. coli* cells/ml (Leonard, Hearty et al. 2004, Wang, Ye et al. 2011).

4.6 CONCLUSION

The SPRi aptamer-based titration assay in this study builds the ground for detecting *Lp* cells in a rapid, simple manner by eliminating multiple processing steps and circumventing the penetration depth of the evanescent electromagnetic field of a conventional SPR biosensing system. The assay strategy is quick compared to conventional culture methods (7-15 days) and qPCR (60-90 minutes) since it requires only 30 minutes to give a result. Furthermore, the use of aptamers also minimizes costs and tedious handling and manipulation steps associated with this method. While a detection limit of $10^{4.3}$ CFU/ml of *Lp* could be achieved without any labeling or signal amplification strategies, the development of a signal amplification strategy would push this simple SPRi-based titration assay over the competitive edge to enable real-time monitoring of bacteria on several

fields such as environmental monitoring or point-of-care diagnosis. In future work, we expect to further decrease the LOD of the present titration assay by means of SPR signal amplification strategies, using AuNPs-labelled aptamers or a sandwich aptamer assay format (Melaine, Saad et al. 2017, Castiello and Tabrizian 2019). These signal amplification strategies would help further reduce the LODs to 50 CFU/ml, which is the desired standard for *Lp* detection in environmental water, thus allowing the application of this technology for direct monitoring of *Lp* in environmental water samples. (National Academies of Sciences and Medicine 2020).

ETHICS STATEMENT: The aptamer used in this manuscript is the subject of patent applications filed in USA, patent application no US 16/850,355; and in Canada – patent application number pending at the time of revised manuscript submission. The inventors are Mariam Saad, Maryam Tabrizian and Sebastien P. Faucher. At the time of submission of the manuscript, the applications were under review. The authors declare no other competing interest.

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CHAPTER 5: CONNECTING TEXT

In Chapter 3 it was observed that the length of sequences and thus complexity of the secondary structures decreased with increasing rounds of SELEX. To minimize the loss of key secondary structural regions, we performed Branched-SELEX which comprised just one round of parallel selections followed by an examination of the resulting sequence pools using HTS data. To identify potential *Lp* specific aptamers, we incubated the initial input pool across different "branches" of non-target bacteria and then narrowed down the pool by eliminating sequences that were present in the pools from non-target bacteria. These non-target bacteria were subject to one round of cell-SELEX in parallel with the target *Lp* bacteria.

Chapter 5 shows the usefulness of the Branched SELEX methodology for large cellular targets as well the search for higher-order aptamers that could have enhanced binding properties and thus be more robust for downstream applications. The supplementary data for this chapter is found in the Appendix (Appendix, Chapter 5, File A2).

Branched SELEX: Investigating a parallel selection method for identifying aptamers that bind to *Legionella pneumophila* cells

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Running Title: Branched SELEX for *L. pneumophila*

5.1 ABSTRACT

Aptamers are short oligonucleotides with complex secondary structures that enable them to bind to a wide variety of targets. The classical SELEX method used to identify aptamers, however, requires several rounds of selections, and hence the use of multiple PCR steps, which result in an increase of sequences amenable to amplification rather than sequences with desired structural and binding properties. The Branched SELEX method can mitigate this unwanted effect by utilizing only one round of selection in combination with high throughput sequencing (HTS). In the Branched SELEX method, the input sequence pool is divided across four branches to identify aptamers binding to cells of the waterborne pathogen, *Legionella pneumophila (Lp)*. One branch contained the target *Lp* strains whereas the remaining three branches were a *Lp* serogroup 5 strain, a *Lp* mutant strain (Δ*fliC*) and a cocktail of water-associated bacteria. Candidate sequences could then be identified by eliminating sequences that were enriched in non-target branch pools. HTS data revealed that after one round of selection the proportion of unique sequences was low and that only a small subset of sequences was enriched in each of the branch pools. A large proportion of sequences are common to both the target *Lp* pools and non-target bacterial pools. Sequence enrichment analysis revealed four aptamers (11547,19654, 5210 and 68339) that were enriched in the target pool and depleted in the respective non-target pools. Three aptamers bind specifically to *Lp* whereas aptamer 68339, despite being depleted in the non-target (Δ*fliC*) pool, exhibits non-specific binding to the *Lp* mutant variant (Δ*fliC*). The study demonstrates the profile of sequence pools after one round of selection and the feasibility of using the branched SELEX method for identifying aptamers to bacterial cells isolated from specific water environments.

Keywords: Branched SELEX, aptamer, HTS, *Legionella*

5.2 INTRODUCTION

Traditional bacterial detection methods are labour intensive and require multiple processing steps to enumerate and monitor bacteria (Ramírez-Castillo, Loera-Muro et al. 2015). A rapid, quantitative cost-effective biosensing platform that could detect the bacteria would facilitate monitoring and surveillance (Ahmed, Rushworth et al. 2014). Aptamers, which are short, folded strands of either DNA or RNA, are a promising alternative to antibodies for use in biosensing platforms (Morales and Halpern 2018). These antibody analogues are developed using Systemic Evolution of Ligands (SELEX).

Systemic Evolution of Ligands (SELEX) is a library screening methodology where a large diverse pool of sequences (up to 10^{16}) is exposed to targets in order to select and enhance a subset of sequences that have affinity for said targets (Komarova and Kuznetsov 2019). The SELEX process consists of iterative rounds of selection and PCR amplification of the pool in order to isolate target binding sequences known as aptamers (Komarova and Kuznetsov 2019). This strategy has proven successful in isolating aptamers with high affinities and specificities against a wide array of targets ranging from whole cells to small molecules (McKeague, De Girolamo et al. 2015, McConnell, Nguyen et al. 2020, Saad and Faucher 2021). Indeed, we have previously used cell-SELEX to identify aptamers binding to *Legionella pneumophila* (*Lp*) bacterial cells (Saad, Chinerman et al. 2020).

One of the drawbacks of the traditional SELEX procedure is the use of multiple PCR amplification steps (Tolle, Wilke et al. 2014). Besides being laborious and increasing the time involved with the SELEX process, PCR amplification steps are prone to PCR bias and the production of artefacts (Tolle, Wilke et al. 2014). This means that sequences that are amenable to amplification are enriched rather than those possessing desired structural-functional properties. These highly complex aptamers may have delayed enrichment as well, meaning that earlier rounds of SELEX pools may contain potentially useful aptamers that are lost or missed, not because they are not binding to the targets, but because of methodological biases.

Reducing SELEX rounds and minimizing the number of PCR steps alone, however, would make it difficult to select and characterize high-affinity aptamers given the extensive size of the pool. To overcome this problem, a combination of high-throughput sequencing (HTS) and parallel selection, rather than iterative selection, could be used to streamline potential aptamers and select

for sequences with desired properties. This is because HTS enables deeper insights into the evolution of the SELEX sequence pools (Komarova, Barkova et al. 2020). This enables better comparative analysis on variations between different pools generated from parallel selections (Komarova, Barkova et al. 2020). Several studies have demonstrated how the use of HTS has improved SELEX outcomes (Dupont, Larsen et al. 2015, Stoltenburg and Strehlitz 2018). Indeed, the benefits of HTS has led to the development of several aptamer specific HTS data processing softwares such as FASTAptamer, APTANII and Aptasuite (Alam, Chang et al. 2015, Caroli, Taccioli et al. 2016, Hoinka, Backofen et al. 2018).

Parallel selection can also help SELEX outcomes by narrowing down the sequence pools. The principle behind parallel selection is that by exposing a series of different targets to the same initial library (input pool) the sequences in each specific target pool would evolve differentially, enabling the identification of unique aptamers that would provide insight on specific target properties and functionalities. For example, Dupont et al used a single round of parallel selection and exposed their sequence pools to different variants of the target protein plasminogen activation inhibitor -1 (PAI-1) (Dupont, Larsen et al. 2015). The pools that evolved in the presence of different variants of the target protein provided detailed information about aptamer binding sites, and the functional effects of these aptamers. Another group split their pool at the 4th and 11th selection rounds to perform parallel selections on target renal cell carcinoma (ccRCC) RCC-MF cell line and non-target RC-124 cell line from healthy kidney tissue (Pleiko, Saulite et al. 2019). They were subsequently able to evolve and select aptamers with differential binding properties.

In the present study, we explored the utility of using HTS and parallel selections, also referred to as branched SELEX, for identifying aptamers binding specifically to serogroup 1 *Lp.* This bacterium causes a pneumonia in humans and is transmitted via aerosols generated by engineered water systems (Lin, Vidic et al. 1998). Incidences of *Lp* infections have increased over recent years with serogroup 1 of *Lp* accounting for most infection cases (Canadian Notifiable Diseases Surveillance System (CNDSS) 2020, (ECDC) 2021, CDC 2021).

For the branched SELEX method conducted in this study, a pre-enriched library of sequences-input pool- was exposed to four different "branches"; (i) Two *Lp* serogroup 1 strains-referred to as target pool, (ii) *Lp* serogroup 5 strain-referred to as Sg5 pool, (iii) an *Lp* mutant lacking a gene (Δ*fliC*) that expresses filament subunit proteins of the flagella (Heuner, Bender-Beck et al. 1995,

Dietrich, Heuner et al. 2001), referred to as the Δ*fliC* pool, (iv) a cocktail of water-associated bacteria isolated from environments where *Lp* was present (Paranjape, Bédard et al. 2020), is referred to as the non-*Lp* pool (Figure 1). The objective of the latter branch was to isolate aptamers that would potentially have specific affinities for *Lp* in representative water environments. Each differentially evolved pool from each "branch" was then subjected to HTS. This combination of HTS and branched SELEX enabled the evaluation of aptamer pools that evolved in the presence of different *Lp* variants as well as non-*Lp* water bacteria. The pools were then examined for sequences that were enriched in the presence of target *Lp* and depleted in the presence of non-target bacteria. Additionally, since the method consists of doing four cell-SELEX processes in parallel, branched-SELEX significantly reduced the number of PCR rounds from the classical cell-SELEX method. To our knowledge this is the first study demonstrating the use of a single round of parallel selections i.e. branched-SELEX, with whole cell bacterial strains relevant to the application source environment.
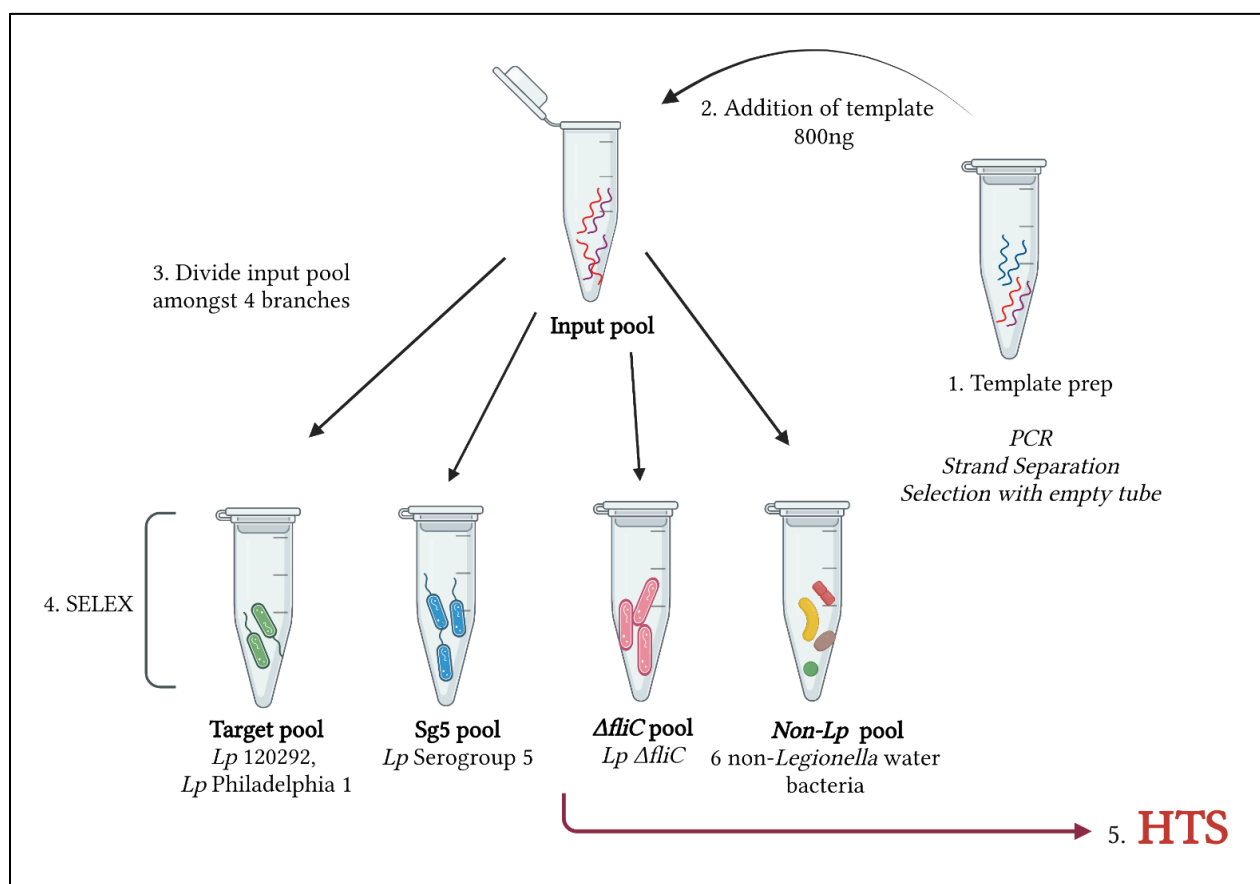
Figure 1: Branched-SELEX schema. Image created in Biorender (biorender.com)

5.3 MATERIALS AND METHODS

5.3.1 Bacterial Strains and Culture Conditions

A cocktail of the environmental *Lp* strain *lp120292*, isolated from a cooling tower implicated in the 2012 outbreak in Quebec City, (Lévesque, Lalancette et al. 2016) and *Lp* strain Philadelphia-1 (ATCC 33152) (Fraser, Tsai et al. 1977), were used as the target branch pool for aptamer generation. *Lp* serogroup 5, and Δ*fliC* were used as strains for each individual branch of the SELEX procedure. All *Lp* strains were cultured on CYE (ACES-buffered charcoal yeast extract) agar plates supplemented with 0.25 mg/ml L-cysteine and 0.4 mg/ml ferric pyrophosphate, at 37 °C for 3 days. For liquid culture, *Lp* were suspended in AYE (ACES-buffered yeast extract) broth supplemented with 0.25 mg/ml L-cysteine and 0.4 mg/ml ferric pyrophosphate until post-exponential phase (OD$_{600}$ of 2.5). A cocktail of non-*Lp* bacteria, which included isolates from cooling towers, namely, *Brevundiomonas sp., Bacillus licheniformus., Stenotrophomonas sp.* and *Sphingomonas sp.* as well as *Pseudomonas putida* KT2440 and *Pseudomonas fluorescens* LMG1794, were used as strains for another branch of SELEX (Paranjape, Bédard et al. 2020, Saad, Chinerman et al. 2020). These strains were first cultured on nutrient agar plates (Difco) at 30 °C for 24 hours and then grown in nutrient broth medium (Difco) overnight until the cultures reached post-exponential phase (OD$_{600}$ of 2.0-2.5). For each strain the concentration of cells was determined by evaluating plate counts to its corresponding OD$_{600}$.

