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Examination Of Virulence-Associated Lipoproteins In Novel Strains Of Haemophilus Ducreyi

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Examination of Virulence-Associated Lipoproteins in Novel Strains of *Haemophilus ducreyi*

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Biographical Sketch

Quantil M. Melendez was born August 10, 1988, in Burlington, New Jersey. She was partially raised in Greenville, NC and Long Island, NY. She received a Bachelor of Science degree in Biology from St. Augustine's College in 2010. She is a candidate for the Master of Science degree in Biology.

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Abstract

Haemophilus ducreyi is a gram negative, sexually transmitted bacterial pathogen. This strict human pathogen causes the genital ulcer disease chancroid, which results in the formation of deep, painful ulcers. Chancroid infection has also been associated with high transmission rates of the Human Immunodeficiency Virus. *H. ducreyi* virulence has been attributed, in part, to the extracellular virulence factors large supernatant protein A (LspA) and large supernatant protein B (LspB). Recently, *H. ducreyi* strains were assigned to two classes, Class I (CI) and Class II (CII). Strains were grouped based on common DNA sequences, expression of several known virulence factors, and differences in lipooligosaccharide production. In 2007, four novel strains of *H. ducreyi*, BE3145, SB5755, SB5756, SB5757, were detected on the lower limbs of children visiting the island chain of Samoa.

In this study, we examined *lspA* and *lspB* in these strains to determine whether the DNA sequences were more similar to those found in CI or CII strains. We hypothesized that the novel strains would contain *lspA* and *lspB* DNA sequences identical to those in CI strains. Therefore, Polymerase Chain Reaction was used to amplify *lspA* and *lspB*, and the PCR products were sequenced and compared to the CI parent strain, 35000HP and CII parent strain, HMC112. Both *lspA* and *lspB* were detected in all four novel strains. In addition, *lspA* was amplified in HMC112; while *lspB* could not be amplified in this strain. It was determined that SB5755, SB5757, and BE3145 contained *lspA* that were 100% identical to 35000HP, while SB5756 possessed 99% identity. However, none of the *lspA* sequences were similar to HMC112. SB5755 and SB5756 contained *lspB* that were 100% identical to 35000HP, while SB5757 *lspB* was 99% identical to 35000HP. Interestingly, BE3415 showed very little identity to 35000HP. Based on these data, strains BE3145, SB5755, SB5756, SB5757 are more similar to CI strains.

CHAPTER 1

Introduction

1.1 *Haemophilus ducreyi*

There are five classical venereal diseases that are responsible for genital ulcerations; gonorrhea, syphilis, lymphogranuloma venereum, donovanosis, and chancroid. These diseases have the ability to facilitate transmission of Human Immunodeficiency Virus (HIV), and are prevalent in many HIV endemic countries such as; Africa, Asia, and South America (Labandeira, Mock, Hansen, 2009). These diseases generate different variations of genital ulceration which appear as sores on the genitalia. Chancroid differs from other genital infections in that chancroid lesions are very deep ulcers that bleed easily and are very painful.

The bacterium responsible for the causing chancroid ulcers is *Haemophilus ducreyi*, a gram negative cocobacillus (Sullivan, 1940). Several virulence factors have been associated with the development of chancroid. These factors have various functions such as resistance to phagocytosis, resistance of killing by antimicrobial peptides, acquisition of nutrients from the host, survival in the presence of innate and adaptive immunity, and attachment to host tissue and proteins (Sullivan, 1940).

A recent study by Ussher (2007) demonstrated the occurrence of novel *H. ducreyi* strains in the Western Pacific island of Samoa. The strains were isolated from young children located on non-genital areas of the body, specifically their lower limbs. These strains were contracted in an uncommon manner, leading to a series of investigations to better understand the characteristics of these strains.

This study focused on two lipoprotein molecules, the large supernatant lipoprotein A (LspA) and the large supernatant lipoprotein B (LspB). Previous studies have shown that the

LspA and LspB molecules work together to contribute to the infectious mechanism of *H. ducreyi* (Labandeira, Mock, Hansen, 2009). LspA and LspB have been found to inhibit phagocytosis by immune cells in the host.

