Emerging and Infectious Diseases: Defending Against a Dynamic Opponent

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by

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DEDICATION

I dedicate

this dissertation to

my daughter, Amaya Belle.

You are my inspiration, my joy, and

my hope for the future. May you always

remember to fearlessly pursue

what sets your heart

on fire,

I love you!

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I would like to thank those who helped me along this journey.

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ABSTRACT

Emerging infectious diseases (EID) pose a growing threat to public health each year. Viruses and bacteria alike are major causes of EIDs and as these pathogens mutate, reemergence occurs, leading to the need for advanced understanding of the pathogens' life-cycle and evolution in order to control outbreaks as well as drug resistances. Tularemia, the disease caused by the highly infectious, bacterium, *Francisella tularensis* (Ft), is commonly acquired via natural infection and is a threat for biological warfare. However, previous data has indicated that Ft may persist in the environment without a host, but the mechanism by which Ft survives in adverse environments is not well understood. Furthermore, HIV, the causative agent of AIDS, has been treated with a combination therapy for the last two decades and has now developed mutations, conveying resistance to drugs within the combined therapy regimen. The goal of this study is to better understand the mechanisms of action that has led to the evolution of each of these very different pathogens in order to provide information necessary to treat these diseases and control the reemergence of these notable pathogens.

1. OVERVIEW OF *Francisella tularensis*

Francisella tularensis (Ft) is a small, fastidious, gram-negative bacteria, found in a wide array of environments and is associated with disease in both vertebrate and invertebrate hosts. Ft is divided into subspecies, in which four subspecies (ssp.) can cause potentially serious disease in humans[1,2]. However, most reported cases of tularemia are caused by *Ft ssp. tularensis* (type A) or *Ft ssp. holarctica* (type B)[3]. Furthermore, type A is found exclusively in North America and is highly virulent, with a mortality rate in humans of 30-60% if untreated[3,4]. Whereas, type B is found predominantly in Russia, Asia, Europe, and Japan with rare instances in North America, and has a low mortality rate in humans. Tularemia is often transmitted by arthropod bites, exposure to infected animals and contaminated food or water. However, the environmental persistence of Ft is not well defined. In 1977, the live vaccine strain (LVS) was utilized as an effective vaccine against tularemia; however, due to the adverse effects experienced by vaccinated individuals, it was not approved for use in the United States[5]. Ft is considered to be one of the most virulent bacteria known and currently there is no licensed tularemia vaccine. This, along with the ability of Ft to adapt to various environments, its wide host range, and ease of dissemination through multiple routes of exposure, the CDC considers Ft a tier 1 select agent with risk of being maliciously employed for use as a biological weapon[6].

1.1. Emergence

Francisella tularensis was originally isolated when a plague-like outbreak occurred in Tulare County, California in 1911[7]. After the 1906 San Francisco earthquake, multiple

cases of, what was thought to, be bubonic plague were reported. However, the United States director of Public Health Services, G.W. McCoy suggested a full bacteriological investigation into the infected individual as well as squirrels and rats within the area of the outbreak[8]. After multiple attempts to isolate the causative agent of the bubonic plague with no success, a more nutrient rich culture medium was used. With these changes to the media, McCoy was able to isolate a novel bacterial pathogen, which was then named *Bacterium tularense*[9]. Although, this bacterium was first isolated in the United States, and has been since considered endemic throughout North America, Europe and Asia[7]. Since its discovery, outbreaks have occurred globally and every state in the United States had experienced an outbreak, except Hawaii[7].

In 1913, tularemia, the disease caused Ft, was first identified in humans at the University of Cincinnati in Cincinnati, Ohio by Dr. William B. Wherry [10]. Later, it was determined by Dr. Edward Francis that serval diseases and syndromes previously reported in North America, Europe and Japan were significantly similar to tularemia. He determined that the causative agent of these diseases was, in fact, *Bacterium tularense*[3]. Soon after this discovery, taxonmists determined that the bacterium causing this disease in humans did not fit the previous description of the bacterium isolated from squirrels and rats. It exhibited different unique properties making it more efficient for human infection[11]. In 1947, a novel genus, named *Francisella,* and species *tularensis* was coined to better describe the pathogen responsible for the human disease, tularemia[3]. Twelve years later, in 1959, it was proposed that there were two distinct strains of Ft[11]. These varieties were originally referred to as ”New World” and ”Old World,” but were later

denoted as Type A and Type B, respectively. The strain differences were determined through virulence assays in rabbits, where the lethal dose for Type A was determined to be 1-10 CFU (colony forming units) and Type B was 108-109 CFU[12]. These reports in 1965, concluded that there were ecological and mammalian host preferences, as well as difference in virulence between the two strains. Type A was occurring only in North America and is highly virulent in rabbits and humans, whereas Type B is less virulent in humans and occurs in North America, Europe and Asia[12.]

1.2. Strains

Ft belongs to the family Francisellaceae, proteobacteria subclass γ and phylogenetic studies of *Francisella* isolated from infected humans show that it is distantly related to the human pathogens *Coxiella burnetii* and *Legionella spp*[13]. Ft is further divided into four distinct subspecies: *F. tularensis* subsp. *tularensis* (type A), *F. tularensis* subsp. *holarctica* (type B), *F. tularensis* subsp. *mediasiatica*, and *F. tularensis* subsp. n*ovicida*[13,14]. Subspecies *tularensis* and *holarctica* cause the most frequent human infections. Furthermore, ssp. *tularensis* (type A) strains are again divided into clades A.I and A.II, which differ in geographical location, modes of transmission and virulence, with clade A.I being the most virulent[14]. Subspecies *holarctica* is spread throughout the Northern hemisphere, whereas ssp *tularensis* is confined to North America[14,15]. Conversely, ssp *novicida* is considered, evolutionarily, the oldest strain, and has a strong association with water and water borne infection of animals[15]. Three of the four subspecies listed above, specifically *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, and *F. tularensis* subsp. *mediasiatica* are select agents based on their rate of

infection, virulence, and mode of transmission. Subspecies *novicida* is not a select agent along with the attenuated live vaccine strain (LVS) of *ssp holarctica,* which was derived in the 1940s in ex-Soviet Union countries13,14. The majority of studies conducted on Ft have used either ssp *novicida* or LVS as model systems for tularemia infection due to the need for biosafety level – 3 (BSL-3) containment needed for the more virulent strains. In this study, I have elected to use the F. *tularensis* ssp tularensis Schu S4 strain, which is the most virulent prototypic strain, and gives the most accurate results for human infection.

1.3. Tularemia

The epizootic disease caused by Ft is known as tularemia. Tularemia can affect both animals and people with rabbits, hares, and rodents, being especially susceptible to the disease and often die in large numbers during outbreaks. There are six clinical manifestations of the disease: ulceroglandular, glandular, oropharyngeal, oculoglandular, typhoidal, and pneumonic (Table 1)[16]. The type of tularemia is determined based on the route of infection.

Table 1: Six clinical manifestations of tularemia[16]		
Types of tularemia	**Symptoms**	**Route of exposure**
Ulceroglandular	Localized lymphadenopathy and cutaneous ulcer at infection site	Tick bite Fly bite Handling infected animals
Glandular	Localized lymphadenopathy	Tick bite Fly bite Handling infected animals
Oropharyngeal	Severe throat pain, exudative pharyngitis or tonsillitis, Cervical, preparotid, and/or retropharyngeal lymphadenopathy	Eating or drinking contaminated food or water
Oculoglandular	Photophobia, excessive lacrimation, conjunctivitis, preauricular, submandibular and cervical lymphadenopathy	Contaminated water or infected animal fluids splashing into the eye
Typhoidal	Any combination of the general symptoms (without the localizing symptoms of other syndromes)	Secondary to other untreated forms of tularemia
Pneumonic	Non-productive cough, substernal tightness, pleuritic chest pain, hilar adenopathy, infiltrate, or pleural effusion may be present on chest X-ray	Breathing dust or aerosols containing Ft or secondary to other untreated forms of tularemia

Because Ft can infect a wide variety of immune and non-immune cells, infection often leads to necrosis and ulceration in multiple tissues throughout the body with extensive damage at the site of infection[16,17]. Macrophages seem to be a primary target for Ft infection and may spread the infection to the regional lymph nodes resulting in lymphadnopathy[17]. Pneumonic tularemia is a severe form of the disease, associated with exposure to aerosols, and has a mortality rate of 30-60% [16,17]. If left untreated, tularemia carries a mortality rate of 10-50%. Typhoid tularemia, affecting multiple organ systems,

carries the highest mortality and even those who survive left with lung, kidney damage and muscle loss post-infection[17].

Diagnosis of tularemia is difficult and often requires a high degree of clinical suspicion based on recent patient activity including travel. A serological diagnosis is the most used method to confirm infection, However, false negatives may occur if antibodies are not yet present[17.] Ft can be cultured from samples taken directly from infected ulcer scrapings, sputum, lymph node biopsy and stool samples. However, in a recent study serology confirmed 84% of cases in infected individuals, but Ft was isolated from only 10% of these cases[17]. Furthermore, isolation of Ft on culture plates must be conducted in a controlled environment, as accidental inhalation of Ft by a laboratory technician presents significant risks. Currently, there have been no clinical trials to define the optimal course of antibiotics against Ft infection. There is no FDA approved vaccine for Ft. However, the CDC suggests the antibiotic regimens listed in (Table 2).

Table 2: Tularemia treatment regimen				
Age Category	**Drug**	**Dosage**	**Maximum**	**Duration (Days)**
Adults	Streptomycin	1 g IM twice daily	2 g per day	Minimum 10
	Gentamicin*	5 mg/kg IM or IV daily (with desired peak serum levels of at least 5 mcg/mL)	Monitor serum drug levels	Minimum 10
	Ciprofloxacin*	400 mg IV or 500 mg PO twice daily	N/A	10–14
	Doxycycline	100 mg IV or PO twice daily	N/A	14–21
Children	Streptomycin	15 mg/kg IM twice daily	2 g per day	Minimum 10
	Gentamicin*	2.5 mg/kg IM or IV 3 times daily**	Monitor serum drug levels and consult a pediatric infectious disease specialist	Minimum 10
	Ciprofloxacin*	15 mg/kg IV or PO twice daily	800 mg per day	10
* Not a U.S. FDA-approved use, but has been used successfully to treat patients with tularemia. ** Once-daily dosing could be considered in consultation with a pediatric infectious disease specialist and a pharmacist				
(Table by Center for Disease control and Prevention. © 2012 –2021) [16]				

1.4. Transmission and Spread

1.4.1. Natural infection

In humans, Ft infection is acquired through the bite of an infected blood-feeding arthropod such as a biting fly or mosquito, ingestion of contaminated food or water, and

inhalation of aerosolized bacteria. During tularemia outbreaks, arthropod-related transmission peaks in the summer and fall months[18]. More than 250 distinct mammalian species have been reported to have acquired a naturally occurring infection of Ft[19]. Therefore, predicting increased instances and potential outbreaks for Ft are complex and often misrepresented. However, human infections are associated with environmental exposure and can be predicted in association with significant zoonotic and environmental events. Furthermore, there is limited knowledge of the complete natural lifecycle of the pathogen and the natural reservoir of the bacterium is still unknown. It has been hypothesized that Ft can persist in the environment outside of a host. Despite the continuous research effort over the last 100 years, the specific mode of this environmental persistence remains unknown[20]. Several studies over the last 20 years have revealed that Ft Type B persists in water, and sediment for a minimum of 16 months[21,22]. Additionally, there have been genomic studies conducted on Ft strains collected from individuals who had been infected between 1947 and 2012 in Western Europe, the results from this study indicated that tularemia migrated from Eastern to Western Europe and that Ft has a ”long-range geographical distribution” but maintain slow and variable replication rates. Collectively, these conclusions support a hypothesis that Ft has a ”resting survival phase” or persistent state within the environment, outside of a host[21–23].