5.3.2 Preparation of SELEX input pool

The input pool was created by PCR amplification of the original SELEX template that was generated after three rounds of positive selection, (R3) (Saad, Chinerman et al. 2020) . The PCR amplification conditions are outlined below in the section Branched-SELEX procedure. The forward and reverse primers were conjugated with fluorescein (FITC) and biotin, respectively. The forward primer (FP) sequence is 5'-fluorescein-GCAATGGTACGGTACTTCC-3'. The reverse primer (RP) sequence is 5'-biotin-TTAGCAAAGTAGCGTGCACTTTTG-3'. Following amplification, PCR products were purified using a MinElute PCR Purification Kit (Qiagen). The total amount of amplified template following purification was 3500 ng. The purified, biotinylated PCR products were then used in conjunction with streptavidin coated magnetic beads (Promega) to generate ssDNA. Streptavidin coated magnetic beads (Promega Technology) were used, according to the manufacturer's recommendation. Briefly, 600 µg of magnetic beads were washed

twice and then resuspended in 900 µl of washing buffer (phosphate buffered saline with 0.05% Tween 20). Next, approximately 3500 ng of PCR product was incubated with the magnetic beads for 10 min, mixing gently by inversion after every few minutes. The mixture was then washed in 1 ml of washing buffer. Finally, the beads were incubated with 500 µl of 200 mM NaOH for 5 minutes. The supernatant was then collected, and the FITC-labelled ssDNA was purified using ethanol precipitation as mentioned previously and quantified with a Nanodrop spectrophotometer (Thermofisher). The total amount of ssDNA recovered was 800 ng. Consequently, 800ng of ssDNA was used as the input pool for the branched-SELEX procedure. Prior to being used for SELEX, the input pool was incubated using a tube rotator with mild shaking for 1 hour at 25 °C in 13ml tubes (Sarstedt) with the absence of any bacteria in order to eliminate any nonspecific sequences that would bind to the reaction tubes.

5.3.3 Branched-SELEX procedure

Briefly, cell-SELEX was performed as previously described (Saad, Chinerman et al. 2020). One round of selection was performed for each branch (Figure 1). Each selection consisted of three steps: Binding, elution, and amplification.

5.3.3.1 Binding and Elution:

SELEX was performed with cells suspended in an artificial freshwater medium (Fraquil) to replicate the physiological state of nutrient-limited environmental conditions Fraquil was prepared as described previously with a final iron concentration of 10 nM and filter-sterilized using a 0.2 µm filter (Sarstedt) (Morel, Westall et al. 1975). Post-exponential phase cultures of all bacteria were rinsed twice with Fraquil ($6000 \times g$, 15 minutes) and suspended in Fraquil. *Lp* cells were suspended in Fraquil at an OD_{600} of 1 corresponding to a concentration of 10^9 CFU/ml for. The concentration of cells was confirmed by CFU counts. For bacteria from each branch (target *Lp* cocktail, *Lp* serogroup 5, $\Delta fliC$ and non-*Lp* bacteria cocktail) the suspensions were adjusted to 10^8 CFU/ml. The suspensions were incubated at room temperature for 24 h in Fraquil. Following Fraquil exposure, the cell suspensions were adjusted to obtain 10^7 cells per selection reaction. The Fraquil exposed cells were washed three times in 1X binding buffer (phosphate buffered saline with 0.1 mg/ml salmon sperm DNA, 1% bovine serum albumin, and 0.05% Tween 20) at room temperature (25°C) using $6,000 \times g$ for ten minutes. The cell pellets were then suspended in 330 µl of 1X binding buffer. The aptamer pool was denatured by heating at 95 °C for 10 minutes,

cooled immediately on ice for 10 minutes, and added to the cell suspension. Finally, 1X binding buffer was added to a total volume of 1 ml. The final mixture was incubated at 25 °C for 1 hour with mild shaking using a tube rotator at 50 rpm. Following incubation, the mixture was centrifuged at $6000 \times g$ for 10 minutes and washed twice with wash buffer (phosphate buffered saline containing 0.05% Tween 20) to remove unbound sequences. To elute the bound sequences from the cells, the final cell pellet was resuspended in 100 µl nuclease free water (Ambion) and heated at 95 °C for 10 minutes and immediately placed on ice for 10 minutes. After centrifuging at $6,000 \times g$ for 10 minutes at 25°C, the supernatant was collected and purified using overnight ethanol precipitation at -20 °C with 5 µg of glycogen as a carrier to recover the eluted ssDNA. The pellet was recovered, dried and suspended in nuclease free water (Ambion). The concentration and quality of the ssDNA was determined using a Nanodrop spectrophotometer (Thermofisher).

5.3.3.2 PCR amplification:

The purified ssDNA pool was then amplified by PCR with One Taq DNA polymerase (NEB), according to the manufacturer's protocol. All primers were used at a final concentration of 0.5 µM. PCR conditions were as follows: initial heat activation at 95 °C for 5 min and 25 cycles of 95 °C for 30 s, 56.3 °C for 30 s, 72 °C for 10 s, and a final extension step of 10 min at 72 °C. After amplification, the concentration and size of the PCR products were confirmed by gel electrophoresis using a 2.0% agarose gel. PCR products were then purified using a MinElute PCR Purification Kit (Qiagen). Samples were then sent for sequencing to the McGill Genome Centre. To verify that the characteristics of the pool change in response to selection pressure, a pool following ten rounds of positive selection, (R10), from a previous study was also sequenced and analyzed as a super-enriched control (Saad, Chinerman et al 2020).

5.3.4 High-throughput sequencing of branched SELEX samples

All high throughput sequencing steps were conducted by the McGill Genome Centre. For each PCR sample forward and reverse standard selection primers- (overhang followed by indexing PCR)- containing Nextera specific barcode sequences were used. Samples were then sequenced by amplicon sequencing using the ILLUMINA MISEQ platform with a v2 ,150 PE, kit (300 cycles).

5.3.5 Sequence data processing and analysis

Forward and reverse reads were sorted and parsed using the AptaSuite demultiplexing module. Reads included were (i) flanked by the constant primer binding regions (Forward 5'-GCAATGGTACGGTACTTCC-3' and Reverse 5'-CAAAAGTGCACGCTACTTTGCTAA-3'), (ii) had a variable region between 10 and 45 nucleotides and (iii) had a forward reverse sequence pair. The read pairs were then combined for each branched SELEX sample pool as well as the input pool. The AptaSuite Demultiplexer program was used to generate a text file of the sequences, each with a specific ID code along with sequence frequencies listed as counts per million (CPM)(Hoinka and Przytycka 2016, Hoinka, Backofen et al. 2018). This dataset was imported into Microsoft Excel for further analysis. For each sample pool approximately one million read pairs were obtained. Since Aptasuite processes reads for linear selections rather than across parallel selections, sequence pools were run and analyzed in batches, and the non-target pools were treated as "negative selection" rounds to enable parallel comparisons. For example, in one batch the input pool was the starting pool or round 0 of the selection process, the target *Lp* pool was the first round of selection, and the Non-*Lp* pool was a negative selection round. In another batch run, the input pool was the starting pool or round 0 of the selection process, the target *Lp* pool was the first round of selection, and the Sg5 pool was a negative selection round. In the third batch run, the input pool was the starting pool or round 0 of the selection process, the target *Lp* pool was the first round of selection, and the Δ*fliC* pool was a negative selection round.

5.3.5.1 Global characterization of sequence pools:

The proportion of sequences that were enriched, singletons or unique to a specific pool was determined using Aptasuite version 0.9.7 (Hoinka, Backofen et al. 2018) and plotted on Graphpad Prism 9.1.2 (Figure 2).

For Figure 3, sequence enrichments of all sequences present in a pool were calculated, by taking the ratio of a specific sequence's CPM in the branch pool to its CPM in the input pool. This ratio/enrichment of that specific sequence in the target pool versus the non-target pool was then plotted to globally visualize the characteristics of the pool and observe if there was a trend where a subset of sequences present in *Lp* target pools, are absent or depleted in non-target pools. All statistical analysis (Levene's Test, White's Test for heteroscedasticity, Standard Deviation determination) on sequence enrichment data were done using Excels Analysis ToolPak and XLstat.

5.3.5.2 Enrichment Analysis:

Next, to narrow down the pools and eliminate the probability a sequence was amplified due to random chance, sequences with low frequencies (1 copy per million sequences) and singletons were removed from the analysis. If a sequence was not present at least 2 times more in the target pool as compared to the input pool, then it was not analyzed further. Sequences that met the criteria were evaluated further for their enrichment across their respective branches.

To enhance identification of potential aptamers against target *Lp*, sequences that were present at least 2 times more in the target strain pool (*Lp* strains *lp120292* + Philadelphia-1), than in the corresponding branch pools, were selected and analyzed further. Amongst these selected candidates, full length sequences of 88 nucleotides were chosen for further investigation.

5.3.5.3 Alignment and secondary structure analysis:

T-COFFEE webserver (default settings) was used for multiple sequence alignment of target *Lp* candidate sequences selected following enrichment analysis (Notredame, Higgins et al. 2000). Secondary structures of candidate sequences were obtained using UNAfolds, DNA folding program with adjusted settings. (Zuker 2003).

5.3.6 Candidate aptamer binding assays

All aptamers were individually synthesized with FITC at the 5′ end by IDT (Integrated DNA Technology). Candidate aptamers were tested for their binding to *Lp* cells as well as to non-*Lp* bacterial isolates, by flow cytometry. Candidate aptamers were also tested alongside with *Lp* aptamer R10C5 as a positive control (Saad, Chinerman et al. 2020). All cells were suspended in Fraquil and prepared as described above for the SELEX procedure. Briefly, 100 nM of each aptamer was incubated in 1X binding buffer with 10^7 CFU/ml of the strain used for SELEX (*lp120292*), or with the strains used in the non-*Lp* cocktail (*Pseudomonas sp., Brevundimonas sp., Bacillus licheniformis., Sphingomonas sp.,* and *Stenotrophomonas sp.)* for 1 hour at 25 °C with mild shaking. As a negative control, bacteria were also incubated with 100 nM of a FITC-labeled scrambled sequence of aptamer R10C5 (5′-FITC-ACAGAATCAGTTCGAGTACATACGCGCGAAGACTCCTAAGGCCGTAGCGTTCTTCCC GGTAATACCATG). The suspension was centrifuged for 10 minutes at 6000 *g* to eliminate excess aptamer and resuspended. These suspensions were then analyzed using flow cytometry.

Analysis was performed on a Guava easyCyte (Millipore) using the green fluorescence channel. A total of 5,000 events were recorded. Cells suspended in binding buffer without any aptamer added were used as controls to measure autofluorescence. For analysis, a gate was first defined based on the forward and side scatters that included most of the cells. Then, a histogram of the number of cells vs the fluorescence intensity was used to define a region where cells were considered positive for green fluorescence and therefore stained with candidate aptamers. This region was setup to include very few cells of the unstained control and therefore represent fluorescence above the autofluorescence. Each experiment was done in triplicate and the percentage of stained cells was recorded. Statistical differences were assessed using ANOVA and Dunnett correction for multiple comparison using GraphPad Prism 9.1.2.

5.4 RESULTS

5.4.1 Choice of input pool

Analysis of sequence pools from our previously reported SELEX methodology (original SELEX) confirmed our prior findings where a noticeable decrease in sequence length was observed as the number of SELEX rounds increased (Figure S1). Figure S1 shows that the proportion of 88 nucleotide sequences, which correspond to the full length of the original randomized library, decreases as the number of SELEX rounds increase. This could lead to a loss of important secondary structures because of shortened sequence lengths. To reduce this adverse effect and ensure high diversity, we used a pre-enriched ssDNA pool, after three rounds of selection to *Lp*, as the input pool for our branched SELEX protocol. As shown in Figure 2, the pre-enriched pool is still diverse given it has a high proportion of sequences that are unrelated to sequences present in other pools (Unique fraction) and low levels of enrichment (Enriched fraction).

5.4.2 Evaluation of SELEX pools

Analysis of SELEX sequence pools show that the proportion of sequences that are enriched using the Branched SELEX protocol are low whereas multiple selection rounds result in a higher fraction of enriched sequences (Super-enriched pool, Figure 2). The branch pools are not significantly more enriched than the input pool (Figure 2). A likely reason for this low enrichment is due to the fact

there was only one selection round. The low enrichment also means there is a high proportion of unique sequences present, suggesting a highly diverse pool of sequences.

To confirm these observations and evaluate if a proportion of sequences were differentially amplified or present in target *Lp* pools versus the non-target branch pools, we plotted the enrichment of sequences in the target pool versus the enrichment of those same sequences in a non-target pool (Figure 3). A highly dispersed scatterplot would indicate that sequences were differentially enriched in the target *Lp* branch versus the corresponding non-target branch. Overall, it is observed that the sequence population of the three branch pools does not dramatically differ from the sequence population of the target *Lp* pool. However, the absence of a completely linear trendline in the plots signifies that there exists a small subset of sequences in the target pool that are differentially enriched in the non-target pools (Figure 3 a,b,c). The Whites test and Levenes test indicate that across each selection branch, the variances of the target pool are unequal to variances of the non-target pool. The two population pools are thus not correlated and evolve independently of each other.

Figure 2. The proportion of reads of different types of sequences from target *Lp* pool, Serogroup 5 *Lp* pool, Δ*fliC* mutant *Lp* pool and non-*Lp* bacteria pool. The Unique Fraction of each branch's pool refers to the proportion of sequences unrelated to sequences in the input pool. Singleton reads are sequences that appear once in each pool whereas Enriched reads refers to sequences that are present in the pool and increase in abundance from the input pool. Super-enriched pool serves as a control and refers to the sequences generated following 10 rounds of selection.