1.1.1 Statement of Problem. Two major goals of infectious disease research are to detect and prevent the acquisition and spread of infectious organisms. Furthermore, the identification of specific factors that contribute to the development of disease may aid in the discovery of promising vaccines. The research presented in this study aims to determine whether novel strains of *H. ducreyi* possess virulence factors identical to those found in the Class I or Class II parent strains. In 2007, a novel acquisition of chancroid was detected on the non-genital skin of New Zealand children visiting the island chain of Samoa (Ussher, 2007). The causative agent of the disease was confirmed to be *H. ducreyi* via gram staining and colony isolation analysis. Investigations were conducted which ruled out the spread of *H. ducryi* to the children by sexual abuse or mal-treatment. Three bacteria isolates were cultured from the children and the 16S rDNA genes sequenced. To verify that these strains were indeed *H. ducreyi*, a DNA sequence comparison against known *H. ducreyi* strains was performed. All isolates were determined to possess a 16 S rDNA sequence that was 100% identical to the Class II *H. ducreyi* strain 33921 (Ussher, 2007).

1.1.2 Statement of Purpose and Hypothesis. So far, four novel strains have been isolated from the children whom visited Samoa and were available for investigation. Previous studies in our laboratory have determined that although Ussher (2007) reported strains SB5755, SB5756, SB5757, and BE3145 to possess a 16 S rDNA sequence 100% identical to Class II strain 33921, the DNA and predicted protein sequences of as many as five virulence-associated factors are most similar to those in Class I strain 35000 HP. The purpose of this study is to

further explore other known virulence associated factors in the novel strains. Our ultimate goal is to use this data to characterize the Samoa strains as Class I or Class II. Therefore, *lspA* and *lspB* were examined in all four novel strains. We hypothesized that the strains would contain *lspA* and *lspB* sequences most similar to the genes in Class I strains. The specific aims of the study were as follows:

Specific Aim 1: To amplify *lspA* and *lspB* in the novel strains by polymerase chain reaction.

Specific Aim 2: To determine the percent identity of *lspA* and *lspB* in the novel strains compared to *H. ducreyi* CI and II parent strains through DNA sequence comparison.

CHAPTER 2

Literature Review

2.1 History of *Haemophilus ducreyi*

Chancroid was first distinguished from syphilis by French scientists Bassereau and Ricord in 1852. They established that syphilis infections resulted in the production of a hard chancre, while chanroid infections resulted in a soft chancre (Bassereau, 1852). They demonstrated that only the soft chancre infected patients possessed the ability to be re-infected at other skin sites by autoinoculation of purulent, pus containing, ulcer material (Albritton, 1989; Trees & Morse, 1995). The causative agent of these soft chancres was determined to be a bacterium, and not a virus like some sexually transmitted diseases such as the Human Immunodeficiency Virus.

Scientists felt that it was imperative to understand what pathogen was capable of separating chancroid infections from other genital ulcer diseases. At the University of Naples, a scientist named Ducreyi studied the cause of the soft chancre through serial inoculations of patients with ulcers present on their genitalia. There was a common single microorganism in the ulcer exudates observed in and outside the neutrophils of each patient. The organisms were incapable of growing *in vitro*. Therefore, cutaneous inoculations were used to determine the infectious agent.

A gram negative coccibacillus was isolated from the patients and termed *Haemophilus ducreyi*. The organism was named *Haemophilus* for its blood loving quality and *ducreyi* for the discovering scientist (Sullivan, 1940).

2.2 *Haemophilus ducreyi* Characteristics

Haemophilus species are small gram negative bacilli that can be found as members of the normal flora of the upper respiratory, gastrointestinal, and genital tracts of humans and animals (Maza, Pezzlo, Shigei & Peterson, 2004). Interestingly, *H. ducreyi* is the only *Haemophilus* species that is a strict human pathogen transmitted through sexual contact.