The persistence of Ft outside of a host would require the remarkable ability of the bacterium to survive variable environmental conditions such as changes in temperature and nutrient availability. This amount of variable stress may have driven the formation of

a symbiotic relationship with nearby protozoa[24,25] in tandem with a close association with different arthropod species[26,27]. Furthermore, there are several environmental conditions that are favorable for increased survival and induction of Ft into the persistent state such as decreased temperature, salinity and nutrient availability. Studies conducted on cold water contaminated with Ft infected animal carcasses and/or feces, concluded that viable and infectious Ft was detected for up to 10 weeks (about 2 and a half months) post contamination[22,28]. It has also been suggested that increased salinity may promote the survival of Ft. Studies conducted on both oceanic salt water and brackish water concluded that the longest survival time for Ft (42 days) occurred in water containing 3.6% NaCl with the lowest survival time in fresh water[22,29]. In accordance with these studies, in February 2021, Golovliov et al. investigated the survival of Ft in a combination of low temperatures and low-nutrient water. They concluded that low temperature (4°C) and 0.9% NaCl increased survival times greatly when compared to the earlier studies that only examined either at low temperature or the effects of saline at higher temperatures[19].

1.4.2. Biological warfare

With the unique ability of Ft to cause infection via airborne transmission along with a low infectious dose and the pathogen's remarkable survivability, it has been considered a potential biological weapon since the early 1900s. Between 1932 and 1945, Ft was studied at the Japanese germ warfare research unit as well as research programs in the former Soviet Union[30,31]. It is up for debate whether Ft has ever been used as a biological weapon in the past. A former Soviet Union Scientist, Ken Alibeck and a group of epidemiological historians claim that tularemia outbreaks that effected tens of

thousands of German and Soviet soldiers in eastern Europe during World War II, may have been the result of intentional use[27]. At the end of the war, there were continued military studies of Ft to include the development of a weapon, by the United States, that would disseminate Ft via aerosol to a widespread population[33]. During this time, the United Sates also conducted research on virulence of the pathogen as well as treatment options and vaccines, which will be discussed later in this chapter. By the late 1960s, The US had stockpiled Ft as well as other biological agents to be used as weapons. In response, the Soviet Union also stockpiled Ft and began research on modified versions of the pathogen[32,33]. Because these efforts were made public by both countries, the World Health Organization (WHO) gathered an expert committee to determine the result of an aerosol dispersal of Ft to a population. It was determined that intentional dispersal of 50kg of virulent Ft over an area with a population of 5 million, would result in 250,000 incapacitating casualties, which would include 19,000 deaths[33]. Due to the course of infection and the mode of transmission associated with Ft, the illness would persist for weeks and even months after initial exposure. Upon further review of this model proposed by WHO, the Centers for Disease Control and Prevention (CDC) estimated that the economic impact of this bioterrorist attack would incur a total base cost of $5.4 billion for every 100,000 people exposed to aerosolized Ft[34,35]. After these grim statistics were revealed, the United State decided to move forward with destruction of their biological weapons and encouraged other countries to do the same.

In 1972, the Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and their Destruction was

signed by the United States and 183 other states parties including: Palestine, and four signatories (Egypt, Haiti, Somalia, and Syria). However, after its implementation, ten states neither signed nor ratified the legally binding treaty, now known as the Biological Weapons Convention (BWC), to include Chad, Comoros, Djibouti, Eritrea, Israel, Kiribati, Micronesia, Namibia, South Sudan and Tuvalu[36]. Furthermore, the Soviet Union, though they had signed the convention, continued their program with the focus on development of Ft strains that were modified to include antibiotic-resistant genes increased virulence factors[6]. Likewise, in 2019, the State Department Report on Compliance with Arms Control, Nonproliferation, and Disarmament Agreements and Commitments indicated that China was continuing to engage in the development and use of pathogens, including Ft, for ”dual-use applications” pertaining to use as a biological weapon[37]. Under the same compliance report in 2019, the United States also claims that Iran, North Korea, and Russia have not abandoned their efforts to develop biological weapons to use offensively, each of these claims did include stockpiles and modified versions of Ft[36,37.]

Since the signing of these conventions and intended destruction of biological weapons, the US Army Medical Research Institute of Infectious Diseases (USAMRIID) has been responsible for defensive medical research on potential biological warfare agents to better protect the US military, including but not limited to decontamination protocols in the field, in the lab and in the clinical setting, prophylaxis, recognition and diagnosis, and medical management. Furthermore, the CDC operates a national program for preparedness and response to bioterrorism and has initiated a broad range of public health partnerships. Universities and industries studying potential bioterrorism agents

must adhere to strict guidelines. In 2002, The *Public Health Security and Bioterrorism Preparedness and Response Act* enlisted the Department of Health and Human Services (HHS) and to establish and maintain a comprehensive list of biological agents and toxins that are a potential threat to public health and/or safety[38]. Comparably, *The Agricultural Bioterrorism Protection Act of 2002* has enlisted the United States Department of Agriculture (USDA) to establish and maintain a list of biological agents that may have the ability to pose a severe threat to animal health and safety, plant health and safety, or to the safety of animal or plant products, these have been termed ”select agents”[38]. The laws require HHS and USDA to keep the select agent list up to date on a biannual basis via the Fedaral Select Agent Program (FSAP). Laboratory facilities that research select agents must register with the FSAP and CDC as well as establishing a workspace appropriate for use of select agents often including biosafety level 3 and/or 4 conditions that are subject to annual inspection. Though working with these pathogens has potential dangers, research with these select agents provides important and urgent scientific discoveries that have led and will lead to filling large considerable gaps in knowledge. Ft is a select agent and requires BSL-3 laboratory conditions to conduct research. It is considered highly infectious and is still considered to be in the top 5 of laboratory acquired infections and is therefore often bypassed by researchers. As a result, there are many gaps in knowledge about the lifecycle, environmental persistence, vaccine development, etc.

1.5. Life cycle

Francisella tularensis is a facultative intracellular pathogen, i.e., it has an intracellular phase within a host that is required for pathogenicity, but also has an

extracellular growth phase[39]. The intracellular portion of the Ft life cycle provides protection for the bacterium and ensures survival and proliferation but is complex and not well understood. With numerous studies conducted on many different strains of Ft in host cell models, it is widely accepted that Ft is a cytosolic pathogen. However, there are discrepancies surrounding other phases of the intracellular life cycle. Additionally, tularemia has been reported in more than 250 species of mammals via natural infection and all naturally occurring human cases have been acquired directly from the environment[4041]. In the majority of human cases, infection was associated with arthropod bite, direct contact with an infected animal, inhalation of aerosolized bacteria or ingestion of contaminated food or water. However, Ft appears to have the ability to persist in the environment without an infected mammal or arthropod host, but, despite a century of research, little is known about the specific mechanisms of this environmental persistence or the natural reservoir between outbreaks.

1.5.1. Intracellular life cycle

As mentioned above, the intracellular phase of the Ft lifecycle is thought to be essential for the survival, proliferation, and virulence of the pathogen. This phase is also necessary for the development of tularemia within the host. Unlike other facultative intracellular pathogens, Ft has the unique ability to infect a variety of different host cell types including macrophages, dendritic cells, polymorphonuclear neutrophils, hepatocytes, endothelial, and type II alveolar lung epithelial cells[42,43]. Much research has been devoted to characterizing the specific steps of the Ft intracellular life cycle from entry to proliferation. Because of this extensive research, it is clear that Ft replication and

virulence is reliant on phagosomal escape and entry into the host cytosol[43]. Utilizing several different strains of Ft and a variety of host cell lines, researchers have defined the intracellular life cycle of Ft. Based on the research in various labs, the intracellular life cycle is as follows: 1) phagocytic uptake, 2) residence in the phagosome - termed the Francisella-containing phagosome (FCP), 3) interaction with early endocytic (EE) and late endocytic (LE) compartments, 4) phagosomal membrane disruption, 5) entry into the host cytosol, 6) bacterial replication, 7) apoptotic and pyroptotic death of host cell[42–45]. The culmination of this research supports the claim that Ft is a cytosolic pathogen, however, it cycles through multiple intracellular compartments with varying environments, making this portion of the life cycle particularly complex. The cycling through these environments also leads to the conclusion that Ft has evolved specialized mechanisms to not only survive but thrive and reproduce within dynamic, stress inducing environments.

Ft entry into nonphagocytic host cells has yet to be defined, however phagocytic uptake within macrophages has been extensively studied and seems to involve the interaction with different phagocytic receptors, which appear to differ based on opsonization[43,46,47]. Antibody opsonized Ft enters macrophages through the FC gamma receptor[47], whereas bacteria that are opsonized by complement binds to the complement receptor 3 (CR3) or the scavenger receptor A (SRA)[45,48]. In contrast to both of these entry events, Ft that has not been opsonized can bind to the mannose receptor to gain entry to the host macrophage[47]. However, it is still unknown which Ft surface receptor is responsible for binding to the mannose receptor. Though several host receptors have been identified as important to Ft attachment, the bacterial factors are not well

characterized. The type IV pilus is an adhesin that contributes to host cell attachment and virulence in several other bacterial species such as *Pseudomonas aeruginosa*, *Neisseria* spp, *Vibrio cholerae*, and *Moraxella catarrhalis*[49,50]. Schu S4, LVS and *F. novicida* all have genes encoding for a Type IV pilius, however, each of these strains have differences within the structure and functional role of the Type IV pilus[50]. In addition, the Ft outer membrane protein (FsaP) may also contribute to host cell attachment. FsaP was identified in LVS but was also found to be upregulated in Type A strains including Schu S4 when isolated from infected mice[51]. Finally, the surface-expressed Ft elongation factor-Tu (EF-Tu) was determined to interact with the host surface receptor, nucleolin and mediate attachment to THP-1 cells[50]. Unfortunately, none of these Ft surface receptors have been determined to be entirely responsible for the attachment of Ft to the host cell and each receptor seems to differ based on the Ft strain.

If opsonized by either complement or antibody, Ft binds to the host cell and is engulfed by a unique form of uptake known as looping phagocytosis[45.] Proceeding engulfment, Fcγ-mediated phagocytosis is activated in macrophages, and neutrophils[47,52]. Once inside the host cell, Ft resides transiently within the FCP before entering the cytoplasm[44,45]. In order to escape the phagosome, the Ft must trigger modifications to the FCP, including acidification of the phagosome like that of a late endosome-like compartment[44,52–54]. In turn, the acidified environment activates virulence genes within Ft and allows disruption of the phagosomal membrane and entry into the host cytoplasm[44,53,54].

Thus far, at least 270 genes have been identified across all infectious strains of Ft as important for replication within the mammalian host cell[55]. However, for the purpose of this dissertation the highly virulent Shu S4 strain will be used, in which it has been determined that there are 453 genes essential for growth *in vitro*[56]. These identified genes include key metabolic pathways, the Francisella pathogenicity island (FPI) proteins and their regulators as well as 34 genes that had never been associated with Ft virulence before this 2019 study[56]. One example is RNase R encoding gene *rng*. This gene has been determined to influence virulence gene expression and inactivation of *rng* has been linked to the upregulation and expression of heat-shock genes involved in stress response in *Stenotrophomonas maltophilia*[56,57]. A large number of these gene products are hypothetical proteins but have been thought to be necessary to survive and escape the phagosome for entry into the cytoplasm. However, it is conceivable that they too serve a dual purpose to not only survive the nutrient depleted, stressful environment associated with the phagosome, but may also provide protection from extracellular environmental stressors as well.