(a)

Serogroup 5 pool CPM/Input pool CPM

Std : 0.302
(Levenes Test, Whites Test)
[(α= 0.05, p<0.0001) , (α=0.05, p<0.0001)]

Target pool CPM/Input pool CPM

(b)

fliCΔ pool CPM/Input pool CPM

Std : 0.329
(Levenes Test, Whites Test)
[(α= 0.05, p< 0.0001) , (α=0.05, p<0.0001)]

Target pool CPM/Input pool CPM

(c)

Non Lp pool CPM/Input pool CPM

Std : 0.368
(Levenes Test, Whites Test)
[(α= 0.05, p=0.0001) , (α=0.05, p<0.0001)]

Target pool CPM/Input pool CPM

Figure 3. Evaluation of global sequence enrichment in target versus non target branch pools. X-axis indicates the enrichment of sequence in target pool while Y-axis shows enrichment of

195

sequences in non-target pool. Each point represents an individual sequence. (a) Enrichment of sequences in Serogroup 5 versus target pool. (b) Enrichment of sequences in mutant Δ*fliC* pool versus target pool. (c) Enrichment of sequences in non-*Lp* pool versus target pool.

5.4.3 Pool reduction and selection of candidate sequences

After eliminating singletons and low copy number sequences, sequences that were present at least 2 times more in the target pool as compared to the input pool, were selected. These sequences were then analyzed for enrichment across their respective non-target branches. The target enriched sequence was further selected if it was enriched at least 2 times more in the target pool than the non-target pool. The selection criterion was based on the premise that the sequence must be present or enriched in the target *Lp* pool and reduced or "lost" in the non-target bacterial pools. This approach reduced our candidate pools and enabled us to identify (i) 39 sequences that were present in the target *Lp* pool but "lost" in the non-*Lp* bacteria cocktail (ii) 30 sequences that were present in the target *Lp* pool but "lost" in the Δ*fliC* pool and (iii) 34 sequences that were present in the target *Lp* pool but "lost" in the Serogroup 5 pool (Table S1, Excel). The proportion of 88 nucleotide sequences in these pools were 70%, 48.4% and 64.7% respectively. Full length (88nt) sequences (corresponding to the length of the original template library) were mostly selected for further analysis. The list of candidate sequences, the non-target branch in which they were "lost" and their enrichments are given in Tables 1-3. Alignment analysis shows that there are no significant regions of similarity between candidates "lost" from or depleted in a common non target branch (Figure S2).

Aptamer ID	Sequence	Input pool CPM	Target pool CPM	Sg5 pool CPM	Target pool CPM / Input pool CPM	Sg5 pool CPM / Input pool CPM	Target enrichment / Sg5 enrichment
125251	GCAATGGTACGGTACTTCCCCATCCGCCCCCGTATGCACGTGTCCACCTCCCCTCACGCCGCACCAAAAGTGCACGCTACTTTGCTAA	1.03E-05	3.55E-05	5.48E-06	3.45	0.53	6.48
5210	GCAATGGTACGGTACTTCCCCTCCCACACCTGACGTCACGTACATCCTCTGCCCCCGCCAGCGCCAAAAGTGCACGCTACTTTGCTAA	0.000018	4.26E-05	8.22E-06	2.36	0.46	5.18
68337	GCAATGGTACGGTACTTCCCCAGCCCCCATCACATGCAGCCTGCCACTCCCGCCCCAGCACATCCAAAAGTGCACGCTACTTTGCTAA	1.03E-05	2.37E-05	5.48E-06	2.29	0.53	4.32
91549	GCAATGGTACGGTACTTCCCACGTCCACACTGACTGCCAGGTTCTGCTCCCGCCCACAAAAGTGCACGCTACTTTGCTAA	1.29E-05	0.000026	5.48E-06	2.02	0.43	4.75

Table 1. List of candidate sequences that met threshold criteria and were "lost" or less abundant in Serogroup 5 selection pool but enriched in target selection pool. CPM refers to counts per million. To determine enrichment the ratio of CPMs were calculated. The enrichment/increase in target pool versus non-target pool was calculated by taking the ratio of the target enrichment to non-target enrichment.

Aptamer ID	Sequence	Input pool CPM	Target pool CPM	ΔfliC pool CPM	Target pool CPM / Input pool CPM	ΔfliC pool CPM / Input pool CPM	Target enrichment / ΔfliC enrichment
72190	GCAATGGTACGGTACTTCCGGCCACCGCCACCGTTAGCACAACTTGTATGTCCTTGCCCACCGCCAAAAGTGCACGCTACTTTGCTAA	1.03E-05	3.08E-05	3.82E-06	2.99	0.37	8.05
68339	GCAATGGTACGGTACTTCCCCAGCCCCCATCACATGCAGCCTGCCACTCCCGCCCCAGCACATCCAAAAGTGCACGCTACTTTGCTAA	1.03E-05	2.37E-05	3.82E-06	2.29	0.37	6.19
34372	GCAATGGTACGGTACTTCCCCCCTTACGCCTGCTGCCCTGTACTTTGGTCCCCCAAAAGTGCACGCTACTTTGCTAA	1.03E-05	2.37E-05	3.82E-06	2.29	0.37	6.19
28172	GCAATGGTACGGTACTTCCCACGTGTATACACCCCGGTACTCCCACCCACTTCCCGTCCGCCCCCAAAAGTGCACGCTACTTTGCTAA	1.8E-05	3.79E-05	3.82E-06	2.10	0.211	9.92
90478	GCAATGGTACGGTACTTCCCCGCCCCGCCCACGCTCCACTATGATCCAAGCCTTGACCCTGTGCCAAAAGTGCACGCTACTTTGCTAA	1.03E-05	2.13E-05	3.82E-06	2.07	0.37	5.58

Table 2. List of candidate sequences that met threshold criteria and were lost or less abundant in Δ*fliC* selection pool but enriched in target selection pool. CPM refers to counts per million. To determine enrichment the ratio of CPM was calculated. The enrichment/increase in target pool versus non-target pool was calculated by taking the ratio of the target enrichment to non-target enrichment.

Aptamer ID	Sequence	Input pool CPM	Target pool CPM	Non-Lp pool CPM	Target pool CPM / Input pool CPM	Non-Lp pool CPM / Input pool CPM	Target enrichment / Non-Lp enrichment
77293	GCAATGGTACGGTACTTCC GCACGCCCGCCGAACGCC ACATACCCTATCGTAGTAC CCACGCCCCAAAAGTGCAC GCTACTTTGCTAA	1.29E-05	4.5E-05	6.81E-06	3.49	0.53	6.60
19654	GCAATGGTACGGTACTTCC CCACACCCTGGGCGCCTAT GTAGACTGCACACGTACCA CTCGTCCCAAAAGTGCACG CTACTTTGCTAA	1.29E-05	4.02E-05	3.41E-06	3.12	0.26	11.81
79396	GCAATGGTACGGTACTTCC CCCCCGCACGTGCCTCGCT ACCCTGGCAAAAGTGCAC GCTACTTTGCTAA	1.03E-05	2.6E-05	6.81E-06	2.52	0.66	3.82
11547	GCAATGGTACGGTACTTCC CCCACACCCGCTATCGCAT GTCACCTTCTGCACTACCT CCACTGCCAAAAGTGCAC GCTACTTTGCTAA	1.29E-05	3.08E-05	3.41E-06	2.38	0.26	9.03
39728	GCAATGGTACGGTACTTCC CCACGGCCATATCCCACGT CCAGTACTTCTTACCGACC CGCCACCCAAAAGTGCAC GCTACTTTGCTAA	1.29E-05	2.6E-05	3.41E-06	2.02	0.26	7.64

Table 3. List of candidate sequences that met threshold criteria and were "lost" or less abundant in non-*Lp* selection pool but enriched in target selection pool. CPM refers to counts per million. To determine enrichment the ratio of CPM was calculated. The enrichment/increase in target pool versus non-target pool was calculated by taking the ratio of the target enrichment to non-target enrichment.

5.4.4 Enrichment Profile of Candidate Sequence Across non-target branches

The selected candidates in Tables 1-3 were further analyzed for their abundance/enrichment in other non-target branch pools. Aptamer candidates that were absent or most reduced in specific non-target pools were only chosen for further testing. For example, although sequence 125251

(Figure 4a) is highly enriched in the target pool and depleted in the Sg5 pool it was not selected for further analysis because it is partially enriched in non-*Lp* pools. This means they would bind to a broader array of non-target bacteria since the non-*Lp* pool has several water bacteria strains.

The selected candidates, therefore, were aptamer 5210 (Figure 4a), 68339 (Figure 4b), 19654 and 11547 (Figure 4c). Aptamer candidate 19654 and 11547 were enriched in the *Lp* target pool and absent in the non-*Lp* branch pool (Figure 4c). Both 19654 and 11547, however, are present in Sg5 and Δ*fliC* branch pools. Aptamer candidate 68339 is enriched in the target *Lp* pool, significantly depleted in the Δ*fliC* branch pool and depleted in the Sg5 and non-*Lp* branch pools (Figure 4b). Aptamer candidate 5210 is highly enriched in the target *Lp* pool but significantly depleted in Sg5 branch pool and the non-*Lp* branch pool (Figure 4a). Candidate 5210 is also slightly enriched in the Δ*fliC* branch pool.

All the candidates also have complex stable secondary configurations under low salt and high salt conditions, with aptamer 5210 retaining the same secondary structure conformation in both low and high salt settings (Figure 5). Indeed, almost all candidates also possess a distinct bulge region, which is identified as the dominant structural element/motif, following multiple rounds of selection with target *Lp* (Figure S3).
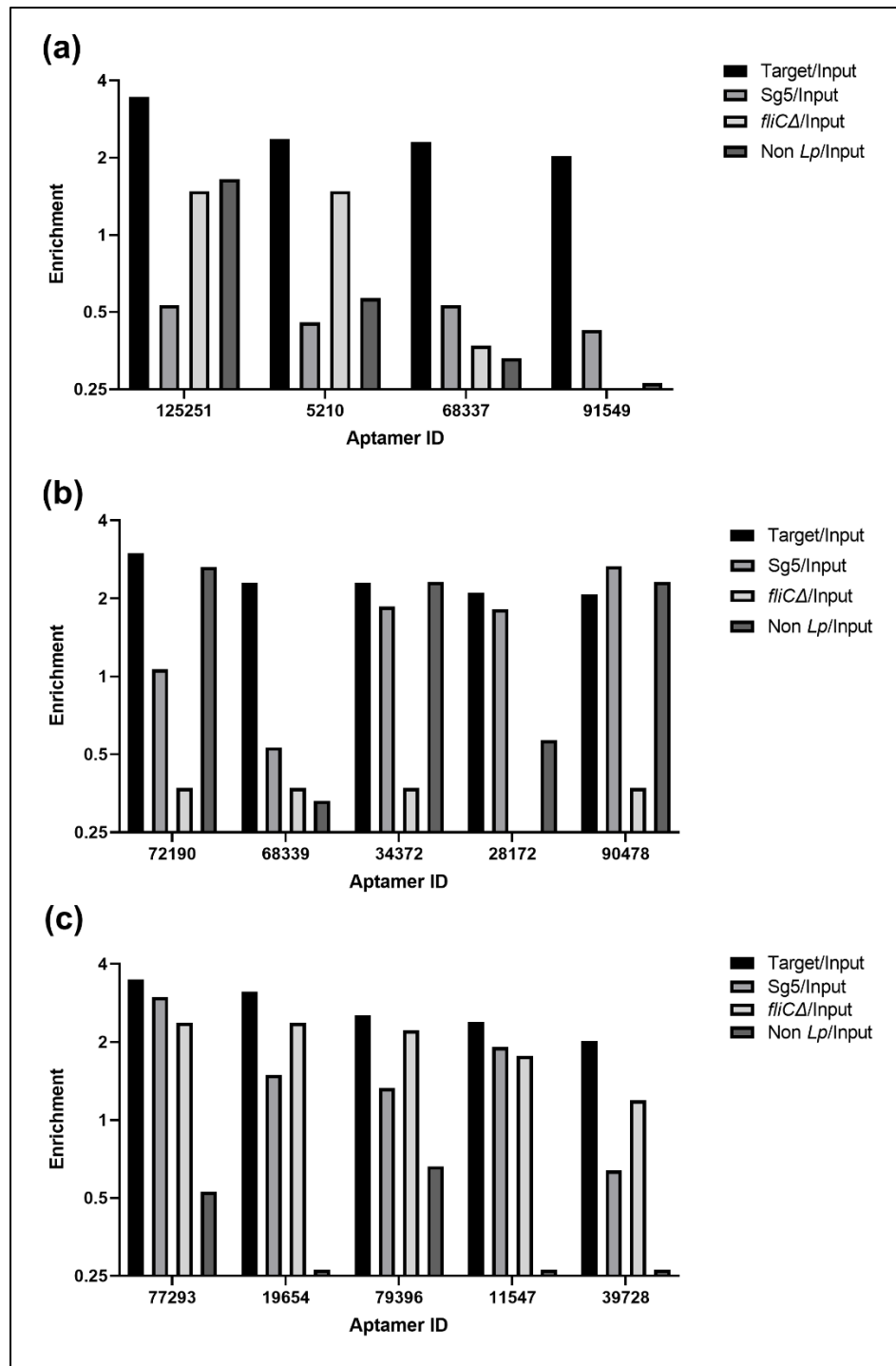
Figure 4. Enrichment profile of candidate sequences across different branches. The enrichment is a ratio of sequence present in a specific branch versus the input pool**.** The enrichment is presented on a logarithmic scale. (a) The enrichment of candidate sequences (abundant in the target pool but "lost" or depleted in Serogroup 5) across all branches. (b) The enrichment of candidate sequences

(abundant in the target pool but "lost" or depleted in Δ*fliC*) across all other branch pools. (c) The enrichment of candidate sequences (abundant in the target pool but "lost" or depleted in non-*Lp*) across all other branch pools.