1.5.2. Extracellular life cycle

The terrestrial and/or aquatic survival of Ft has been described throughout history as persistence in the environment but remains to be fully characterized despite decades of research. As mentioned previously, multiple studies have detected the presence of Ft strains in water and sediment and have experienced outbreaks that do not correspond with arthropod or transmission by infected animal[17–23.] These studies along with the fact that most mammals are susceptible to infection, and some animals can survive and clear

the infection naturally, leads to the theory that Ft may persist in the environment in a non-mammalian, non-arthropod reservoir. The majority of studies suggesting long-term survival of Ft in the environment have detected the pathogen by either culture or PCR in environmental water samples[3,7,17–23,56]. Long-term detection of Ft has been reported from countries in which water-borne cases are frequent. For example, in Turkey, two independent studies were conducted on fresh water that was collected from a water source near a village and from the water supply for the same village in 2008, 2009 and 2012, Ft was detected in each water source, for every recorded year, these studies confirmed two different water sources as persistent reservoirs[57,58]. In Sweden, Ft was detected in water and sediment over a three-year period, during outbreak and non-outbreak periods, this study determined that Ft is persistent in water and/or sediment between tularemia outbreaks[20]. Similarly, in Ukraine, Germany and the Netherland, after reemergence of tularemia cases, ecological studies were conducted, which showed that the natural waters are an aquatic reservoir for Ft[59–61]. Taken together, these studies suggest that Ft is either present in the environment due to repeated contamination by infected animal carcasses and waste or persistence in the environment without a host.

Laboratory studies have described the ability of both type A and type B Ft to survive in water microcosms from 1 to 70 days[27,62,63]. Upon further analysis, both water temperature and salinity seem to affect the persistence and survival of Ft within the environment. In a 2012 study, ssp *holarctica* was cultivable for one day at 25°C but was cultivable for up to 28 days at 8°C[63]. Likewise, ssp *holarctica* and ssp *tularensis* were cultivable in fresh water for up to 10 days at 21°C but remained cultivable for up to 42 days

in brackish water (3603.5mg/L NaCl) at 21°C 27. In 2000, Ft LVS was described as persistent in tap water for 140 days[56]. Most recently, in 2021, while studying formation of biofilms in Ft, it was concluded that ssp *holarctica* remained, not only culturable, but infectious in a mouse model after a 24-week incubation period in freshwater at 4°C, however it was determined that SchuS4 lost viability between 14 and 20 weeks and was also determined to be avirulent after 24 weeks[39]. Interestingly in the two former studies[26,57], the researchers utilized colony-forming units (CFU) on nutrient agar plates to determine viability of the bacteria, and determined that Ft was still metabolically active in the water microcosm after it was no longer culturable on nutrient agar plates. This state is defined as ’viable but non-culturable' (VBNC). The VBNC state is a unique strategy utilized by many bacterial species to survive adverse conditions. In addition, bacteria in the VBNC state may be “resuscitated” and become culturable under specific, yet unknown, conditions[62,64]. The VBNC state may be an adaptive strategy used by Ft that supports long-term survival, though the specific trigger for inducing this state remains unknown.

1.5.3. **Viable but non-culturable state**

The VBNC state was first reported in *Escherichia coli* and *Vibrio cholerae* in 1982. Since its discovery, 85 species of bacteria have been found to have a VBNC state[56]. Within these 85 species, 67 species are considered to be pathogenic and 16 of the pathogens are known to cause human infection[56]. Pathogenic bacteria that have a VBNC state are of major concern for public health and safety. This is due to the fact that these bacteria can contaminate food and water sources but are not detectable by conventional testing methods. This leads to false negatives and underestimation of total viable cells within a

sample. Therefore, the identification of pathogens with a VBNC state, as well as understanding the mechanism used to induce and maintain this state is of high importance. Furthermore, humans and/or other mammals that are infected with a VBNC pathogen may take weeks or months to manifest disease symptoms, this is concerning in terms of exposure to natural infection or intentional release, due to the delayed response time for treatment, decontamination, and reduction of transmission.

Despite the "non-cultruability" of bacteria within the VBNC state, the cells are not regarded as non-viable or dead due to some key characteristics. These characteristics include an intact membrane that retains undamaged DNA, metabolic activity, respiration, active transcription and continued nutrient uptake[58,59]. Likewise, VBNC cells often exhibit physiological and molecular differences when compared to viable, culturable cells. These differences may include morphology, rate of metabolism, composition of cell membrane, gene expression, resistances to physical and chemical stressors (including drug resistances), and virulence potential. However, these differences are not consistent across all species that have a VBNC state.

Some species experience cell dwarfing, or reduction in size upon entry into the VBNC state, while other species experience a complete change in shape[58,59]. These changes are common in cells within the VBNC state but are also associated with cells under stressful conditions that do not enter the VBNC state, therefore change in morphology cannot be used alone to determine whether a cell is in the VBNC state or not[58,60]. A morphological change during VBNC state has not been recorded in Ft. VBNC cells also show distinct differences in cell membrane composition, usually including

proteins, fatty acids and peptidoglycan[58]. Each of these changes in membrane characteristics may affect host cell attachment, detection by the immune system and entry into host cells for intracellular pathogens such as Ft, but cell membrane/wall composition while in the VBNC state has never been described for Ft. Metabolic rate decreases in VBNC cells to conserve the available nutrients and to not expend energy, however, though active metabolism has been observed in VBNC cells of Ft, metabolic rate differences have never been measured or compared to culturable cells[29,61,62]. In addition, cells within the VBNC state have a completely different gene profile compared to cells within exponential growth. This has been observed in both *E. coli*[63] and in *V. cholerae*[64] as well as other human pathogens, but gene expression has not been studied in Ft cells within the VBNC state. One of the most important properties associated with the VBNC state is the ability to withstand chemical and physical stressors. These stressors may even serve as the inducer of the VBNC state within many species. In terms of physical stressors, some species have been observed to withstand sonication and extreme temperature change[58]. In terms of chemical stressors, greater tolerance to varying salinity, pH, ethanol, chlorine, and antibiotics has been observed in VBNC cells[58,60]. However, though Ft has been confirmed to persist for extended periods when exposed to similar stressors, it has not been confirmed that these cells were indeed in a VBNC state[40,41,61,65,66].

2. CHARACTERIZATION OF *Francisella tularensis* VIABILITY AND CULTURABILITY WHEN EXPOSED TO ENVIRONMENTAL STRESSORS

2.1. Significance and Aims

Ft has evolved adaptive mechanisms to persist in adverse extracellular conditions, transition to and from vertebrate and invertebrate hosts and replicate intracellularly. Consequently, Ft has been associated with vector-borne, water-borne and food-borne disease. However, the adaptive changes undergone by Ft, including gain and loss of virulence, during transitions from one environment to another are not well defined. Also, environmental reservoirs for Ft persistence between outbreaks have not been identified. There is a critical need to understand the adaptive strategies employed by Ft in order to survive in adverse conditions and maintain or regain virulence in the mammalian host. Understanding these strategies will aid in identification of areas where Ft may persist between natural outbreaks or intentional dissemination. Furthermore, gaining a better understanding of the pathogen's life cycle may also lead to new targets for antimicrobial agents and therapies. To begin addressing these gaps in knowledge, viability and culturability has been evaluated in the highly virulent strain of Ft, ssp. t*ularensis,* SchuS4, after exposure to a variety of different stressors. Data from other researchers has also been evaluated to determine which genes may be up or down regulated in response to the environmental stressors.

<u>Specific Aim 1:</u> Evaluate the effect of environmental stressors on viability and culturability of Schu S4

Hypothesis: Environmental stressors such as temperature and nutrient deprivation within a microcosm will result in a subpopulation of Sch S4 cells entering a VBNC state.

<u>Specific Aim 2</u>: Establish a quick and accurate way to distinguish between and estimate VBNC, VC and dead Schu S4 cells.

Hypothesis: Membrane integrity along with culturability can be used to estimate the number of cells within a population that have entered the VBNC state.

2.2. Overall hypothesis and Innovation

It was hypothesized that *Francisella tularensis ssp. tularensis* Schu S4 enters a viable but non-culturable (VBNC) state when exposed to environmental stressors. The results from these studies not only provide new information to the field about Ft life cycle and persistence, but also provide insights into novel detection methods that are rapid and accurate.

2.3. Materials and methods

2.3.1. Biosafety Statement

All assays in this study were carried out in containment conditions within a BSL-3 laboratory in accordance with guidelines set forth by the MU responsible official and Institutional Biosafety Committee, University of Missouri as well as federal guidelines provided by the Centers for Disease Control and Prevention (CDC), Department of Defense (DoD), and the National Institute of Allergy and Infectious Diseases (NIAID). The

culture assays were conducted in a tier-1 suite at the Laboratory of Infectious Disease Research (LIDR) at the University of Missouri. The viability and membrane integrity assays were carried out within the immunology core at LIDR operating at teir-1 conditions.

2.3.2. Bacterial strain and culture conditions

The Ft *ssp tularensis* type A Schu S4 strain used for this study was obtained from the Biodefense and Emerging Infections (BEI) Research Resources Repository in Manassas, VA. The stock Schu S4 was first inoculated onto a BBL™ Chocolate II Agar (GC II Agar with Hemoglobin and IsoVitaleX™) and grown overnight at 37°C with 5% CO_2. A single colony was then isolated from the plate and used to inoculate 10ml modified Mueller-Hinton (MHM) broth containing Mueller-Hinton broth powder (VWR cat# 101320-364), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), glucose, ferric pyrophosphate and IsoVitaleX™. This liquid culture was grown overnight at 37°C, 180 rpm. 1ml of overnight culture was used to inoculate 100ml of fresh MHM and was grown for 48 hours at 37°C, 180 rpm. This culture was then concentrated by centrifugation, resuspended in fresh MHM with 5% glycerol, aliquoted into 1ml samples and stored at -80°C to be used as working stock for the remainder of the study. Routine Schu S4 cultures were grown overnight at 37°C, 180 rpm after inoculating 10ml of MHM with 1ml of working stock.

For the environmental stressor experiments, a routine culture of Schu S4 was grown overnight as mentioned above and was used to inoculate a 50 ml stock of MHM which was grown at 37°C, 180 rpm for 48 hours Nine different microcosms were defined with 9 aliquots from the same stock culture. This culture was washed with PBS and

adjusted to 10^9 CFU/ml before resuspending into 10ml of one of the following: MHM, sterile tap water, or sterile dH_2O at 4°C, 18°C or 37°C. Each of these conditions were tested in triplicate.

2.3.3. Determining culturability of Ft

Culturability of SchuS4 was assessed every day for the first seven days followed by once a week for a total of 8 weeks. Culturability and CFU count was determined by an optimized, drop plate method. 10X Serial dilutions of the cultures were performed using sterile PBS in a 96 well-plate in triplicate. 10uL drops from each dilution were plated on BBL™ Chocolate II Agar. The drops were left to dry on the plates for approximately 5 minutes and the plates were inverted and incubated for 48hrs at 37°C and 5% CO_2. A countable dilution was determined to be the dilution that gives 3-30 countable colonies. The colonies were counted for each dilution and then scaled up and expressed as CFU/ml. If no colonies were present at any dilution, it was considered to be non-culturable.

2.3.4. Determining viability of Ft

To determine viability of Schu S4 I used the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen). This assay uses a mixture of SYTO® 9 green-fluorescent nucleic acid stain and propidium iodide red-fluorescent nucleic acid stain. SYTO 9 penetrates the membrane of all bacterial cells, where propidium iodide can only penetrate those cells with a damaged membrane. Therefore, SYTO 9 (green) fluorescence is visible in live cells and propidium iodide (red) fluorescence is visible in dead cells. The BacLight assay was used to stain Schu S4 in accordance with the product information sheet. The stained samples were stabilized by suspension in a low melting temp agarose in a 1:1 ratio for

image analysis. BioTek's Lionheart™ LX Automated Microscope was used to view and analyze the stained samples. The images acquired with scanning were performed in brightfield using a 4x objective, then the A 20x objective with a correction collar setting was used for image capture and for analysis. Brightfield and fluorescence with a combined FITC/GFP/LED (SYTO 9) filter and a PI/LED (propidium iodide) filter were used to view stained cells. Z-stack images were obtained via vertical acquisition of 50 focal planes at 11uM per slice for a total depth of 550uM. Image processing was applied using a rolling ball size of 2uM. To obtain cell counts, a primary mask was used to distinguish SYTO9 labeled objects that range from 0.2-0.7uM was selected. A secondary mask was defined for Propidium iodide labeled objects ranging from 0.2-0.7uM. The primary and secondary masks were used in tandem to determine dually staining cells. A volume calculation was made based on the dimensions of the Z- stack in order to determine cell count.

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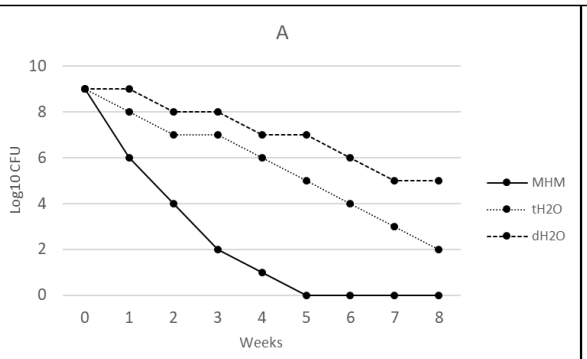
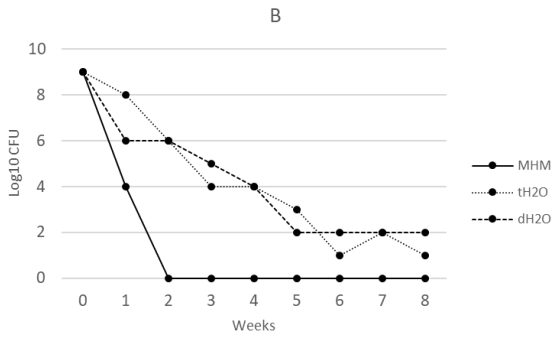
2.4. Results

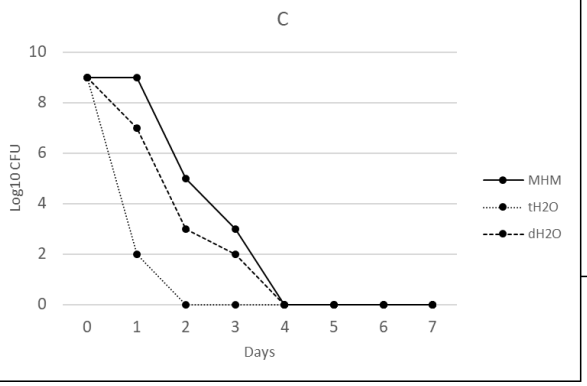
2.4.1. *F. tularensis* Schu S4 remains culturable in nutrient rich conditions and nutrient depleted conditions for at least 8 weeks at low temperatures.

Within the first four days after incubation in supplemented nutrient media at 37°C, culturability of Schu S4 rapidly declined from 10^9 CFU to complete loss of culturability (Figure 1C). However, when incubated in the same supplemented nutrient media at 18°C and 4°C, the culturability was extended to 2 and 4 weeks respectively (Figure1A-B). When incubated in nutrient depleted, sterile tap water and dH_2O at 37°C, Schu S4 lost culturability two- and four-days post inoculation respectively (Figure1A). However, when the temperature of these microcosms decreased to 18°C and 4°C, culturability declined slowly but remained culturable for the remainder of the experiment (Figure1A-B).

Figure 1: Culturability of SchuS4 in Nutrient-Rich and Nutrient-Poor Conditions





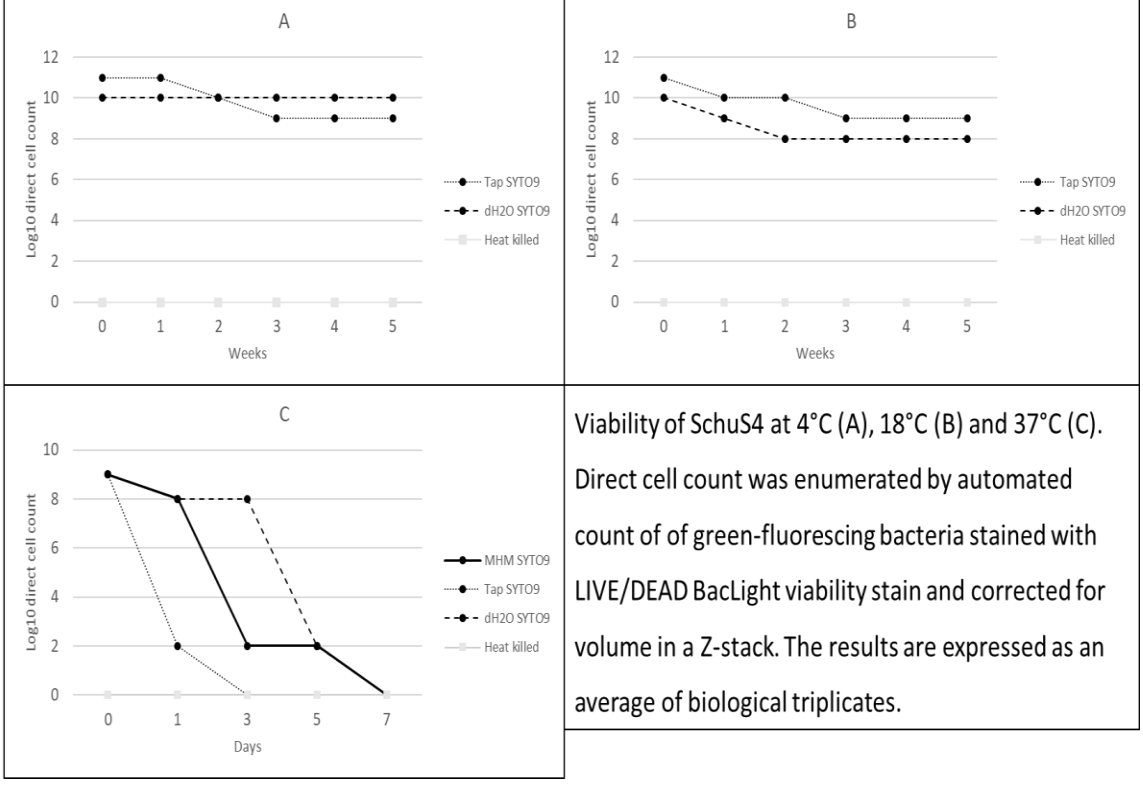


Culturability of SchuS4 in supplemented growth media (MHM), sterile tap water (tH20) and sterile DI water (dH2O) at 4°C (A), 18°C (B) and 37°C (C). CFU count was enumerated using serial dilutions and the drip plate method on CHOC II agar plates. The results are expressed as an average of biological triplicates.

2.4.2. *F. tularensis* Schu S4 loses viability at high temperatures in a nutrient rich and nutrient depleted environment within six days

In a nutrient rich environment, at 37°C Schu S4 lost viability within five days post inoculation. For this condition, viability and culturability were correlated. After five days post inoculation, no cells stained exclusively with Syto9, indicating total cell death (figure 2C). In nutrient poor tap water loss of viability was indicated at three days post inoculation which also correlated with culturability (figure 2C). However, in nutrient poor dH_2O, complete loss of viability was not observed until seven days post inoculation, which was three days after the loss of culturability (figure 2C).

Figure 2: Viability of SchuS4 in Nutrient-poor conditions



Viability of SchuS4 at 4°C (A), 18°C (B) and 37°C (C). Direct cell count was enumerated by automated count of of green-fluorescing bacteria stained with LIVE/DEAD BacLight viability stain and corrected for volume in a Z-stack. The results are expressed as an average of biological triplicates.

2.4.3. *F. tularensis* Schu S4 remains viable for at least 5 weeks in nutrient poor conditions at low temperatures

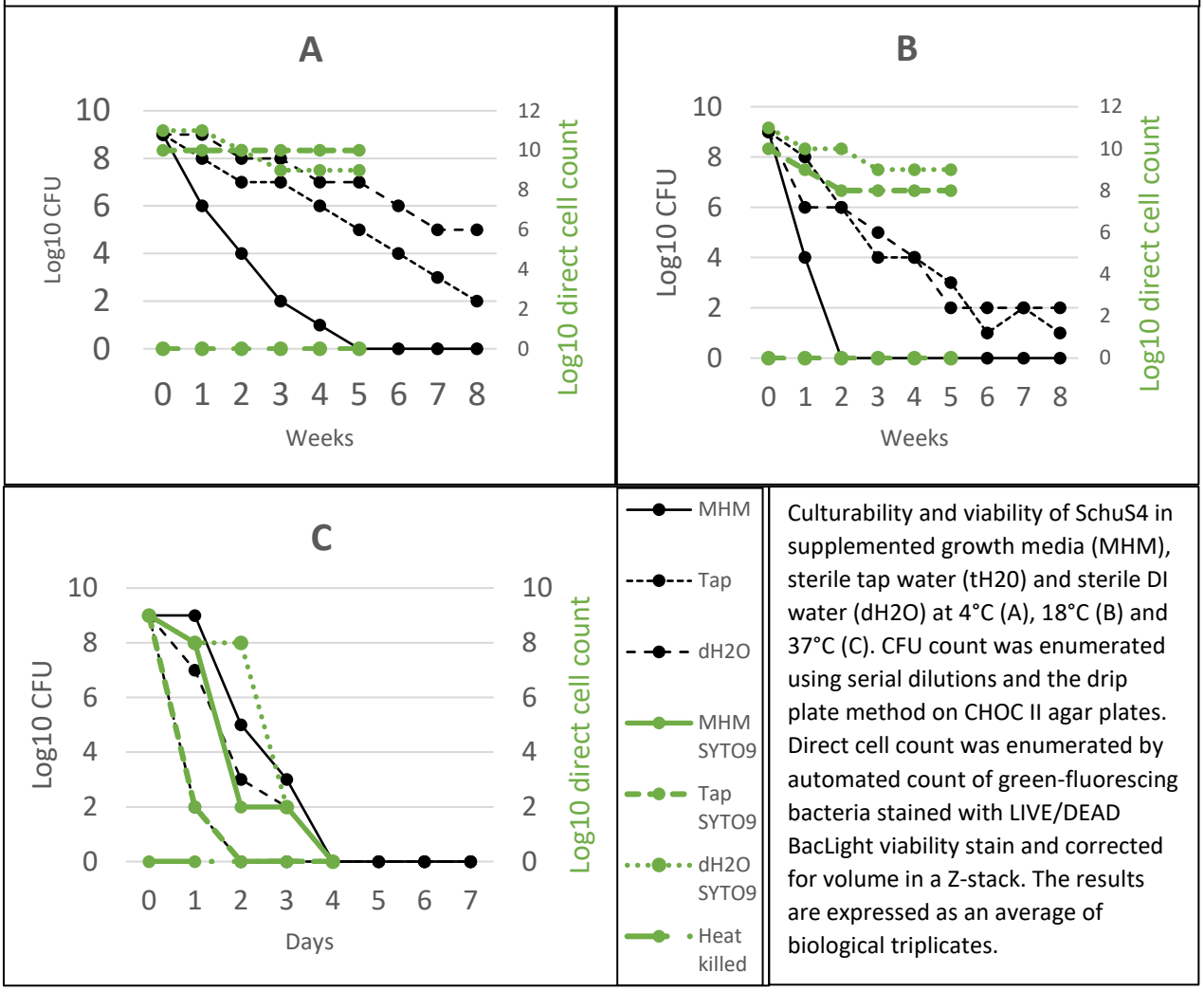
When incubated in nutrient poor tap water and dH_2O at both 18°C and 4°C almost all cells stained exclusively with SYTO 9, indicating intact cell membranes and viable cells. This result continued through the remainder of the experiment. Results indicate that roughly the full bacterial inoculum remained viable for at least 5 weeks (Figure 2A-B) at 18°C and 4°C.