(a)
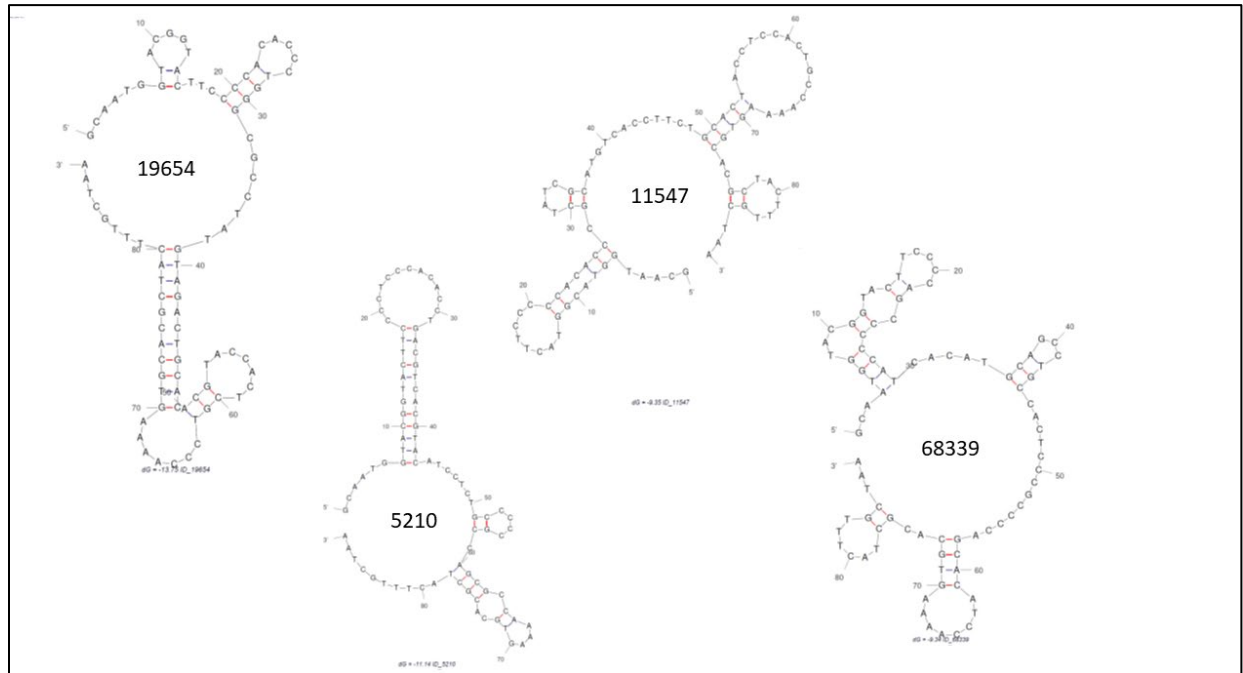
(b)
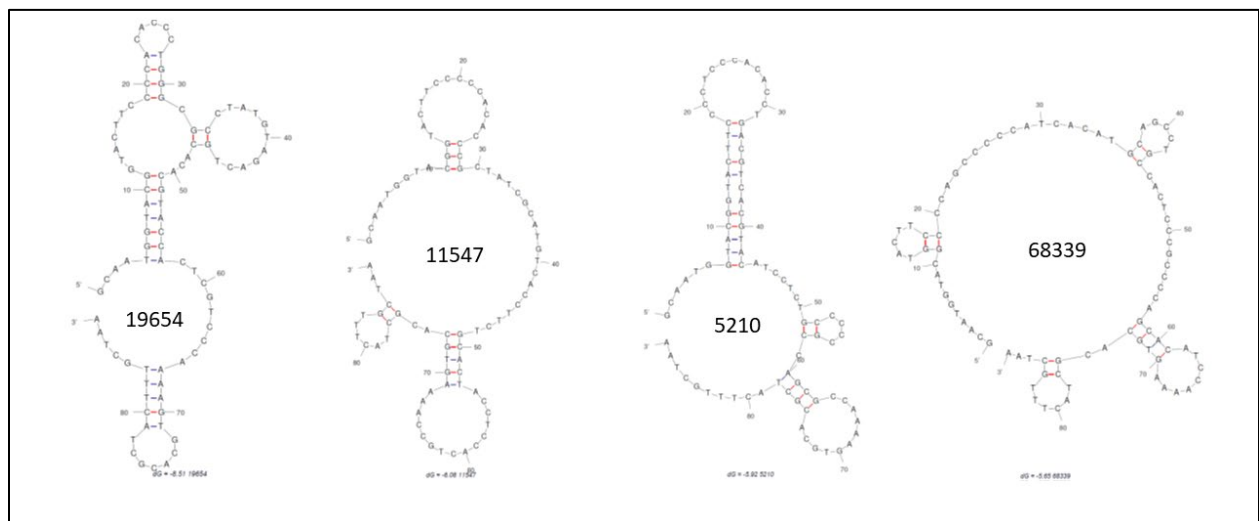
Figure 5. Secondary structures of selected candidate sequences as predicted by UNAfolds, DNA folding module, under modified settings, (a) 0.7-1M [Na$^+$], 25° C and (b) 100mM [Na$^+$], 25° C.

5.4.5 Candidate Aptamer Binding Assays

Figure 6 shows the binding of aptamers 19654, 11547, 5210, and 68339 to *Lp* and non-*Lp* environmental isolates. Neither aptamer stains more *Lp* cells than the positive control R10C5. Scrambled is shuffled R10C5 sequence and serves as a negative control indicating a random sequence would not bind better. All four candidate aptamers show very low binding (less than 10%) to non-*Lp* environmental isolates. Though aptamers 5210 and 68339 abundance were reduced in the non-*Lp* pool, in contrast to 11547 and 19654 that were completely depleted and "lost", they also exhibited minimal binding to environmental isolates (Figure 6c and d).

Given that 5210 was significantly reduced in the Serogroup 5 pool and 68339 was significantly reduced in the $\Delta fliC$ pool we investigated the binding ability of 5210 to Serogroup 5 and 68339 to the $\Delta fliC$ (Figure 7). Aptamer 5210 shows reduced binding to serogroup 5 *Lp* compared to target serogroup 1 *Lp*. Aptamer 68339 binds $\Delta fliC$ strain and does not seem to significantly discern between target *Lp* and $\Delta fliC$.

Figure 6. The specificity of FITC labelled aptamers (a) 19654 (b) 11547 (c) 5210 (d) 68339 binding to target *Lp* strain *lp120292* and to non-*Lp* cooling tower isolates, was analyzed by flow cytometry. The percentage of cells stained by each aptamer are presented. The binding of a scrambled sequence of aptamer R10C5 was used as a negative control whereas R10C5 was used as a positive control. The values of three experiments are shown with the mean and standard deviation. A two-way ANOVA with a Dunnett correction for multiple comparisons was used to infer statistical significance compared to positive control namely, R10C5 aptamer+ *Lp* strain *lp120292*. No significant difference was observed between R10C5 labelling of *Lp* or aptamer candidates of *Lp*. No significant difference was observed between R10C5 labelling or aptamer candidates for each non *Lp* environmental isolate   ****$P < 0.0001$; ns, not significant.

Figure 7. The binding of aptamer (a) 5210 to target *Lp* strain *lp120292* and to Serogroup 5 strain was analyzed by flow cytometry. Similarly, the binding of aptamer (b) 68339 to target *Lp* strain *lp120292* and to Δ*fliC* strain was analyzed by flow cytometry. The percentage of cells stained by each aptamer are presented. The binding of a scrambled sequence of aptamer

R10C5 was used as a negative control whereas R10C5 binding to *Lp* was used as a positive control. Cells refer to no aptamer added. The values of three experiments are shown with the mean and standard deviation. A two-way ANOVA with a Šídák correction for multiple comparisons was used to infer statistical significance. ****$P < 0.0001$; ns, not significant. (b) 68339 binds Δ*fliC* strain and target *Lp* strain whereas (a) 5210 binds target *Lp* strain but shows minimal binding to serogroup 5 strain.

5.5 DISCUSSION

A single round of parallel selecion (brached SELEX) was combined with HTS to reduce the sequence pools and select for sequences that are absent or depleted in the presence of non-target bacteria but amplified in the presence of target *Lp*. This strategy circumvents the need for multiple selection rounds or synthesis of thousands of individual sequence candidates for preliminary characterization as potential aptamers.

Initial analysis shows that the fraction of unique sequences decreases as the number of selection rounds increase (Super-enriched pool, Figure 2) in an iterative classical SELEX strategy. One round of selection, as in the Branched SELEX method, results in higher proportion of unique sequences in each pool (Figure 2). This is in part due to the fact that cellular targets are multimeric,complex and thus have muliple binding sites (Kalra, Dhiman et al. 2018). It is observed that one round of selection, as in Branched SELEX, results in highly diverse sequences given the diversity of epitopes of the target cells. While doing selection on renal cell lines Pleiko et al observed similar trends where the 4th round of selection had a higher proportion of unique sequences as opposed to the 11th round of selection (Pleiko, Saulite et al. 2019). The high complexity and diversity of the cell targets also means that the proportion of enriched sequences, after one round of selection, are significantly smaller compared to the proportion of enriched sequences following multiple rounds of selection (Figure 2). Large multimeric targets such as cells also have a higher chance of common aptamer binding targets (Figure 3) (Kalra, Dhiman et al. 2018).This is observed when the enrichment of sequences in the target pools are compared to the enrichment of sequences in the non-target pools (Figure 3). A widely distributed scatterplot would signify that a large number of sequences evolve differentially, however we observed only a subset of sequences (0.004%)- ~$40/10^6$- that are differentially enriched. The proportion of this subset was

relatively similar regardless of the type of non-target used. In fact only a small fraction of sequences ~0.03% ,from the target pool was enriched by at least 2 times . These observations highlights a limitation of this technique. Reduced number of rounds for large targets means that sequences retreieved are present in low abundance and this makes it difficult to search for statistically signficant, differentially binding aptamers. Proteins and small molecules, by contrast, have a smaller and finite number of binding pockets and interactions, as comapared to cells, which can facilate the streamlining of a large pool of sequences (Kalra, Dhiman et al. 2018).

The broad diveristy of the type of sequences, isolated from just one round of selection, is also illustrated in the alignment analysis (Figure S2). Despite being similarly enriched in the target *Lp* pool and depleted or absent in a common non-target branch pool, the selected candidate sequences had no regions of similarity or common motifs amongst them.

 One round of selection also means that the probability of identifying non-specific aptamers also increases. Indeed, our enrichment analysis shows that the majority of the candidate sequences were present across the different branches/non-target pools despite being selected for their absence and reduction in a specific non-target (Figure 4). For example aptamer ID 90478 , was enriched in the Non – *Lp* cocktail pool and Serogroup 5 pool despite being reduced in the Δ*fliC* pool (Figure 5b). This is similar to observations made by Plieko et al 2019 who found that after 4 rounds of selection, there were sequences that bound to their negative selection renal cell lines (Pleiko, Saulite et al. 2019).

Stochastic variations due to handling steps can also affect sequence enrichment analysis (Spill, Weinstein et al. 2016, Alam, Chang et al. 2018). When identifying broad spectrum RNA aptamers using a poly-target, paralell selection approach, Alam et al 2018 noted that replicates of their sample pools, following three rounds of selection, showed a multimodal distribution for many sequence populations. This means that despite the similar selection protocol given the same target, a different subset of sequences was either enriched, reduced or stayed neutral (Alam, Chang et al. 2018). The target for this study was lentiviral reverse transcriptases. Given the complexity and large diversity of our target pools –mulitmeric, live bacterial cells at 10^7 cells/ml- random variations in sequence frequencies could be similar to stochiastic variations due to handling steps, however a replicate analysis would be interesting to observe if variations in sequence popoulation pools are similar across replicates.

Given the small subset of enriched sequences,the stringency of the Branched SELEX protocol was ehnanced by leveraging the parallel selections and selecting candidate aptamers that were absent or reduced in other non-target pools as well (Figure 4). These aptamers were 19654, 11547, 5210 and 68339. All four aptamers bound to target *Lp* and neither significantly bound to strains from the non *Lp* pool (Figure 6). Aptamers 19654 and 11547 were expected to be selective for *Lp* given they were absent in the non- *Lp* cocktail. Aptamer 5210 is also selective for target *Lp* and even discerns between Serogroup 5 and serogroup 1 *Lp* given that the target *Lp* (*lp120292*) is serogroup 1 strain (Figure 7a). Bacteria belonging to a specific serogroup have a common surface expressed antigen. Different serogroups occur due to variations in the O-antigen chain of the lipopolysaccharide (LPS) structure of the bacteria (Lerouge and Vanderleyden 2002). LPS is also considered to be one of the most abundant surface molecules present on the bacterial membrane (Le Brun, Clifton et al. 2013). Given this result, it is possible that aptamer 5210 binds to LPS of serogroup 1 *Lp*, but additional experiments, beyond the scope of this manuscript will be required to confirm this. Structural analysis also reveals that the secondary structure of 5210 remains unchanged in high and low salt conditions, illustrating the potential stability of this aptamer (Figure 5). However,examining the enrichment profile does not guarantee specific aptamers. Indeed, aptamer 68339 does not discern between Δ*fliC* mutant *Lp* and target *Lp* (Figure 7b) despite being reduced in the Δ*fliC* pool and enriched in the target *Lp* (Figure 4b). This could be a consequence of the minimal number of selection rounds leading to non specific sequences (Pleiko, Saulite et al. 2019).

All aptamer candidates had complex secondary structures in both high and low ionic strength conditions (Figure 5). The secondary structures were analyzed in both conditions since the ionic strength of water can vary depending on the source environment, minerals, presence of disinfectants and residuals etc. The secondary structures all possessed an internal bulge region. This is interesing since structural prediction analysis with Aptasuites AptaTrace module shows that following 10 rounds of positive selection, the most common secondary structural element that becomes enriched in later rounds is the bulge region (Figure S3). Tracking the enrichment of secondary structural elements/motifs improves aptamer selection since aptamers recognize and bind to their targets by way of secondary structural elements. However a challenging aspect of structural analysis is the extremely high diversity of the sequence pools following one round of selection and the subsequent inability of several computational softwares (MEME, Aptasuite) to

identify an extensive dataset of non-canoncial secondary structures (Song, Zheng et al. 2019, Komarova, Barkova et al. 2020). Furthermore these programs were developed using RNA libraries (Komarova, Barkova et al. 2020). Both these factors make it difficult to analyze structural motif enrichment in a meaninful way.