2.4.4. The VBNC state is induced in F. tularensis Schu S4 in nutrient depleted conditions at low temperatures

The viability of Schu S4 incubated in nutrient poor, sterile tap water or dH_2O for 5 weeks at 18°C and 4°C was compared with the control, heat killed Schu S4. A comparison of fluorescence between the control and experimental cells is shown (Figure 2A-B). For Schu S4 cells incubated in tap water at 18°C, culturability decreased at a steady pace from 10^9 to 10^2 CFU/ml within 8 weeks (Figure 1B). However, the number of viable cells decreased from 10^{11} to 10^7 cells/ml within 3 weeks but remained constant through the remainder of the 5 weeks (Figure 3B). Likewise, culturability of Schu S4 incubated in tap water at 4°C, decreased at steady rate but remained culturable through 8 weeks (Figure 3A), where the number of viable cells decreased slightly, but remained at 10^7 cells/ml for the remainder of 5 weeks (Figure 3A). Culturability and viability of Schu S4 incubated in dH_2O at 18°C, followed a trend similar to that of the tap water incubated SchuS4 (Figure 3B). Interestingly, viability of Schu S4 incubated in dH_2O at 4°C never decreased (Figure 3A) and remained constant though 5 weeks even though culturability decreased at a

steady rate (Figure 3A).

Figure 3: Viability and Culturability of SchuS4



Culturability and viability of SchuS4 in supplemented growth media (MHM), sterile tap water (tH20) and sterile DI water (dH2O) at 4°C (A), 18°C (B) and 37°C (C). CFU count was enumerated using serial dilutions and the drip plate method on CHOC II agar plates. Direct cell count was enumerated by automated count of green-fluorescing bacteria stained with LIVE/DEAD BacLight viability stain and corrected for volume in a Z-stack. The results are expressed as an average of biological triplicates.

2.5. Conclusion

Although *Francisella tularensis* has been studied for over 100 years, and the presence of the pathogen within the aquatic environment has been identified in several studies, the mechanism of persistence in the environment, and the aquatic reservoir for Ft has yet to be determined. In line with recent studies that have shown induction of the VBNC state in Ft *ssp holarctica,* as a result of reduced nutrients and low temperature, it was hypothesized that *Francisella tularensis ssp. tularensis* Schu S4 also enters a viable but non-culturable (VBNC) state when exposed to environmental stressors. It was observed that in nutrient depleted tap (tH20) and deionized (dH2O) water at lower temperatures (4 and 18°C) Schu S4 remained culturable for at least 8 weeks, with ~6 Log decrease in CFU. However, within the 8-week period in which culturability decreased, viability remained constant. These results indicate that a VBNC state was induced in a subpopulation of Schu S4 in the nutrient-depleted, low temperature microcosms. It was further observed that at 37°C in nutrient-depleted tH20 or dH2O, culturability and viability were both lost by day 4. This indicates that temperature is important for the induction of the VBNC state in Schu S4. Our results support the previous data that Ft persistence and randomized infections in humans are often found in areas with water temperatures ranging from 4 to 30°C. Likewise, seasonal temperature change could pay a major role in the induction of the VBNC state within the aquatic environment. With this confirmation that highly infectious SchuS4 may survive in water for months while remaining undetectable by conventional methods, it is imperative that alternative methods of detection, such as PCR or more advanced technologies are used when

monitoring aquatic environments during potential outbreaks or intentional release. Further studies will be necessary in order to determine subsequent environmental conditions that may also lead to induction of the VBNC state such as change in pH, salinity, presence of other microbes, etc. It is also important to further understand if Schu S4 can infect animals while in the VBNC state. Similarly, it is necessary to understand what is required for resuscitation of VBNC to culturable Schu S4. As suggested by our findings, it may be necessary to reconsider the standard temperature for growing Schu S4 cultures in both the laboratory and clinical settings. Ft is notoriously difficult to culture and only 10% of samples retrieved from infected individuals test positive via culture testing. These discrepancies may be due to incubation at a temperature that is too harsh to support "healthy" growth of Schu S4.

3. Overview of HIV

The human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS) belongs to a subgroup of retroviruses and was first recognized in humans 1981[67,68]. However, the origin of HIV is clearly defined as a zoonotic transmission event between non-human primates to humans un the early 1900s[69]. Since then, 76 million people have been infected with HIV and approximately 35 million people are currently living with HIV. HIV has been devided into types, groups and subtypes. There are two major types of HIV, HIV type 1 (HIV-1), which causes ~95% of human infections, and HIV-2, which accounts for ~5% of all human infection[68–70.] Since the beginning of the HIV epidemic, considerable progress has been made towards the treatment of individuals diagnosed with HIV and AIDS. For example, the development of combination antiretroviral therapy (cART) has increased the life-expectancy of people living with HIV to a life-expectancy similar to that of an uninfected person[71]. However, the development of cART comes at price of new challenges. The virus develops resistance to drugs of first-line regimen and are therefore the patient must be switched to costly, second- and third-line treatment regimens[72]. Furthermore, transmission of HIV with these drug resistances mutations reduces the effectiveness of the first-line regimens requiring continuous monitoring of viral load. Therefore, it is important to understand the mechanisms behind the drug resistances and develop new treatments against mutated viruses.

3.1. HIV Genome Organization

A single positive stranded RNA molecules make up the HIV genome. However, the core of the mature virion contains the two identical copies of RNA genome. A series of events including annealing of the Primer Binding Site (PBS) with the 3'end of $tRNA^{Lys3}$, cleavage by RNase H, two (first and second) jumps of partial double-stranded DNA, and the cleavage of flaps results into the formation of a dsDNA genome of virus that exists in complex with HIV-1 integrase called pre-integration complex (PIC) generates a integration-ready DNA copy of the HIV-genome. The HIV proviral DNA is the product of reverse transcription of viral RNA into DNA and the double stranded DNA product in then inserted into the host genome[73]. The HIV genome contains several genes, which encode structural proteins that are common among the retroviruses, however, it also includes overlapping open reading frames that encode accessory regulatory proteins[74]. The 5'LTR region of the HIV genome has the transcriptional promoter of the viral genes[74]. The genes that code for the HIV structural proteins are *gag, pol,* and *env*. The *gag* gene encodes the following proteins in, order of 5' to 3' directional reading: outer core membrane proteins (MA, p17), capsid protein (CA, p24), nucleocapsid (NC,p7) and a nucleic acid-stabilizing protein[73,74]. The *gag* reading frame is followed by the *pol* reading frame which encodes for the following enzymes: protease (PR, p12), reverse transcriptase (RT, p51), and RNAse H (p15) and integrase (IN, p32)[74]. Following the *pol* in the *env* open reading frame. The *env* gene encodes two envelope glycoproteins proteins: gp120 and gp41[73,74]. HIV-1 also produces short, spliced mRNAs that encode for important regulatory proteins including

the transactivator protein (Tat) and the RNA splicing-regulator (Rev), which are necessary for the initiation of HIV replication. Incompletely spliced mRNAs encode more regulatory proteins: negative regulating factor (Nef), viral infectivity factor (Vif), viral protein r (Vpr) and virus protein unique (Vpu), which impact either viral replication, budding or pathogenesis[73–75].

3.2. HIV structure

The mature HIV viral particle measures ~100 nm in diameter and is surrounded by a lipid envelope. The envelope contains 72 spikes that are made up of trimers of linked heterodimers that consist of gp120 and gp41[73,76]. Furthermore, the viral envelope is composed of a lipid bilayer that surrounds a series of structural shells that hold the viral genome and its necessary enzymes[76]. The outermost shell is formed by matrix proteins (MA) which interact with the inner layer of the envelope[77]. A conical core is located inside of the matrix layer and is composed of ~1000-1500 copies of the capsid (CA) protein[77.] The ribonucleoprotein (RNP) is located inside of the capsid and contains two copies of the positive-sense RNA genome[76,77]. Several (~30 to 40) copies of the RT, RNAse H, and IN enzymes are also packaged within the capsid engulfing the nucleic acid[73,76,77].

3.3. Replication of HIV

The initial step of the HIV replication cycle is binding to the host cell. During this step, the viral spike proteins on the envelope bind to a surface protein (CXCR5 and CCR4) on a host cell[76]. Once HIV is bound to the host cell, the viral membrane begins to fuse with the host cell membrane, and the HIV core is released into the cytoplasm of the cell [76,78.] Once inside the cell, the capsid protein disassembles, but the nucleoprotein complex

remains intact[78]. Reverse transcription takes place within this complex and it is termed the pre-integration complex[78]. To initiate reverse transcription, a cellular tRNA ($tRNA^{Lys3}$) is required to act as a primer[75,76,78]. Therefore, a tRNA is incorporated during assembly of the viral particle in its origin cell. This tRNA is then annealed to the viral RNA genome at a primer binding site (PBS)[78]. This tRNA, termed $tRNA^{Lys3}$, primes the reverse transcriptase-catalyzed synthesis of cDNA[78]. The double-stranded cDNA product is then translocated into the host cell nucleus and integrated into the host genome by HIV integrase. The cellular machinery makes the RNA copies of the HIV genome to be used for protein synthesis. The newly synthesized RNA then is exported out of the nucleus either as spliced RNA which codes for envelope protein and several viral regulatory proteins or full-length RNA, which is either translated to *produce gag* and *pol* polyproteins or is packaged into new virions[78]. Both viral genomic RNA and $tRNA^{Lys3}$ together with many additional host factors are packaged into the new virions during the assembly process. Once immature virus-like particles are assembled, they begin the process of budding at the host cell membrane[76]. Once the buds are released from the cell membrane, the viral protease self-cleaves, to cleave mature viral proteins that are encapsulated within the core, and particles begin the maturation process. The steps of HIV replication are visually represented in Figure 4.