5.6 CONCLUSION

Branched-SELEX in combination with HTS is a rapid way to enable identification of application-specific aptamers ,binding to a particular target, from a diverse pool of sequences. However this method has several caveats for large complex targets , such as cells, which primarly include the limited evolution of the sequence pools given the vast diverisity of targets. Examining the enrichment of candidate sequences across non-target branch pools alone is challenging for identifying specific aptamers. Additional strategies are needed that would narrow down the pools further and improve aptamer selection. An additional round of selection with replicates would meaningfully reduce the pool and identify statistically significant, differentially binding aptamers. In addition to sequence enrichment, the enrichment of motif/structural elements should also be considered , to potentially enhance this Branched SELEX approach and highlight aptamers with key secondary structures that are selected for and thus contain the desired binding properties.

5.7 ACKNOWLEDGMENTS

5.8 SUPPLEMENTARY DATA
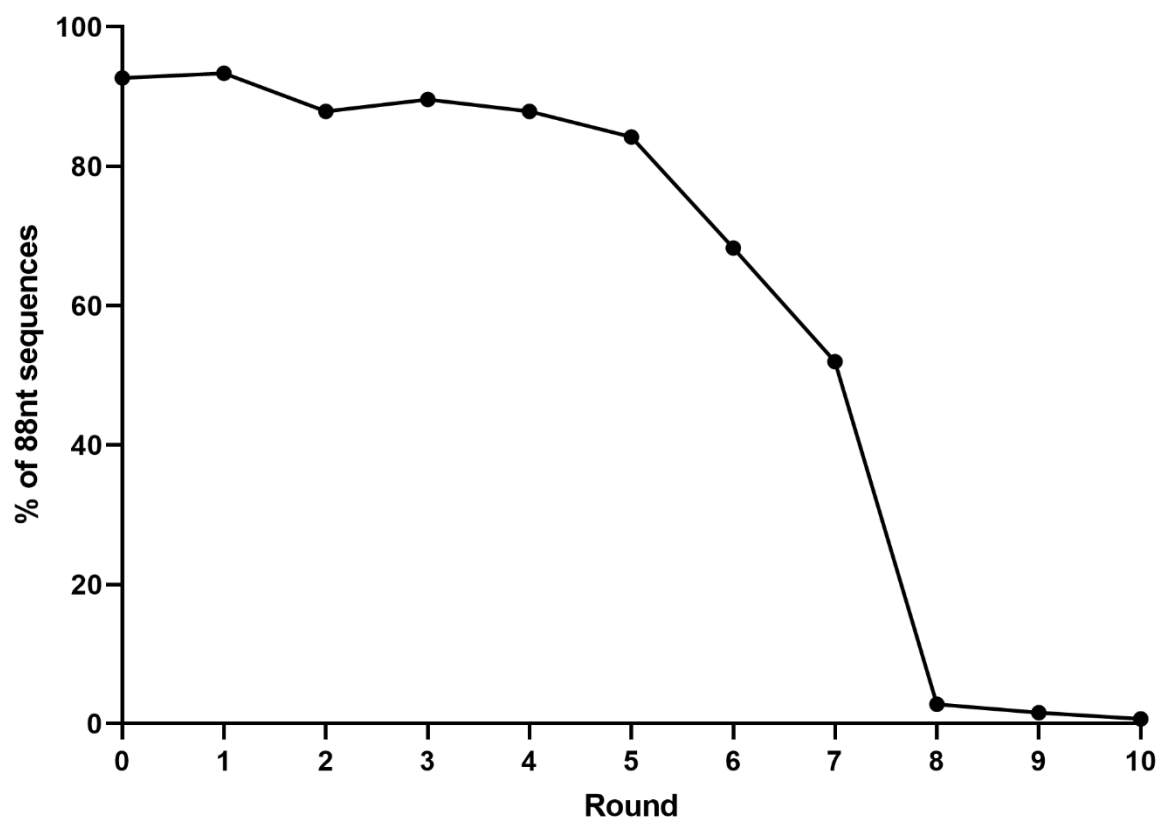
Figure S1: The proportion of 88 nucleotide (nt) sequences present in each SELEX round. The fraction of sequences that are 88 nt decrease with increasing SELEX rounds.

```
SCORE=466
*
 BAD AVG GOOD
*
SgV5210       :    47
SgV68337      :    47
SgV91549      :    44
SgV125251     :    47
cons          :    46

SgV5210       CCTC-CCACACCTGACGTCA-CGT-ACATCCTCTGCCCCCGCCAGCGC
SgV68337      CCAG-CCCCCATCACATGCAGCCT-GCCACTCCCGCCCCAGCA-CATC
SgV91549      CACGTCCACAC-TGACTGC-------CAGGTTCTGCTCCCGC---CCA
SgV125251     CCAT-CCGCCCCGTATGCA-CGTGTCCACCTCCCCTCACGCC-GCAC

cons          *    ** *        *        *      *   *  *   **
```

```
SCORE=343
*
 BAD AVG GOOD
*
fliC28172     :    32
fliC68339     :    31
fliC72190     :    33
fliC34372     :    35
fliC90478     :    34
cons          :    34

fliC28172     CACGTGTATA-CACC-CCGGTACTCCCACCCACTTC-CCGT---CCGCCCC
fliC68339     CCAGCCCCCATCACA--TG-CAG--CCTGCCACTCC-CGCCCCAGCACATC
fliC72190     GGCCACCGC--CACCGTTAGCACA-ACTTGTATGTCCTTGC---CCACCGC
fliC34372     CCCTTACG-----CC--TG-CTG--CCCTGTAC-TT-TGGT------CCCC
fliC90478     CCGCCCCGCC-CACG-CTC-CACTATGATCCAAGCCTTGAC---CCTGTGC

cons            *          *              *             ***    *
```

```
SCORE=443
*
 BAD AVG GOOD
*
Non-Lp11547   :    45
Non-Lp19654   :    44
Non-Lp77293   :    45
Non-Lp79396   :    47
Non-Lp39728   :    45
cons          :    44

Non-Lp11547   CCCACACCCGC-TATCG--CATGTCAC---CTTCTGCA-CTACCTCCACTGC
Non-Lp19654   CC-ACACCCT--GGGCGCCTATGTAGA---CTGCA-CACGTACCACTCGTCC
Non-Lp77293   GC-ACGCCCGCCGAACG-CCACATACC---CTATCGTA-GTACCC-ACGCCC
Non-Lp79396   CC-CC---C-------G--CACGTGC----CT----CG-CTACC----CTGG
Non-Lp39728   CC-ACGGCCA--TATC--CCACGTCCAGTACTTCTTAC-CGACCC-GCCACC

cons          *  *   *     *       *  *     **        ***
```

Figure S2: Multiple sequence alignment of randomized region of candidate sequences that were abundant in target pool but diminished in non-target branch pools namely Serogroup 5, Δ*fliC,* and non-*Lp* pools (T-coffee webserver, default settings).
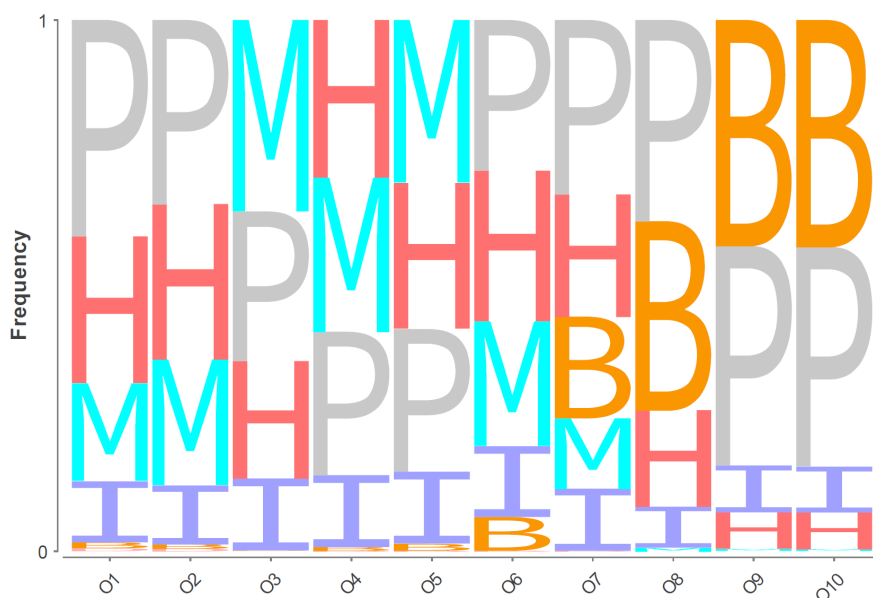
Figure S3: Type and frequency of motifs over ten selection rounds. Each column corresponds to a selection round whereas the letters H, B,I,M,D, and P correspond to the elements; Hairpin, Bulge Loop, Inner Loop, Multiple Loop, Dangling End, and Paired. Each letters height is proportional to the probability of its occurrence in the pool. (Data generated in Aptasuites AptaTrace module v.0.9.7)

Table S1: Excel File

List of Sequences Enriched in Target Pool & Depleted in Non-Target Pool

TableS1_Shortlist_Sequence_EnrichedinTargetpool_DepletedinNontarget

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CHAPTER 6: DISCUSSION

6.1 General Discussion

The detection and characterization of infectious microbial agents is key for investigating outbreaks and perform epidemiologic studies (van Seventer and Hochberg 2017). Timely detection and characterization of infectious microbial agents also augments public health, food safety, well as water and environmental quality control (van Seventer and Hochberg 2017). Propagation of infectious microbial agents has heavy socioeconomic implications that extend far beyond the metrics of number of deaths or mortality as was all too evident in the recent global SARS-COV-2 pandemic (Gadermann, Thomson et al. 2021, UNDP 2022). The presence of effective detection and diagnostic tools, therefore, plays a crucial role in the spread of infectious microbial agents such as viruses and pathogenic bacteria.

Detection methods for bacterial pathogens such as *Legionella* have been heavily reliant on traditional methods such as the growth of organisms on selective, artificial growth media followed by characterization of this growth using biochemical and/or nucleic acid-based testing (Rajapaksha, Elbourne et al. 2019). The time, labour, cost, and complexity associated with traditional methods necessitates the development of new tools and methods that would facilitate detection and thus timely interventions to minimize the spread of any bacterial pathogen (Rajapaksha, Elbourne et al. 2019). To be viable and surpass traditional detection methods these new tools would need to be automated, sensitive, and rapid. Biosensors are the only analytical devices that come close to fitting the bill (Turner 2013). Ideally, the category of biosensors which, along with the aforementioned properties, are user-friendly, portable devices easily used by non-specialists, for point of care or in situ analysis (Turner 2013).

A biosensor incorporates a biorecognition element and harnesses the specific, sensitive interaction of said element with its cognate target to transduce physico-chemical phenomenon, the result of which is a meaningful bioanalytical measurement (Turner 2013). Given its multiple components, the development of a biosensor is a very complex undertaking and requires consideration and validation of many elements prior to being a meaningful tool for real world applications such as *Lp* detection.

The goal of this thesis was to develop a strategy to detect *Lp*, using aptamers with a SPR sensor, that would supplement the traditional plate count and nucleic acid amplification detection methods. This biosensing set-up would be further developed for field applications i.e portable, rapid, sensitive, specific, automated device. Indeed, much of the work in this thesis is preliminary, requiring an understanding of and thus manipulation of target properties -to develop aptamers as our biorecognition elements- as well as the physical optical phenomenon of the SPR biosensor to detect *Lp*.

To achieve this goal the first objective was to develop aptamers, the biorecognition element, binding specifically to *Lp*. To ensure that the target for the *Lp* aptamer was in its native state, Cell-SELEX was used. The target *Lp* was a strain from the Quebec City 2012 outbreak and was grown to post-exponential phase followed by exposure to Fraquil (nutrient poor media) to ensure the bacteria were in their transmissive phase and representative of the application environment. The enrichment of the pool for *Lp*-binding sequences was observed via flow cytometry. After 10 rounds of positive selection, interspersed with two rounds of negative selection, just two aptamers were identified by cloning and sequencing. These two aptamers were then characterized using flow cytometry and microscopy.

In Chapter 3, we chose Cell-SELEX to develop aptamers against *Lp*. To generate robust aptamers against whole cells, Cell-SELEX has been a widely used method given that it eliminates the arduous task of selecting a specific biomarker to isolate, purify and enrich as targets for selection (Kaur 2018). This is important given *Legionella's* broad species diversity in the environment which makes it more complex to select a single molecular biomarker (Duron, Doublet et al. 2018). For clinical use, since serogroup 1 is the most dominant isolate (National Academies of Sciences and Medicine 2020), it would have been useful to create aptamers using LPS of serogroup 1 strains, but steps would have to be undertaken to ensure the target remains in its native conformation following the cumbersome purification and isolation of the LPS target. Furthermore, in an environmental detection context, the stability of LPS molecules means that non-viable *Legionella* could still be detected following heat or biocide-based water disinfection protocols (Gao, Wang et al. 2006, Xue, Zhang et al. 2019) . This has important implications when assessing the risk or hazard of *Legionella* burden. It would be costly and impractical to shut down a whole water system on account of dead nonpathogenic *Legionella* in a system.

Broad affinity aptamers would have been an attractive option for detecting various serogroups of the *Legionella pneumophila* subspecies as well as other environmental members of the *Legionella* genus. A modification of the Cell-SELEX method, called Sequential Toggle-SELEX was used to identify aptamers against bacteria of different genera- *Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae, Citrobacter freundii, Bacillus subtilis, and Staphylococcus epidermidis* - by "toggling" or introducing different bacteria at each selection round (Song, Nguyen et al. 2017). The authors were successful in identifying aptamers with broad affinity to six bacterial taxa. To isolate such aptamers, one could envision a Cell-SELEX procedure where the selection rounds would "toggle" between cocktails/mixtures of *Lp* of varying serogroups as well as other members of the *Legionella* genus. However, in this case, an extensive number of negative selection rounds with non-target bacteria would need to be strictly included to minimize the number of selection rounds and the probability of obtaining non-specific aptamers (Hamula, Peng et al. 2015).