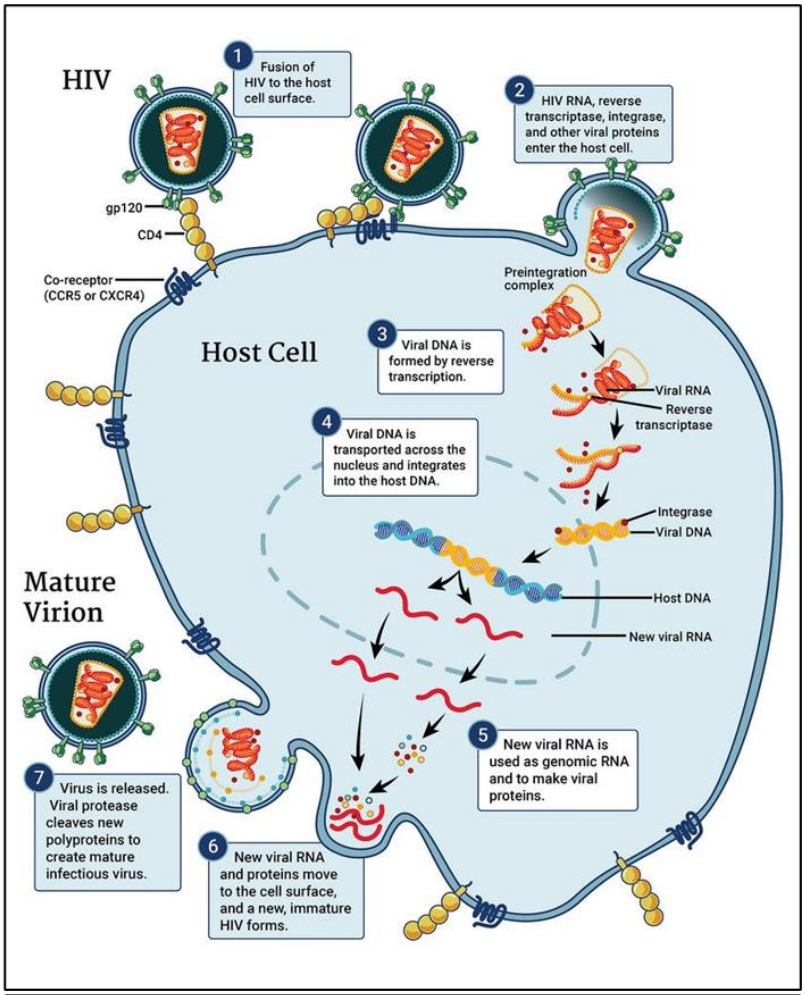


Figure 4: Illustration of the HIV replication cycle. (Adapted from NIAID: https://www.niaid.nih.gov/diseases-conditions/hiv-replication-cycle)

3.4. Structure and Function of HIV-1 Integrase (IN)

The HIV enzyme IN has two important catalytic functions: 3'processing and DNA strand transfer, both functions are essential for the insertion of the viral cDNA into the genome of host cells[79,80]. HIV-1 IN is a 32 kDa enzyme, consisting of three functional domains: N-terminus domain, catalytic core domain (CCD) and C-terminus domain (Figure 5)[79].

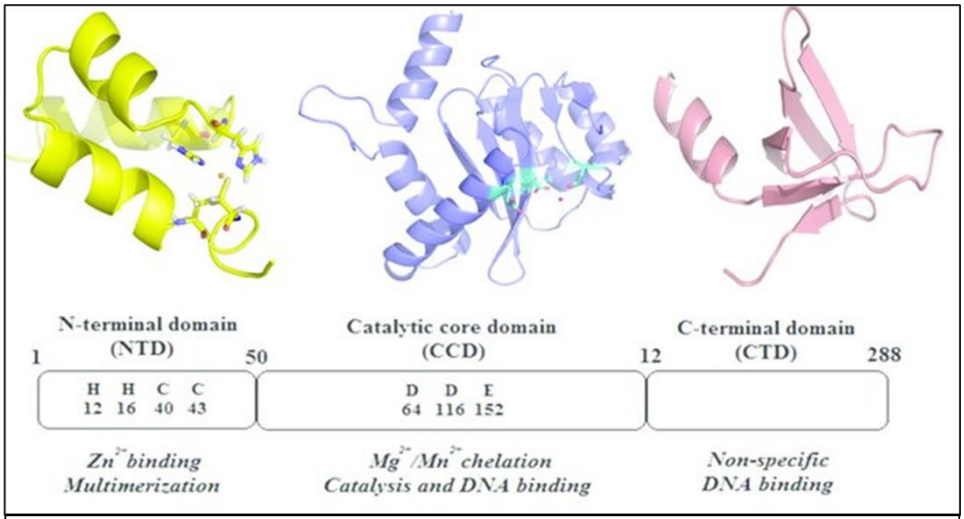


Figure 5: HIV Integrase Structural Domains (Adapted from: Hajimahdi et al)

Within the N-terminus domain, there is an HHCC binding motif that binds Zn^{2+} and stabilizes the proteins quaternary structure (Figure 5)[79,80.] The CCD contains a motif known as the catalytic triad (DD35E) that coordinates one Mg^{2+} between D116 and D64 and the other Mg^{2+} between D64 and E152 (Figure 5)[79,81]. The CTD binds DNA non-specifically and stabilizes the bond between IN and vDNA (Figure 5). Though the CCD contains the enzymatically active site, full catalytic activity requires all three domains.

Integration is required for the replication of HIV because both transcription of the viral genome and production of viral proteins are dependent on the integration of full-length viral cDNA into the host chromosome[79,81]. After reverse transcription, the viral cDNA is prepared for integration integrase-mediated trimming of the 3'-ends of the viral DNA[81]. Ate each end of the viral DNA are long terminal repeats (LTR). The LTR is cleaved adjacent to invariant nucleotide sequence and exposes a recessed 3' terminus[79,80]. With this region exposed, IN cuts the chromosomal DNA strand across the major groove and joins the viral DNA ends to the targeted DNA 5' - phosphates[79,80]. Single strand gaps are

repaired via host enzymes (DNA polymerases, flap endonuclease and DNA ligase) to complete the integration process and establish a stable provirus[79,81].

3.5. HIV Antiviral Therapy

Six different classes of drugs are FDA approved for treatment of HIV-1[82]. However, the foundational antiviral drugs that were first implemented in the clinic are the nucleoside reverse transcriptase inhibitors (NRTIs)[83]. Generally, NRTIs act as chain-terminators leading to the stalling of the HIV RT[83]. The nonnucleoside RT inhibitors (NNRTIs) also affect the function of the HIV RT but do so by binding to a specific "pocket" distinct from the catalytic site[83]. These RT targeting drugs were soon followed by integrase strand transfer inhibitors (INSTIs), protease inhibitors (PI), fusion inhibitors, and coreceptor antagonists[84]. The first antiviral drugs for HIV-1 were given as a monotherapy, but the standard of care evolved in 1996 , with the administration of combination of drugs known as HAART (highly active antiretroviral therapy)[84]. This combination therapy included a cocktail of three fully active antiviral agents that targeted different steps of the HIV life cycle[84]. With the proper implementation of HAART for HIV patients, viral replication can be suppressed for decades and greatly increases life expectancy[84]. In recent years (2010), the terminology, HAART has evolved into a more simplified version, cART. Typically, cART consists of a combination of NRTIs, as backbone plus either NNRTIs, PIs, or INSTIs. Though cART is highly effective at keeping viral loads under control and improving quality of life for the patient, persistent viral replication within reservoirs may still occur[83,84]. This may be due to poor adherence to drug schedule, poor drug tolerability, and unfavorable interactions between antiviral agents and or other drugs[84]. Each of these

instances may lead to the evolution of drug resistance mutations within the persistent virus population[84]. Drug resistance has been documented for all antiviral drug classes and has been the cause of failed therapy in patients. For the purpose of this dissertation, I will focus on drug resistances within the INSTI drug class.

3.6. INSTI Resistance

Integrase is one of the most recent HIV enzymes to be targeted for the drug development[81,84.] First integrase inhibitor Raltegravir (RAL) was approved by the FDA in 2007, followed by Elvitegravir (ELV) in 2012, and Dolutegravir (DTG) in 2013[81,84] and Bictegravir (BIC) in 2018. AS mentioned previously, the function of integrase is to catalyze 3' processing and viral DNA strand transfer. All drugs that target HIV integrase, target both 3'endonuclease and the strand transfer function. However, the efficacy of these drugs more prominent against strand transfer function and are therefore, these drugs are termed integrase strand transfer inhibitors (INSTIs)[82,84]. INSTIs inhibit the IN function by specifically binding between the IN and the DNA and by chelating essential Mg^{2+} ions at the active site[84]. Because these mechanisms of actions are conserved within the INSTI class of drugs, viral mutations that lead to resistance to INSTIs are often found in the integrase active site that is responsible for coordinating the two Mg^{2+} cofactors[81,82,84]. In clinical studies, resistance mutations that appear in patients taking RAL often involve one of three primary genetic mutations: Y143H/R/C, Q148H/R/K and N155H[81,84]. This study will focus on the resistance mutations N155H and its secondary mutation E92Q.

4. Molecular Models of HIV-1 E92Q Mutation and Assessment of Resistance to HIV-1 Integrase Inhibitors

4.1. Materials and methods

4.1.1. PFV IN/DNA structure preparation for validation of docking protocols

Utilizing the PrepWizard module of Schrödinger Suite (Schrödinger LLC, NY), the structure of the PFV IN/DNA complex was prepared. After deleting RAL and DTG from the crystal structures of PFV IN/DNA/RAL (PDB entry 3OYA[27]) and PFV IN/DNA/DTG (PDB entry 3S3M[26]) the complexes were deemed suitable for INSTI docking. The PrepWizard module is used to add missing sidechains and hydrogens, assign bond orders, water sample orientation and create heteroatom states of metal ions. Furthermore, LigPrep (Schrödinger Suite) was used to prepare the structure of RAL and DTG and these structures were docked using Induced-Fit Docking (IFD) (Schrödinger Suite).

4.1.2. Structure of wild type and E92Q mutant HIV-1 IN/DNA and docking of RAL

Our lab first constructed the molecular model of the wild-type HIV-1 IN/DNA complex. To complete this model, one subunit (PDB entry 5U1C[25]) of HIV-1 IN was extracted from the cryoEM structure and was subjected to PrepWizard (Schrödinger Suite, NY) where it was superimposed onto the structure of the PFV IN/DNA/RAL complex (PDB entry 3OYA[27]) using Cα atoms of all amino acid residues within 10 Å from the active site carboxylates of the two enzymes. The DNA from the PFV IN/DNA/RAL complex and the HIV-1 IN structures were merged and then minimized using the MacroModel (Schrödinger Suite, NY). Then, the E92Q mutations were generated using Prime (Schrodinger Suite, NY). WaterDock (PMID: 22396746) was used to dock the water

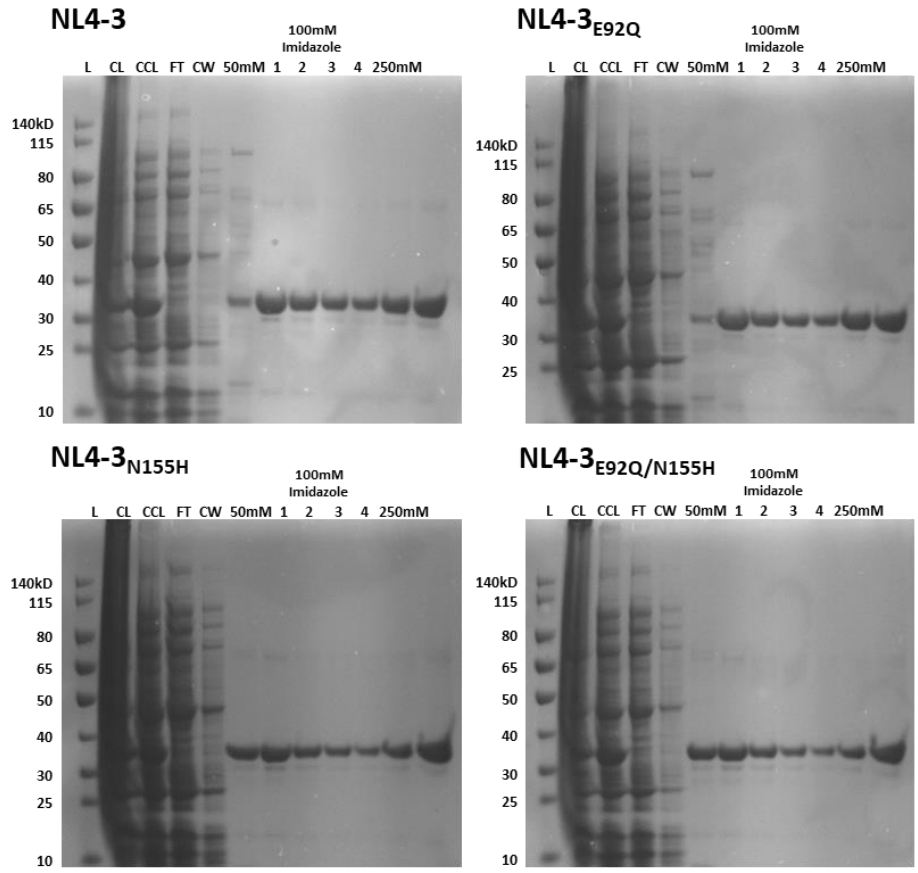
molecules in WT/DNA and E92Q/DNA complexes however, before docking, the water positions seen in the crystal structures of PFV IN/DNA/RAL were validated using WaterDock. Because the WaterDock program only predicts the position of oxygen molecules, the resulting structures were also subjected to the PrepWizard module to add the hydrogens associated with those water molecules. These complexes were used for the docking of RAL using the Induced-Fit Docking (IFD) protocol of Schrödinger Suite. Hybrid QM/MM (quantum mechanics/molecular mechanics) computation was used to optimize and determine electronic properties of the water molecules in the WT-RAL and Q92-RAL complexes with the best docking scores. Finally, Jaguar (Schrödinger Suite) was used to optimize water conformations, determine electronic charge on oxygen atoms and potential energy. All other atoms, except water molecules, were treated with molecular mechanism approximation.

4.1.3. Expression and Purification of WT and Mutant Integrase Enzymes.

The HXB2-derived HIV-1 IN was expressed and then purified as previously described[85]. The coding region of the integrase genes (HXB2: 4230-5096) were cloned into the pRSFDuet-integrase vector with a 6xHis-tag on the N-terminal. The recombinant integrase proteins were then expressed in BL21-DE3-RIL cells and protein expression was induced by the addition of 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) in an incubator-shaker for 3 hours at 37 °C. The cells were harvested via centrifugation and resuspended in 20 mM Tris-HCl pH 8.0, 1 M NaCl, 4 mM 2-Mercaptoethanol (βME), 5 mM imidazole, 10 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate

hydrate (CHAPS), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.15 mg/mL lysozyme. The cells were then homogenized and sonicated and the cell debris was removed via centrifugation. The recombinant IN proteins were then purified by Ni-affinity chromatography. The purified samples were subjected to denaturing polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate to ensure purity of the samples (Figure 6). The mutations were generated using site-directed mutagenesis and nucleotide substitutions were directed by primer mismatches and Phusion High-Fidelity polymerase (ThermoFisher). The methylated template material was subjected to DpnI digestion and transformed into *E. coli* strain HB101 for plasmid repair and expansion. Both WT and mutants were expressed and purified as described previously[85,86].

Figure 6: HIV-1 IN Panel – Expression and Purification



4.1.4. Determination of DNA binding affinity

$K_{d.DNA}$ of WT and E92Q mutant HIV-1 INs were determined using a microscale thermophoresis (MST) assay as described previously[86]. In this assay, the HIV-1 IN/DNA binding was estimated by titrating 20 nM Cy5-labeled DNA (24/19-mer) with increasing concentrations of WT or E92Q mutant HIV-1 IN (7 nM to 12,000 nM) in a buffer containing 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 100 mM NaCl and 0.1% Pluronic-F127. The Cy5-labled DNA substrate consists of 19 nucleotides from the 3'-UTR of HIV-1 subtype C annealed to a 24-mer oligonucleotide. This structure leaves a labled, 5-nucleotide overhang on the 5'-end. The binding isotherms were determined by plotting the

difference in normalized fluorescence against fluorescence associated with the increasing IN concentrations. The data points were fit to a biphasic function (equation 1) to determine $K_{d.DNA}$ of HIV-1 IN in high- and low-affinity DNA binding modes, as previously described[86].

$$Fraction\ bound = A.\frac{(K_{d1}+[IN_0]+[DNA_0])-\sqrt{(K_{d1}+[IN_0]+[DNA_0])^2-4[DNA_0][IN_0]}}{2[DNA_0]} + B.\frac{(K_{d2}+[IN_0]+[DNA_0])-\sqrt{(K_{d2}+[IN_0]+[DNA_0])^2-4[DNA_0][IN_0]}}{2[DNA_0]}$$

(equation 1)

In equation 1: A and B are arbitrary parameters, K_d = [DNA][IN]/[DNA-IN], [DNA] is the concentration of free DNA, [IN] is the concentration of free IN in respective modes and $[IN_0]$ is the concentration of added integrase.

4.1.5. Assessment of 3'-end-processing activity

The 3'- end processing of both the WT and mutants were determined by annealing a 21-mer oligonucleotide to a complementary 5'- Cy3 labeled 21-mer oligonucleotide that contained a 3'-end-CAGT sequence. The cleavage of the GT dinucleotide would result in a 19-mer product. The endonucleolytic cleavage of the 3'-end GT was induced by incubating 300 nM WT or mutant protein, 5 nM DNA substrate, in a buffer containing 20 mM MOPS (3-(N-morpholino) propanesulfonic acid), pH 7.2, 25 mM NaCl, 1 mM $MnCl_2$ and 3 mM βME. The reactions were allowed to proceed for 120 minutes and were stopped at various times by a stop solution containing 95% formamide, 0.01% bromophenol and 50 mM ethylenediaminetetraacetic acid (EDTA). The cleavage products

were resolved on a 20% polyacrylamide 8 M urea gel. To further investigate the 3'-processing activity of IN the presence of RAL, a 3'-processing assay was utilized, as previously described[87], based on the removal of the GT dinucleotide which causes disruption of a quenching probe and fluorescence of an Alexa Fluor ® 488 fluorophore at the 3'end. Inhibition of the 3'-processing results in reduction of the fluorescent signal in comparison with the positive control.

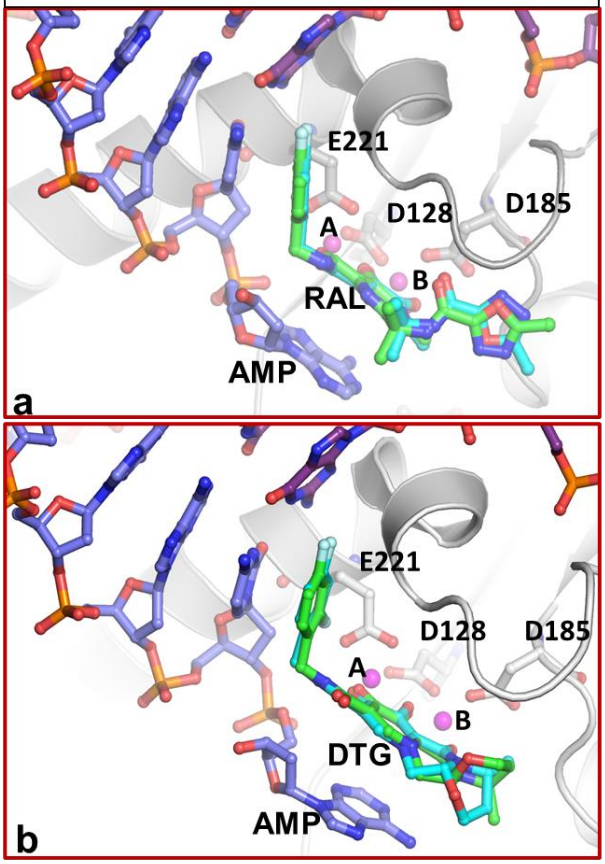
4.2. Results

4.2.1. Validation of docking protocol

Because the crystal structure of the HIV-1 IN in complex with DNA and INSTI has not yet been solved, a docking protocol was used to generate molecular models of this complex. The accuracy of the docking protocol was confirmed by comparison of the PFV IN/DNA/RAL and PFV IN/DNA/DTG complexes generated by the docking protocol to their crystal structures (PDB entries 3OYA and 3S3M)[88,89]. The crystal structures of these complexes contain several water molecules[88], therefore, all water molecules close to the active sites were included in the validation of the docking protocol. The Induced-Fit Docking (IFD) scores (Schrödinger Suite, Schrödinger LLC, New York, NY) of docked RAL and DTG in the PFV IN/DNA complex were calculated and the poses with the best IFD scores were recorded (Figure 7A-B). As determined in Figure 4A, the crystallographic pose of RAL agreed closely with the docking prediction of RAL. However, the oxadiazole moiety was flipped by ~180° due to rotation around a single bond between oxadiazole and the β-hydroxy-ketone structural motif (Figure 7A). Likewise, the crystallographic pose of DTG agreed closely with the docking prediction of DTG, with exception of the carboxamide

moiety (Figure 7B). In both complexes, PFV IN/DNA/RAL (Figure 7A) and PFV IN/DNA/DTG (Figure 7B), the key components such as divalent cation chelating oxygen atoms of RAL and DTG and halogen-substituted phenyl rings, were perfectly superposed (root mean square deviation < 0.3 Å), confirming the accuracy of the docking protocol.

Figure 7 – Docking of RAL and DTG in the PFV IN/DNA complex

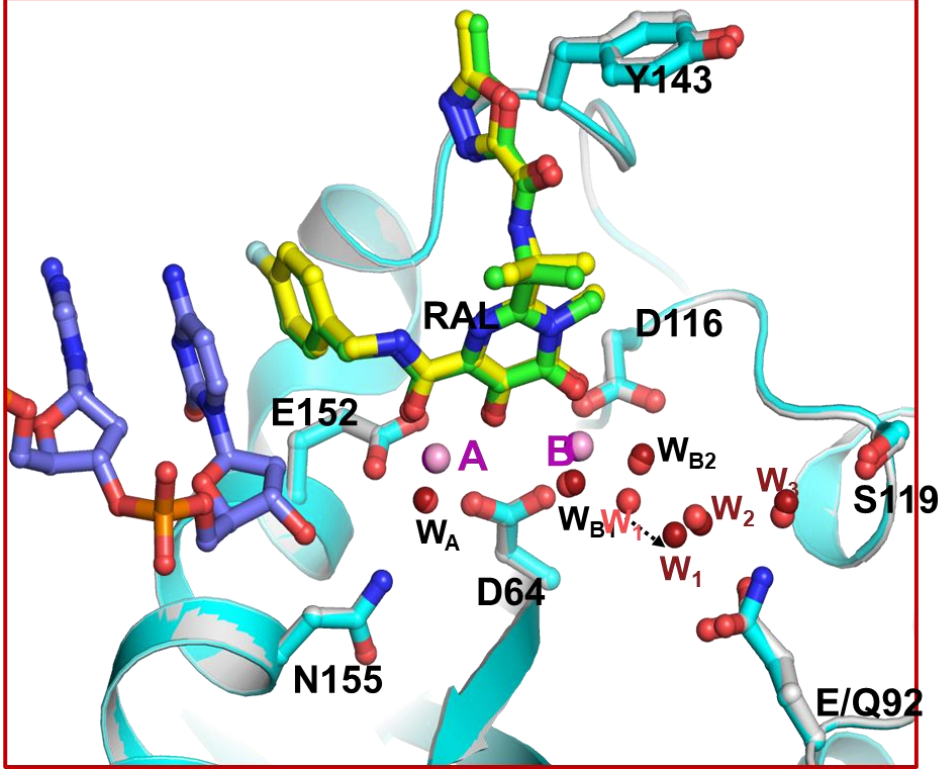


4.2.2. E92Q mutation changes the distribution of waters near INSTI-binding site

Molecular models of the WT and E92Q HIV-1/DNA/RAL complexes were generated to better understand the mechanism of E92Q INSTI resistance. All water molecules near the INSTI-binding sites were included in all simulations. Before conducting RAL docking in HIV-1 IN in the presence of water, the docking of the water molecules was

validated in the PFV IN/DNA/RAL complex and compared to the crystal structure, which was previously determined[88,89.] The IFD scores (Schrödinger Suite, Schrödinger LLC, New York, NY) of docked RAL in the E92Q/DNA (E92Q/RAL) complex superposed onto the WT/DNA (WT/RAL) complex were calculated and the poses with the best IFD scores were modeled (Figure 8). When the two complexes were superposed, The RAL molecules matched with root-mean square deviation of less than 0.3 Å (Figure 8). The position of the waters in the two complexes are also similar near the inhibitor binding site (Figure 8). However, the most striking difference between the two complexes is the position of the water molecule (W_1). In the E92Q-RAL complex, W_1 appears to be displaced by ~1.8 Å from its position in the WT-RAL complex (Figure 5). In the WT-RAL complex, W_{B1} serves as a ligand for the metal ion B and interacts with W_1. Conversely, in the E92Q-RAL complex, W_1 is ~4 Å away from W_{B1}, this indicates a loss of interaction between W_{B1} and $W_{1.}$