As mentioned in Chapter 1, *Legionella* exists in planktonic forms transiently as part of a multispecies biofilm and, predominantly, intracellularly in protozoan hosts. Typically to detect total *Lp* in a system, nucleic acid-based markers are utilized (Wang, Bédard et al. 2017). For example, *Lp* from cooling tower water was previously detected with SPRi by quantifying *Legionella* 16S rRNA (Foudeh, Trigui et al. 2015). In terms of sample processing steps, this method is similar to other nucleic acid-based methods such as qPCR (Foudeh, Trigui et al. 2015). For intracellular microbial detection, previous work has shown it is possible to isolate aptamers that are specific to infected host surface proteins, facilitating detection of host-microbial complexes. Indeed, Oteng et al successfully identified high affinity aptamers against red blood cells that were infected with the malaria causing *Plasmodium falciparum* parasite (Oteng, Gu et al. 2020). The authors hypothesized that the infected erythrocytes encoded specific surface proteins and structures enabling development of such specific aptamers. A project, involving a European consortium from academia and industry, attempted to identify aptamers against the *Legionella*-Amoeba complex but to date no results from this work have been made publicly available (APTARES-Eurostars 2016). Developing a viable aptamer against a *Legionella*-host complex is not a trivial task given the bacterium's broad natural host range, the diversity of *Legionella*-protozoa interactions and the wide array of factors that influence these interactions all of which require further investigation (Boamah, Zhou et al. 2017). These aspects add to the complexity of developing aptamers relevant to intracellular *Lp* in environmental field applications.

Given the extensively studied and established models of *Legionella*-amoeba infection it may be useful to develop aptamers against such a target for preliminary work (Boamah, Zhou et al. 2017), but ,even though they are the natural reservoirs for the bacteria in the environment, developing aptamers against *Legionella's* other protozoan hosts would be impractical given the broad diversity and poor characterization of these hosts (Boamah, Zhou et al. 2017)..

Given the complexity of identifying a single biomarker that would detect all total, membrane intact *Lp* in a system, a single outbreak associated *Lp* strain was chosen as the target. Consequently, for our Cell-SELEX we did 13 rounds of selection (ten positive and two negative) all the while ensuring that our target *Lp* would have properties that are representative of *Legionella* in real water environments. To further streamline our SELEX process, we used two *Pseudomonas* strains – common inhabitants of water systems (Paranjape, Bédard et al. 2020)- for negative selection.

An additional factor we considered for our Cell-SELEX process was the high number of selection rounds which could lead to PCR bias. To mitigate this effect, we performed a preparative PCR step in the first 3 rounds (data not shown) to determine the optimal number of PCR cycles (Sefah, Shangguan et al. 2010).

Following aptamer identification, the next step was to incorporate aptamers on the SPR platform. Given the specific constraints of the SPR sensor discussed in Chapter 1 and 4, such as the limited penetration of the evanescent field where changes in the refractive index are captured as well as the diffusion-limited mass transport of large targets, we developed the titration assay to infer the concentration of *Lp* cells by measuring the amount of free aptamers. Multiple parameters including buffer conditions, aptamer concentrations, complementary aptamer probe (cApt) concentrations and incubation times were evaluated for optimal signal response. Of the three buffers (PBS, Fraquil, SSC) tested in this assay only the high salt SSC buffer facilitated incremental binding of aptamers to *Lp* such that varying concentrations of *Lp* could be determined. With this assay, concentrations of *Lp* down to a limit of 10^4 cells/ml could be detected, without any sample processing or signal amplification steps. The specificity of this assay was also demonstrated using *Pseudomonas*, a common inhabitant of water systems. As mentioned previously, the use of this assay meant that no extraction of microbial surrogate biomarkers would be required, which then eliminates multiple sample processing steps as well as the inability to discern whole, membrane-intact, viable cells. This improves the time, costs and complexity associated with this method. By

eliminating the need to inject whole bacterial cells, we circumvented the SPR problems with limited field range, similar RI and minimal diffusion of large cellular targets to the metal-dielectric surface. An additional advantage of not injecting cells is that any confounding changes in RI, that occur due to morphological features of the bacteria, are not registered. Indeed, one study showed that despite their aptamer binding specifically to target *Pseudomonas aeruginosa* (PAO1) over other non-target bacteria in an LSPR sensor, their SPR assay showed less specificity for PAO1 over *S. aureus* due to sensitivity of the system to the spherical *S. aureus* cells (Hu, Fu et al. 2018).

Our assay in chapter 4 showed that the high ionic strength of the SSC buffer facilitated incremental binding of aptamers to *Lp* such that varying concentrations of *Lp* could be determined down to a limit of 10^4 cells/ml. Given the complexities of aptamer characterization, the configuration of this assay and the fact that the interaction is not a 1:1 stoichiometry, it would be difficult to ascertain K_D values and rash to make broad conclusions regarding aptamer-target binding in real world settings. This assay, however, is attractive given that it is quick to perform, validated at temperatures in environmental settings and requires no signal amplification steps. The incorporation of AuNPs could improve the sensitivities of this assay by enhancing the plasmon resonance effect but this would have to be commensurate with aptamer-target binding for cell concentrations <10,000 CFU/ml (Castiello and Tabrizian 2019). For real-world environmental samples, pre-enrichment and mixing modules could easily be supplemented with this assay to improve sensitivity. Indeed, a study from Japan where *Legionella* was detected onsite, in cooling tower water, used a portable, microfluidic, optical sensing device with the help of fluorescent polyclonal antibodies. Pre-enrichment of the water sample in conjunction with the mixing module enabled detection of *Legionella* down to a limit of 10 cells/ml, in contrast to 1000 cells/ml without pre-enrichment (Yamaguchi, Tokunaga et al. 2017). Another promising aspect of the SPR based titration assay is its specificity. When using *Pseudomonas* there was no gradual binding of aptamers and thus the signal responses obtained for unbound aptamers was similar across different concentrations. Furthermore, the viability test (Chapter 4, Figure 4d), confirmed that the assay was detecting viable, membrane intact *Lp* since bacterial cell concentrations were not affected following 1-hour exposure to the high salt SSC buffer.

The detection limit of our assay of 10^4 cells/ml was not practical for *Lp* field applications which requires a detection limit of 50 cells/ml (National Academies of Sciences and Medicine 2020).

Furthermore, it was also observed, in the cloning and sequencing results from our first study in Chapter 3, that we could only identify very few candidate aptamers and that the complexity of these aptamers with interesting structural features was diminished with increasing rounds. Consequently, in Chapter 5, we set out to rapidly identify more aptamers possessing complex structural elements, using a combination of HTS and parallel selections. The parallel selections were to evolve sequences and isolate aptamers that would potentially have enhanced affinities for *Lp* in representative water environments, by using non *Lp* environmental water-associated bacteria and variants of *Lp*, as separate "branches" of our parallel selection (also referred to as Branched SELEX). The aim of this study was to utilize a combination of HTS and Branched SELEX to obtain deeper insights into these differentially evolved aptamer pools and isolate higher-order, higher affinity aptamers that would perform better in our assay setting. The results of this study revealed that though we were able to identify *Lp* binding aptamers with selective properties, none of these aptamers labeled more cells than R10C5 and thus did not have enhanced binding properties. The study in Chapter 5, however is significant since currently, there is a limited number of datasets that are aptamer sequence pools following SELEX with bacterial cells. Additionally, highly enriched sequences were also observed which were not previously identified in Chapter 3, through conventional cloning and sequencing (Appendix, Table A3). The large dataset improved the potential of our SELEX outcomes, since the cloning and sanger sequencing of the final selection round in Chapter 3 only enabled identification of two aptamers, R10C5 and R10C1 from just 13 sequenced clones. Perhaps unsurprisingly, HTS data confirmed that both these aptamer sequences were also the most enriched (Appendix, Table A3). The benefit of HTS, over cloning and sanger sequencing, was similarly observed in a study to identify aptamers binding to Protein A found on the cell wall of *Staphylococcus aureus* bacteria. The number of candidate sequences identified from cloning and sanger sequencing increased from 88 to 2597 with HTS, which resulted in the identification of two more aptamers (Stoltenburg and Strehlitz 2018).

The Branched-SELEX method coupled with HTS enhanced the selection process by identifying sequences that would have otherwise been missed in target sequence pools or present in non-target aptamer sequence pools. The benefit of parallel selections, in the Branched SELEX method, is that aptamers common to the target bacterial strain and multiple non-target bacterial strains could be eliminated simultaneously, without having to undergo multiple iterative SELEX rounds. This reduces the number of time and steps of the classic SELEX methodology. Additionally, the

increased set of sequences from HTS gives more options for further aptamer and aptamer-based assay development using either post SELEX strategies and modifications or affinity-based assays.

6.2 <u>Limitations and Considerations</u>

A high number of amplification steps can adversely impact SELEX outcomes. To offset this effect, we performed preparative PCR and removed additional salts, such as $MgCl_2$, from our initial binding buffer. This was to minimize the loss of sequences that would form complex structures which are not easily amplified, since the presence of salts aids in the formation of these complex secondary structures (Wang, Chen et al. 2019). Additionally, a previous study had successfully isolated several aptamers against *E. coli* without the use of additional salts such as $MgCl_2$ (Kim, Song et al. 2013). Many groups however have successfully isolated aptamers against bacterial cells despite incorporating $MgCl_2$ in their binding buffers (Bruno, Carrillo et al. 2010, Duan, Ding et al. 2013, Marton, Cleto et al. 2016, Chen, Teng et al. 2020).

Despite the preparative PCR step and salt exclusion it was observed that the proportion of full-length nucleotides and thus more complex structures decreased with increasing number of rounds (Chapter 5, Figure S1). High throughput sequencing of multiple SELEX rounds, with or without $MgCl_2$, could perhaps better illustrate the effect of the salt on SELEX outcomes. If formation of salt- mediated secondary structures causes loss of sequences in later rounds due to PCR amplification bias (Le, Krylova et al. 2019), then HTS would help identify aptamers with interesting structural features in earlier rounds and thus balance out any potential negative effects from salt inclusion.

Though the SELEX methodology in Chapter 3 did result in two *Lp* binding aptamers, they are less structurally complex than sequences from previous rounds such as Round 6 (Chapter 3, Table 1).

A factor that could have impacted the SELEX process was our recovery method i.e the use of magnetic beads for separating the PCR product to recover ssDNA for subsequent rounds of selection. During each round of SELEX, the average loss of product with the magnetic bead-based recovery method was ~77% (Table A1). This is particularly detrimental in initial SELEX rounds where the diversity is high and thus sequences are present in low abundances. A low yield would then mean loss of these low abundance sequences with potentially desired binding properties. The

effect of recovery method can have negative consequences for SELEX outcomes and reduce aptamer enrichment (Wang, Chen et al. 2019, Oteng, Gu et al. 2020). Few works report their yields from recovery methods or list the amount of the ssDNA used in each round of selection. In studies where there are minimal or single rounds of selection, such as in Branched SELEX (Chapter 5), it becomes even more important to list the recovery yield to enable insights into the diversity of the sequence pools. Cloning and Sanger sequencing or HTS would then allow examination of these sequence pools for diversity.

In addition to evaluating diversity of sequence pools, HTS can also validate and examine the effects of the initial library on the SELEX process (Komarova, Barkova et al. 2020). As mentioned previously in Chapter 1, library characteristics also affect SELEX outcomes (Vorobyeva, Davydova et al. 2018). Our study did not utilize any complex library design strategies such as the inclusion of modified or artificial nucleotides or primer-less regions. The randomized/variable region of our library was 45 nucleotides which is average and consistent with other studies (McKeague, McConnell et al. 2015, Vorobyeva, Davydova et al. 2018). A sequence distribution analysis of our initial library, using Aptasuite, shows that 70.2% of the sequences correspond to a randomized region of length 45 nucleotides (Appendix, Figure A1). This suggests that given IDTs reported efficiencies, the synthesis process was adequate and primarily contained the expected products (Pazdernik 2020). The chemical synthesis of a random library can be subject to bias which means that its nucleotide composition is not necessarily in equimolar distributions i.e 25% for each nucleotide (Takahashi, Wu et al. 2016, Scoville, Uhm et al. 2017, Vorobyeva, Davydova et al. 2018). Indeed, sequencing data showed that the nucleotide composition of our library had a bias in the randomized region towards G (~ 29%) as opposed to A, C or T (~26 %, 22%, 23%) (Appendix, Table A2). However, this bias seemed to have a negligible effect on the selection process since by the tenth round of selection, the nucleotide composition in the randomized region shifted towards C (~36%) followed by G (~29%). Nucleotide A was only slightly reduced (~22%) whereas T was significantly depleted (~ 13%) (Appendix, Table A2). This information coupled with the large diversity of our initial library (10^{15} sequences) suggests that our library did not negatively impact our SELEX process.

Characterization of aptamer-target interactions is important in determining an aptamers performance for a specific assay. The identified two aptamers from Chapter 3 were characterized

for their binding affinity and selectivity prior to being used on the SPR platform in Chapter 4. Though SPR is well-established for characterizing ligand-target interactions, the physical limitations of the SPR system prompted the use of flow cytometry to evaluate the binding affinities by determining the equilibrium dissociation constant (K_D). Flow cytometry is also a common method for evaluating aptamer-cell interactions (Tan, Acquah et al. 2016).

For both aptamers, R10C5 and R10C1, the K_D values were in the ~100nM range. These values are not only comparable to high affinity antibodies binding to small protein targets but also consistent with reported dissociation constants of other bacterial binding aptamers, many of which have K_D values in the low nanomolar range (Zhou and Rossi 2017, Trunzo and Hong 2020, Saad and Faucher 2021).These K_D values, though very good, should not be considered definitive when describing aptamer functionality for specific biosensing applications. In a study identifying *E. coli* OMP binding aptamers using competitive- displacement FRET assay, Bruno et al 2010 observed that their aptamer Eco8F, despite exhibiting strong binding affinities with an ELISA-like colorimetric plate assay, performed poorly in SPR affinity analysis (Bruno, Carrillo et al. 2010).The authors also concluded that the highest affinity aptamers identified by their ELISA-like colorimetric plate assay were not the most sensitive aptamers in a competitive displacement FRET assay format (Bruno, Carrillo et al. 2010).These affinity discrepancies were the result of inherent differences of the methodologies (Ostatná, Vaisocherová et al. 2008, Bruno, Carrillo et al. 2010).

A 2013 study further highlights how aptamer binding affinities and thus functionality can be affected by the type of assay. With the aid of an SPRi biosensor, Daniel et al determined that the solution and surface-phase affinities of a well-studied thrombin binding aptamer, $K_{D\text{-Sol}}$ and $K_{D\text{-Surf}}$ respectively, revealed significantly distinct values (Daniel, Roupioz et al. 2013). This is because surface- phase affinities are affected by surface grafting density which impact both aptamer-surface as well as aptamer-target interactions (Daniel, Roupioz et al. 2013, Simon, Bognár et al. 2020). Avidity also affects aptamer binding strength and needs to be considered when evaluating K_D values (Kalra, Dhiman et al. 2018). If there are multiple affinity sites, a single K_D is not sufficient or informative with regards to aptamer binding strength. Consequently, it is critical to consider the method/strategy used to determine K_D or conduct characterization experiments, as that will provide insights into an aptamer's performance in a particular biosensing application (Kalra, Dhiman et al. 2018, Plach and Schubert 2020, Khan, Burciu et al. 2021). One way to obtain

a more accurate K_D would be to identify the exact biomarker of an aptamer cellular target. In the case of *Lp*, not only would identifying the biomarker enable more accurate aptamer-target affinity studies, it would also determine if the biomarker target is indicative of the presence of viable bacteria. For example, if the biomarker is an outer membrane protein (OMP) which is susceptible to denaturation by heat or oxidants, unlike LPS, then such a biomarker would better illustrate the bacterial load and subsequently the efficacy of any disinfection protocols.