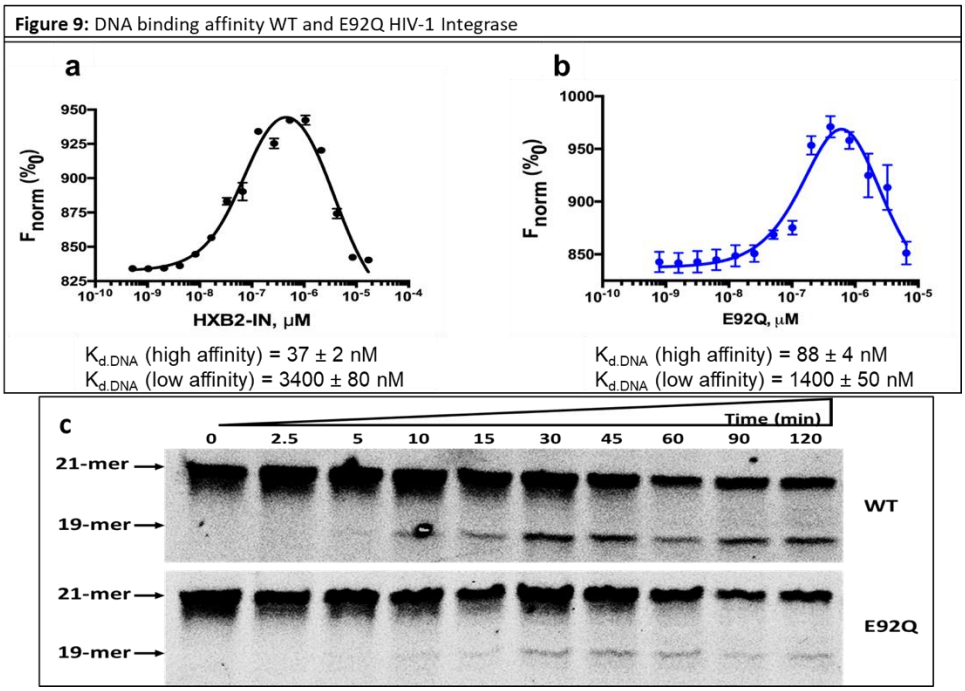
Figure 8: RAL poses in E92Q/DNA complex



4.2.3. E92Q mutation decreases DNA binding affinity and 3'-end-processing activity

When subjected to microscale thermophoresis (MST), the E92 mutation retained the previously reported high- and low- affinity modes of DNA binding, however, The $K_{d.DNA}$ in high-affinity mode was increased by ~2.4 -fold ($p \leq 0.02$), whereas $K_{d.DNA}$ in low-affinity mode was decreased by ~2.4 – fold ($p \leq 0.05$) (Figure 9A and B). These results suggest that the E92Q mutation decreased the DNA binding affinity in high-affinity mode but increased the DNA binding affinity in low-affinity mode. Furthermore, the effect of the E92Q mutation on the 3'-end processing (EP) activity of IN was evaluated. Both the WT and E92Q HIV-1 INs exhibited significant 3' -EP activity overtime (Figure 9C). Conversely, the

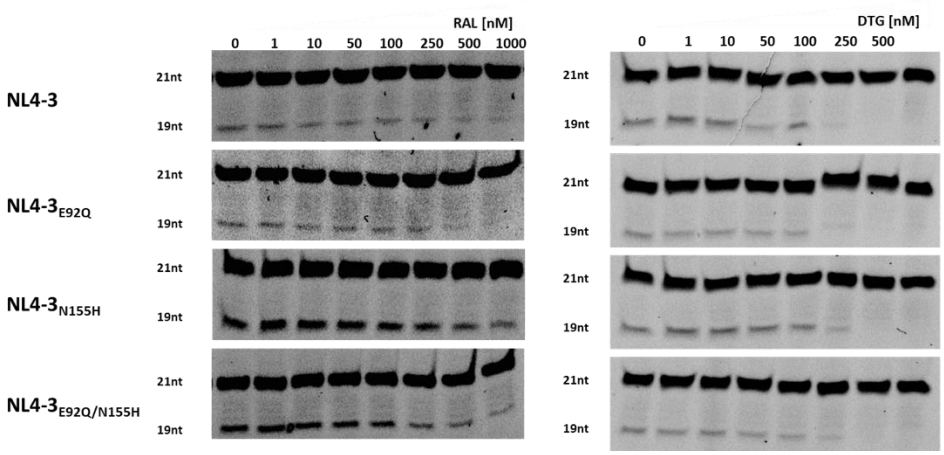
3’-EP activity of the E92Q mutant was significantly decreased compared to the WT enzyme (Figure 9c).



4.2.4. IN Mutations Exhibit resistance to RAL

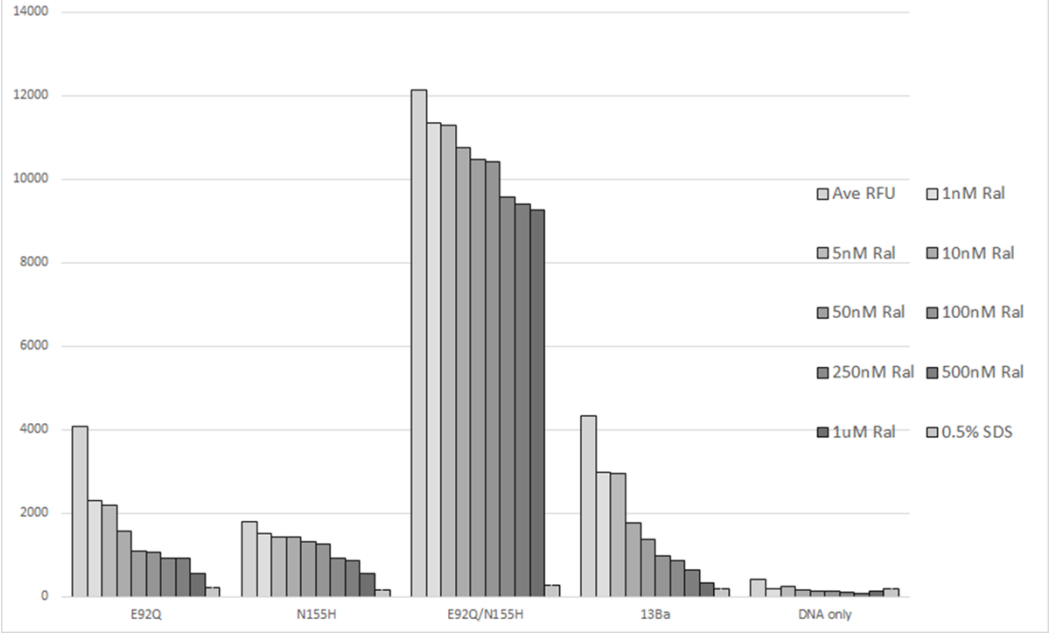
The effects of varying RAL and DTG concentrations on both WT and mutant IN over time were evaluated. Starting at 250nM RAL it was observed a decrease in IN activity in WT and the E92Q mutant, with activity nearly abolished in the E92Q mutant at 100nM (Figure 10). However, In the N155H mutant and the N155H/E92Q double mutant, IN activity remained up to 100nM RAL (Figure 10). Conversely, IN activity was completely abolished in WT and all mutants at ≤500nM DTG. These results suggest that IN with the N155H mutation and the N155H/E92Q double mutation are resistant to RAL but not DTG.

Figure 10: WT and Mutant IN Panel 3'-EP With RAL or DTG



It was further evaluated the effect of RAL 3' processing with a fluorescence based assay in which fluoresces indicates 3' processing activity. It was observed that when compared to the positive control (Ave RFU), both the N155H mutant and the N155H/E92Q double mutant maintained 3' processing in the presence of RAL, indicating resistance. This confirms the previous results from the gel-based assay (Figure11).

Figure 11: E92Q/N155H Double Mutant Resistance to RAL



4.3. Discussion

This study utilized a combination of biochemical techniques and molecular models to better understand the mechanism of RAL resistance for E92Q. RAL and DTG resistance were also evaluated in theE92Q, N155H mutants and the E92Q/N155H double mutant. Our results indicate that in high affinity mode, the E92Q mutation decreases DNA-binding affinity as well as 3' end processing activity. It may also be inferred from these results that, since INSTIs bind to the IN/DNA complex, reduced DNA binding due to the E92Q mutation may also affect the activity of RAL. Furthermore, our results indicate that the mechanism of RAL resistance for E92Q. N155H, and E92Q/N155H differs from the DTG mechanism of resistance as these mutations were not resistant to DTG. This is important for the clinical treatment of patients with HIV-1 that have been taking the cART with RAL and have developed a RAL resistance. Because DTG is still effective at suppressing IN activity in RAL resistant strains. DTG may be used as an alternative INSTI I the cART regimen for these patients. Continuous research on mutated HIV-1 strains will provide the information needed to ensure proper and effective treatment for those living with HIV.

VITA

Melissa Coy was born in Dunedin, Florida on May 24, 1989. She completed her high school education at Fort Gibson High School in Fort Gibson, Oklahoma in 2007. In December 2011 she received her Bachelor's degree in Science Education from the Northeaster State University in Tahlequah, Oklahoma and utilized this degree to teach high school anatomy and physiology at Union High School in Tulsa, Oklahoma for five years. While teaching high school, she returned to Northeastern State University and in 2015 earned Master's degree in Natural Sciences while researching the halophilic bacterium *Halorhodaspira halophila.* In 2016, she was accepted to the University of Missouri to pursue a PhD. After extensive research on the select agent *Francisella tularensis* she began research on HIV. These years of research led to her highest academic achievement thus far, a PhD in Veterinary Pathobiology.

Over the years, Melissa has received the following awards:

University of Missouri Electron Microscopy Core Fellowship

IMSD Fellowship, NIH

Distinguished Honors Graduate

Choctaw Nation Scholarship

NSU Alumni Legacy Scholarship

NSU Green and White Scholarship

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