Affinity based assays are also not sufficient to explain aptamer-target binding. A recent study investigating 3 ampicillin binding aptamers showed no aptamer-target interaction following the use of multiple analytical techniques -namely AuNP gold nanoparticle aggregation assay, isothermal titration calorimetry (ITC), native nano-electrospray ionization mass spectrometry (native nESI-MS), and 1H-nuclear magnetic resonance spectroscopy (1H NMR) (Bottari, Daems et al. 2020). This was even though the aptamers had affinities in the nanomolar range and were independently reported and characterized multiple times in the literature (Bottari, Daems et al. 2020). In addition to the type of assay or analytical technique -ELISA, SPR, Lateral flow assay (LFA) etc.- assay design or configuration also affect biosensor performance and sensitivities (Khan, Burciu et al. 2021). An anti-cholera toxin aptamer in multiple LFA formats, such as sandwich assay and competitive assay, yielded varying detection limits ranging from between 1 and 100 ng/ml (Frohnmeyer, Tuschel et al. 2019).

With regards to the SPR based titration assay, it was observed that there was no gradual binding of aptamers in the presence of *Pseudomonas* and thus the signal responses obtained for unbound aptamers was similar across different concentrations of the bacteria. It would be largely speculative however, to assume that such selectivity would be retained in real world settings given the ionic capacity of the high salt buffer and the complexity of a water system which can contain Gram-positive bacteria, protozoa as well as all sorts of detritus, each with varying surface physico-chemical properties and thus susceptible to the effects of the ionic high salt buffer (Winslow and Haywood 1931, Helander and Mattila-Sandholm 2000, Nagaoka, Murata et al. 2010, Gandhi and Shah 2016, Wang, Bédard et al. 2017, Santos, Chaumette et al. 2019, Kundukad, Udayakumar et al. 2020, Paranjape, Bédard et al. 2020). Indeed, despite the supernatant from 10^8 cells/ml of *Pseudomonas* not containing any aptamer (Chapter 4 Figure 4f), there is still a slight signal response measured. Whether this is because of byproducts released by the *Pseudomonas*

bacterium, that interact with the sensor surface due to the high salt buffer, remains to be investigated.

A further aspect of this assay which complicates its use for real world *Lp* detection is the sensitivity of the assay. The high LOD of 10^4 cells/ml is a far cry from the 50 cells/ml detection limit needed to mitigate any propagation risk (National Academies of Sciences and Medicine 2020). Finding high concentrations of *Legionella* in maintained water systems such as premise plumbing or potable water is may be of limited use as there is some evidence that other biological or abiotic factors would be indicative of water quality long before *Legionella* would reach such amounts (Rhoads, Garner et al. 2017). However, current biological indicators of water quality are not reflective of the presence of opportunistic premise plumbing pathogens (OPPPs) such as *Legionella* (Ashbolt 2015) which has led to the argument to use *Legionella* as a supplementary indicator to determine microbial drinking water quality (Zhang and Lu 2021). Furthermore, to develop a biosensor that is in a portable, user-friendly format instead of large instrumentation that could detect such small quantities of *Legionella,* the assay sensitivity would have to be drastically improved and pre-enrichment/pre-concentration as well as mixing modules would need to be incorporated. The titration assay developed here utilized large analytical instrumentation. The transducer elements of the SPR would need further development to achieve similar sensitivities on a smaller portable scale. The complex plasmonic phenomena means this is more challenging than developing portable detection technology such as lateral flow assays which often employ colorimetric, chemiluminescence or fluorescence phenomenon. Furthermore "signal-off" methods, such as the titration assay, are less practical for real world applications as they can increase the probability of false negatives and have higher LOD's given they have an upper limit to signal suppression (Zuo, Song et al. 2007).

One potential way to improve assay sensitivities is to identify multiple, higher order aptamers, meaning aptamers with more secondary structural elements. These could have enhanced binding properties and thus perform better in a "signal-on" format. In Chapter 5. Branched SELEX in conjunction with HTS was used to identify such aptamers. However a signficant limitation of this technique was the reduced number of rounds for large complex targets such as cells. One round of selection resulted in a high diversity and thus minimal evolution of the pool. This means that the

sequences retreieved were present in low abundance which made it challenging to identify statistically signficant, differentially binding aptamers.

One round of selection also increases the probability of identifying non-specific aptamers. One group found that following 4 rounds of selection, there were still sequences that bound to their negative selection renal cell lines (Pleiko, Saulite et al. 2019). Indeed our own study in Chapter 5, identified a sequence that bound to both the target and non-target cell, despite being depleted in the non-target cells sequence pool.

Yet another limitation of the Branched SELEX protocol is due to the the complexity and large diversity of the targets i.e large, multimeric cells . Given the extensive target diversity random variations in sequence frequencies could be similar to stochiastic variations due to handling steps, This coupled with low sequence abundaances due to the limited number of selection rounds makes it difficult to identify meaninfully enriched sequences.

These limitations also highlight a disadvantage inherent to aptamers. Unlike their proteinaceous counterparts i.e antibodies, aptamers have fewer possible complex configurations to adopt given that there are only 4 nucleotides for nucleic acids as opposed to 20 amino acids for antibodies. This limits their affinity and avidity. Avidity is important for aptamer interactions with complex targets such as bacterial cells. Furthermore aptamer structures are highly dependant on solution conditions. Water systems can differ in disinfectant residuals, total dissolved solids and conductivity as a result of ionic strength, pH and temperature fluctuations all of which can affect aptamer structure formation (McKeague, McConnell et al. 2015). Furthermore complex water matrices may have compounds or nucleases that could promote degradation of aptamers. These parameters are significant to consider when developing an aptamer for detection of *Lp* in complex water matrices.

Given aptamers recognize and bind to their targets by way of secondary structural elements , another aspect of aptamer development to consider is structural analysis. A challenging aspect of this analysis, however, is the extremely high diversity of the sequence pools following one round of selection and the limitation of computational softwares (MEME, Aptasuite) to process an extensive dataset of non-canoncial secondary structures (Song, Zheng et al. 2019, Komarova, Barkova et al. 2020). Additionally these programs were developed using RNA libraries and limited in their capacity to resolve a large number od DNA based secondary structural elements.

(Komarova, Barkova et al. 2020). Both these aspects make it difficult to analyze structural motif enrichment in a meaninful way.The aptamers identified from the parallel selection method possess more secondary structural elements given they are longer. While there are reports of truncated or shorter length aptamers having enhanced binding efficiencies (Hu, Wang et al. 2017, Iqbal, Labib et al. 2015, Hassan, Dixon et al. 2021) there is also evidence that longer sequences result in more sequence complexity which leads to aptamers with higher affinities (Zhu, Li et al 2019). When identifying aptamers against programmed cell death-ligand (PD-L1), using ssDNA libraries with random-region lengths of 15, 30, 40, and 60 nucleotides, Zhu et al. observed candidates' with longer random-region lengths (60 nt) had lower dissociation constant (K_d) and higher affinity compared to shorter length sequences (Zhu, Li et al 2019).

6.3 <u>Future Work</u>

The works developed in this thesis are preliminary and as such leave multiple avenues for further investigation and study.

6.3.1 Branched SELEX

The primary caveat of the Branched SELEX method, in Chapter 5, is the minimal evolution of the sequence pools given the vast diversity of targets. The incorporation of an additional round of parallel selection would reduce diversity and highlight sequences that are meaningfully enriched thereby narrowing down the pool and improving aptamer selection. A replicate analysis would minimize stochastic variations and identify aptamer candidates that are consistently enriched in the presence of targets. The combination of additional selection rounds and replicates would meaningfully reduce the pool and identify statistically significant, differentially binding aptamers. In addition to sequence enrichment. analysis of the enrichment of key secondary structures in the presence of targets would further improve the selection of high functionality aptamers.

6.3.2 SPR Assay

The aptamer based SPR titration assays primary limitation is the high limit of detection for *Lp* cells. To improve the assay sensitivity, AuNP labelled aptamers could be incorporated to enhance the plasmon resonance effect and thus amplify the signal (Castiello and Tabrizian, 2019). However, the effects of AuNP on the conformational folding and subsequent binding to *Lp* would first need to be examined. The unbound aptamers could be isolated and labelled with AuNP but

this adds a layer of complexity and increases the cost of this method. Furthermore, the new AuNP labelled aptamers would have mass bulk properties that would affect any RI changes and could also be subject to steric hindrance. This means that surface grafting densities and chemistries would need to be investigated for effects for effects on signal response.

As previously mentioned, to determine the titration assays robustness in terms of specificity, The use of environmental water samples would provide better insights into the applicability of this assay for field settings. For real world samples, pre-enrichment and mixing modules would need to be incorporated to better isolate *Lp* from a water sample.

6.3.3 Post SELEX Modifications

Post SELEX modifications involve strategies to improve aptamer functionality and stability without having to alter the SELEX process by developing modified libraries or using costly enzymes to amplify selected pools (Gao, Zheng et al. 2016). One such strategy is using a cocktail or mixture of multiple aptamer candidates. This strategy is particularly useful in enhancing affinities for large diversity targets such as cells who can have dynamic membrane surface structures in response to temperature, growth stage, nutrient availability, and oxidative stress agents. One of the earliest reported works using this strategy showed that a mixture of five anti-*S. aureus* aptamers showed enhanced binding efficiencies over the individual aptamers in pyogenic fluid samples (Cao, Li et al. 2009). Similarly, Kim et al 2014 demonstrated that a mixture of previously identified *E. coli* aptamers- E1, E2 and E10- enhanced binding efficiencies and enabled detection of the bacterium down to a limit of 371 CFU/ml using an electrochemical sensor with Au coated electrodes (Kim, Chung et al. 2014).In another study, the polyclonal aptamer library, R16, generated against the bacterium *P. aeruginosa* (PAO1), exhibited significantly higher labelling efficiency for 51 *Pseudomonas* isolates as compared to the individual C1R1 aptamer (Kubiczek, Raber et al. 2020). R16 was also selective for the bacteria over *E. coli* (Kubiczek, Raber et al. 2020). The authors suggested that since the composition of the bacterium's cell wall is tightly regulated and thus dynamic, the diverse polyclonal library (R16) compensates for the reduced labelling efficiency of C1R1, by binding to targets the individual aptamer does not recognize (Kubiczek, Raber et al. 2020). A mixture of aptamers identified from the HTS datasets in Chapter 5 could be used to enhance labelling efficiencies for *Lp* provided the aptamers do not compete for

the same epitope. The effect on target specificity of such a mixture would also need to be investigated.

The use of multiple aptamers to construct a multivalent aptamer is another strategy to enhance capture and labelling of cellular targets. By immobilizing long sequences with >500 aptameric/binding regions on their magnetic nanoparticles, Chen et al 2019 were able to capture and isolate cancer cells from whole blood (Chen, Tyagi et al. 2019). However, steps would have to be taken to ensure the aptameric regions orientation or structural binding component is not compromised. To do this a suitable scaffold/linker length and flexibility would need to be optimized (Vorobyeva, Vorobjev et al. 2016, Kalra, Dhiman et al. 2018).

Aptamer truncation is another post-SELEX modification strategy that has been reported to increase aptamer binding affinities. The rationale for aptamer truncation is to remove non-essential sequence regions that could form secondary structures that would destabilize the aptamer-target interaction (Shangguan, Tang et al. 2007). Two aptamers identified after 12 rounds of Cell SELEX with the bacterium *Bifidobacterium breve* had lower K_D values following removal/truncation of primer binding regions (Hu, Wang et al. 2017). A truncated version of the aptamer, R4-6, named Min_Crypto2, that was developed against water-borne *Cryptosporidium parvum* oocysts, performed better in a fluorescence plate-based assay than the original aptamer (Iqbal, Labib et al. 2015, Hassan, Dixon et al. 2021).There are several works, however, where truncation of the aptamer has had no effect on binding affinities (Duan, Wu et al. 2012, Duan, Wu et al. 2013). This is because, in all likelihood, the key structural regions are conserved and unaffected by primer binding regions. This strategy may not be feasible for aptamers with short, randomized regions (~20 nt) such as R10C1, given the limited number of structural elements but R10C5, sequences from R6 (Chapter 3,Table 1) or the full-length sequences identified through HTS in Chapter 5 may be truncated and further investigated,

Post SELEX strategies are just one avenue of improving aptamer-based assays. The modular nature of the SELEX process means that it can be modified to better align with our SPR biosensing strategy. A type of SELEX that does this, and would be interesting to investigate for future research, is Capture-SELEX.

6.3.4 Capture SELEX

The SELEX design in Chapter 3 does not guarantee high functionality, surface immobilized aptamers. An interesting SELEX strategy would be Capture-SELEX, first described by the Yingfu Li lab in 2005 and further developed by Stoltenberg et al. to identify aptamers binding to aminoglycoside antibiotics (Nutiu and Li 2005, Stoltenburg, Nikolaus et al. 2012). Much of the Capture-SELEX process is similar to the classical SELEX approach in that it includes incubation of the library with the target, elution/extraction of target bound sequences, followed by subsequent amplification of these sequences. The difference however is that during Capture-SELEX, the first step is to immobilize the library onto a solid matrix. This is achieved with the help of special libraries that contain a small region known as "docking" sequences, which are complementary to oligos immobilized on a surface (Stoltenburg, Nikolaus et al. 2012). These sequences help "dock" or tether individual members of the library onto the solid surface matrix. Addition of the target to the immobilized oligonucleotide library will induce a conformational change or switch, which separates the candidate sequences from the surface matrix, leaving only the "weaker" sequences that preferentially hybridize to the immobilized oligos. Consequently, only target bound sequences are recovered. These target bound sequences are then amplified and subjected to repeated rounds of selection until an aptamer pool with desired properties is obtained (Stoltenburg, Nikolaus et al. 2012).

To eliminate any false positives, that would arise from the release of library sequences that are loosely bound to the matrix, Stoltenberg et al. employed numerous, stringent washing steps several of which were included at temperatures slightly higher (~28) than application temperatures (Stoltenburg, Nikolaus et al. 2012). They also incubated their immobilized DNA library-bead complex in selection buffer for 45 min with mild shaking (Stoltenburg, Nikolaus et al. 2012). These steps were to eliminate any background release/elution of hybridized oligonucleotides from the beads that were caused by their protocol's incubation steps. In addition to these steps, it is also very important to include counter or negative selection steps. Given the large diversity of epitopes on bacterial cell surfaces, the negative selection steps would be crucial to enhancing affinities as well the selective properties of the resulting aptamer pools from such a SELEX strategy. The negative selection steps could be interspersed and sequentially included in the Capture SELEX process or even as a separate parallel Capture SELEX selection process. In either selection strategy HTS could be used to monitor the selection process and distinguish between sequences that are highly enriched in the target bound pools versus non-target bound pools.

We know from Chapter 4 and the regeneration steps therein, that both aptamer hybridization and de-hybridization result in an SPR signal response that is not dependent on large bacterial cells penetrating the electromagnetic field and approaching the sensor chip surface. The aptamers identified from Capture-SELEX could be immobilized on the surface of the SPR sensor chip in a specific array to minimize any steric hindrance (Hu, Fu et al. 2018) and used to detect *Lp* in a more direct manner. The release of the sensor chip-bound sequences in the presence of their bacterial target would result in a signal response and thus *Lp* could be detected and quantified. As a result of Capture-SELEX, these surface immobilized aptamers would have functionalities that better align with our SPR sensing application. The aptamers identified from the Capture-SELEX method could be succcesful in a "signal-on" method of detection.

The Capture-SELEX strategy was originally designed to prevent immobilization of targets and thus retain their native conformation. To date, no Capture-SELEX strategy has employed the use of whole cell bacteria as targets (Lyu, Khan et al. 2021). A possible reason for this may be the complexity of the cellular target which has diverse, dynamic epitopes, running the risk of generating low efficiency or non-specific aptamers. One group attempted to circumvent this problem by using LPS of *Salmonella enterica* Typhimurium as the target (Ye, Duan et al. 2017). By combining Capture-SELEX with Toggle-SELEX, referred to as orientation selection by the authors, the broad affinity aptamers EA5, EA7 and EA10 were identified (Ye, Duan et al. 2017). BSA was used for counter selection. The authors "toggled" the selection process after the 6th round by using whole *Salmonella typhimurium* and *Escherichia coli* cells. They subsequently observed that all three aptamers could identify LPS from other Gram-negative bacteria namely *Salmonella enterica serotype enteritidis*, *Escherichia coli* 055: B5 and *Pseudomonas aeruginosa* but minimally labelled *Salmonella typhimurium* and *Escherichia coli* cells. Aptamer EA7 was determined to be the most selective aptamer and used in an assay to detect LPS in drinking water at the limit of 3 ng/ ml (Ye, Duan et al. 2017). This study did not identify aptamers binding to bacterial cells, since the target was just LPS and only one round of selection was done for each whole cell bacterium. However, it does demonstrate how Capture-SELEX can be used to identify broad affinity aptamers for water-borne pathogen detection. As mentioned previously, given the diversity of the *Legionella* genus, broad spectrum aptamers would be useful for environmental monitoring and risk mitigation. Instead of LPS, however, which, as previously mentioned, is resistant to disinfection protocols (Gao, Wang et al. 2006, Xue, Zhang et al. 2019) and not

indicative of viable *Legionella* amounts, outer membrane proteins could be used as a target in the Capture-SELEX protocol. The absence of target immobilization steps means that the target would be in its native state increasing the robustness of the aptamer for detection of viable *Legionella* in water.

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CONCLUSION

The past two decades have seen significant growth in aptamer research and development not least because they are cost-effective and have versatile chemical properties. This versatility has led to an impetus in aptamer-based applications ranging from therapeutics and diagnostics to bioimaging and detection. Commercial interest in aptamer-based sensing is also high given their economic advantage over antibodies. Recently, the UK based company Aptamer Group, plc developed an aptamer against a common epitope found in the spike protein of all SARS-CoV-2 variants. This aptamer is being integrated onto the biotech company DeepVerge's clinically validated Microtox®PD platform for the detection of SARS-CoV-2 in wastewater. Such news highlights the potential of aptamer-coupled sensors for detection of water-borne pathogens.

The goal of this thesis was to develop an aptamer-coupled biosensor that would help overcome the limitations set by traditional methods for detecting whole *Lp* cells. Given this goal, our objectives were to first generate potential aptamers that bind to *Lp*, validate, and characterize these aptamers, and then incorporate them onto the SPRi sensing system to determine their feasibility as a method to detect *Lp* in complex water matrices. Though we were successful in identifying aptamers binding to *Lp* and adapting them onto the SPR platform for *Lp* detection, the platform requires further development for real world applications primarily because of the high LOD for *Lp* as well as the constraints of the SPR system. Furthermore, there is a limited understanding of the aptamer-*Lp* interactions given the simple affinity-based characterization studies described in this thesis. The classical iterative SELEX approach in Chapter 3 coupled with cloning and sanger sequencing also impeded the identification of multiple, structurally complex aptamers with potentially improved binding properties, leading to the use of Branched SELEX in Chapter 5.

For the Branched SELEX method, the use of HTS, in conjunction with parallel selections, streamlined the selection process by identifying more potential *Lp* aptamers that were also absent in non-*Lp* bacteria sequence pools. However, this method is limited by the complexity of the target which led to a minimal evolution of the aptamer pools, given just one round of selection. The resulting high diversity of the pools means that potentially interesting candidate sequences are present in low abundances making it difficult to identify significant, differentially binding aptamers. An additional round of parallel selection coupled with replicate analysis would enable

identification of higher order, structurally complex aptamers that are significantly enriched in the presence of target *Lp* and depleted or absent in the presence of non-*Lp* bacteria.

As discussed in Chapters 1 and 2, the SELEX methodology, must take into consideration many elements which include target properties since these dictate the type of sequences enriched in SELEX pools. The partitioning, recovery and analysis of these sequences must then be carefully optimized in order to minimize the loss of any potential high-affinity aptamers. Additionally, to achieve the ideal aptamer kinetics of a specific assay, the SELEX process should also, if possible, align as close as possible to the end application platform.

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APPENDIX

File A1 (Chapter 2, Table S1):

Characteristics of aptamers and biosensors developed to detect water-borne pathogens

https://www.frontiersin.org/articles/10.3389/fmicb.2021.643797/full#supplementary-material

File A2 (Chapter 5, Table S1):

Shortlisted sequences enriched in the target pool and depleted in the non-target pools.

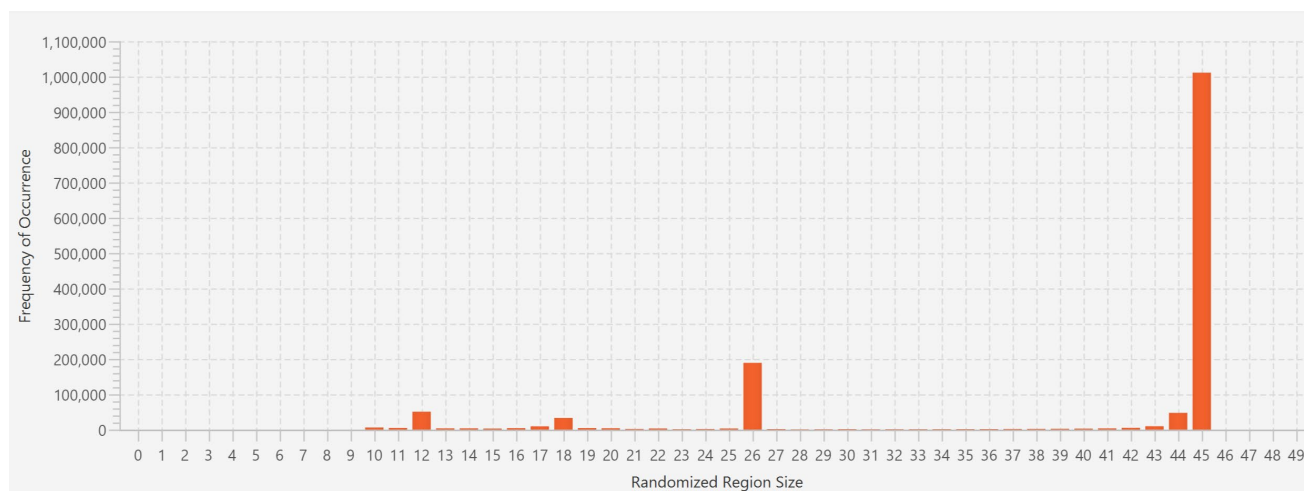TableS1_Shortlist_Sequence_EnrichedinTargetpool_DepletedinNontarget

Figure A1: Frequencies of randomized region sequences of varying lengths. The total number of sequence reads are 1424749. Sequences with randomized region of 45 nucleotides are present in the highest proportion. Data and figure generated in Aptasuite version 0.9.7.

ROUND	Added library/pool(ng)	Elution Eluted ssDNA(ng)	PCR Post PCR purification (ng)	Strand Separation Post strand-recovery purification (ng)
1	32,840	4,560	2,007.90	260.6
2	260.6	4,627.20	2485 / 1,635	427.2 / 313.6
3	427.2	7,869.4	1,685	323.4
4	323.4	12,681.20	1,730	499.8
5	499.8	7369.6	2120	333.2
6	333.2	6634.6	2,323	672
7	672	9,148.80	2,755.20	837.2
NS1	837.2	NA (Buffer mixture)	5,260.80	487.6
8	487.6	9,329.60	2,241.60	872.2
9	872.2	10,291.20	2,424	672
NS2	672	NA (Buffer mixture)	1,809.60	333.2
10	333.2	21,971.60		

Table A1: Nucleic acid yield, listed in nanograms(ng) for each SELEX round following either elution, amplification (PCR) or single strand recovery steps

	Library	Round 1	Round 3	Round 10
A	25.64	24.01	19.75	22.02
G	28.7	26.71	22.6	29.36
C	22.38	25.25	36.1	36.14
T	23.28	24.03	21.55	12.49

Table A2: Nucleotide composition of initial library pool as well sequence pools from rounds 1,3 and 10. The proportion of each nucleotide in different rounds is given.

Randomized region sequence	R10 CPM	Enrichment	R3 CPM	Enrichment	Library CPM
GGACAGTGCTGAAAACTGTGACCCCC	530713.8	68700	7.729	7.729	0
CACGCCGCCCTCCCCCAC	72856.65	14100	5.152	5.152	0
CCACCCCACGCTGCTCC	8846.118	1140	7.729	7.729	0
GGACAGTGCTGAAAACTGTGACCCCC	2507.758	973	2.576	2.576	0
GGGGCGGGGGG	4134.667	802	5.152	5.152	0
GCCCAAACTGTGACCCCTACTG	4582.928	445	10.305	10.305	0
GGGGGGGGGGG	1075.201	417	2.576	2.576	0
GGGGGGGGGGGG	1068.932	415	2.576	2.576	0
GGGGCGGGGGGG	116513.6	243	479.17	479.17	0
GGGGGGGGTGGG	479.609	186	2.576	2.576	0
GGGGCGGGGAGG	445.127	173	2.576	2.576	0
GGGGCGGGTGGG	739.789	144	5.152	5.152	0
GGGGCGGGAGGG	366.76	142	2.576	2.576	0
CACACCCCCGCCCCCAC	708.442	137	5.152	5.152	0
CCACATGCACCCTCCC	341.682	133	2.576	2.576	0
GGGGCGGGGGGC	275.853	107	2.576	2.576	0
GGGGCGGGGGGG	235.102	91.3	2.576	2.576	0
GGGGCGGGGGGG	231.968	90	2.576	2.576	0
GGGGCGGGGGGG	206.89	80.3	2.576	2.576	0
GGGCGGGGGGG	178.678	69.4	2.576	2.576	0
CCCTGCCGCCCTCCTCAC	178.678	69.4	2.576	2.576	0
CCACGCCACGCCCCTCCC	175.543	68.1	2.576	2.576	0
GGGGCGGGGGGG	689.634	66.9	10.305	10.305	0
CACTCCCCACACCGCCC	169.274	65.7	2.576	2.576	0
CAACCGGCCC	9930.723	55.9	177.757	177.757	0
GGGGCGGTGGGG	134.792	52.3	2.576	2.576	0
CACGCCGCCCTCCCCCA	128.523	49.9	2.576	2.576	0
GGGGCGGGGGGG	125.388	48.7	2.576	2.576	0
GGGGCGGGGGAG	247.641	48.1	5.152	5.152	0
CCCCCCACGC	122.253	47.5	2.576	2.576	0
CCACGGCCCCAC	238.237	46.2	5.152	5.152	0
CACACACGCCC	115.984	45	2.576	2.576	0
CACCCCACGCC	103.445	40.2	2.576	2.576	0
GGCGGCATGCACATCGGGGGTCACATG	307.2	39.7	7.729	7.729	0
CACACCCCCCGC	97.176	37.7	2.576	2.576	0
CACCCCACGC	94.041	36.5	2.576	2.576	0
ACCCGGGCTGCC	6849.315	33.2	206.095	206.095	0
CACGCCGCCCCCCTC	84.637	32.9	2.576	2.576	0
CCACGCCCCACCACCCTTCGC	81.502	31.6	2.576	2.576	0
CACCCCGCCC	78.367	30.4	2.576	2.576	0
CCACGCCACCTCATTACCC	78.367	30.4	2.576	2.576	0

CACGCCGCCCTCCCCCAC	75.233	29.2	2.576	2.576	0
CCACACCCCACCACCCTATACGCAC	147.331	28.6	5.152	5.152	0
CACGCCCCTACCCCCAC	144.196	28	5.152	5.152	0
GGGGGAGGGGT	68.963	26.8	2.576	2.576	0
CCACCCCACGCTGCTCC	68.963	26.8	2.576	2.576	0
CACGCCACCCC	68.963	26.8	2.576	2.576	0
CACACCCCCGCCCCCCGC	59.559	23.1	2.576	2.576	0

Table A3: Top 50 sequences enriched in Round 10, listed in descending order of enrichment. Sequences are listed without primer binding sites. CPM refers to counts per million. The underlined sequences correspond to R10C5 and R10C1, respectively. Data generated in Aptasuite version 0.9.7.