Haemophilus species are difficult to detect in direct patient material, such as tissues and topical skin, due to its small size and faint staining. This species is a facultative anaerobe; which requires an atmosphere of 5% CO_2 at 35^{o}C for optimal growth. Most of the *Haemophilus* species require either an X factor provided by hemin and/or a V factor provided by NAD. The bacterium normally acquires these factors from the host given that they are unable to make these critical factors on their own. *H. ducreyi* is unlike any bacterium in its genus. *H. ducreyi* only requires an X factor for ideal growth *in vitro*, which is provided in the chocolate agar culture medium. When visualized by Gram stain, *H. ducreyi* arranges in a common "school of fish" pattern and appears as small pinpoint yellowish tinted colonies, considerably the smallest of its genus (Maza et al., 2004). As a facultative anaerobe, *H. ducreyi* grows below the surface of the broth in tight small clumps in broth medium, unlike other *Haemophilus* species which grow less agglutinated and at higher altitudes in broth medium (Maza et al., 2004).

2.3 *Haemophilus ducreyi* Clinical Progression

H. ducreyi infection may result in the production of very painful ulcers on the skin. Studies to better understand the production of these ulcers have revealed that the bacillus bacterium is restricted mainly to human species and, so far, only found to be obtained through sexual transmission. Transmission of *H.* ducreyi, from human to human, initiates with a break in the integrity of the epithelium. Once *H. ducreyi* enters a break in the skin, the first signs of

pathological changes in the host cells can be observed. These changes consist of edematous and swollen epithelium cells within an infiltration of polymorphonuclear leukocytes (Sullivan, 1940). At this stage of infection small papules are able to be visibly seen on the skin in a narrow inflamed zone. This step is followed by the pustule formation stage that occurs days later. During this phase an abscess-pimple-like small raised inflamed area can be observed. This feature shortly ruptures and results in a restricted ulcer with ragged damaged edges and without indurations or hardening (Sullivan, 1940).

2.4 Chancroid Clinical Progression and Treatment

Human and animal clinical trials have revealed that chancroid ulcers, caused by *H. ducreyi* infection, develop through a serious of stages. Following exposure to *H. ducreyi*, the initial stage of infection lasts approximately 3-10 days. During this stage a pimple like abrasion appears on the genitalia which progresses into a papule. At the end of this period, within 1-2 days, the obstruction on the genitalia progresses from a papule to a pustule. A pustule is a pus filled abrasion that resembles a pimple in its final stages before eruption. Soon after the development of the pustule, an ulcer forms which resembles a ruptured pimple with ragged edges (Bassereau, 1852; Sullivan, 1940).

Patients suffering from chancroid infections can be treated for pain and the infection managed with antibiotics such as azithromycin and penicillin derivatives (Sullivan, 1940). However studies have shown that *H. ducreyi* is considered inherently resistant to both tetracyclines and penicillin (Plourde, 2002). Therefore, the recommended treatment for chancroid is azithromycin or ceftriaxone (Ussher, 2007).

2.4.1 Studies of *Haemophilus ducreyi* in the human model. Given that *H. ducreyi* is a strict human pathogen in nature, the development of a human model was an important step

towards enhancing the understanding of chancroid infection. Laboratory studies have been conducted to observe the infection period in humans through exposure of non-genital skin in areas such as the deltoid. In one study, this human inoculation model was conducted by infecting patients with *H. ducreyi* at multiple sites on the backs and arms, and the progressions of the bacterium in different doses were observed (Janowicz & Ofner, 2009). According to a study performed at Indiana University, "the most important determinant of pustule formation [was] gender" (Janowicz & Ofner, 2009). The study was designed to test differential human susceptibility to the bacterium, in an effort to contribute insight on immunity to the pathogen or developing vaccination strategies.

2.5 Virulence-Associated Factors of *Haemophilus ducreyi*

Chancroid is attributed to extracellular and secreted virulence factors which are responsible for the ability of the bacterium to evade the host immune response (Janowicz et al., 2004). In several studies, the mechanisms that *H. ducreyi* has developed to avoid killing was investigated using macrophage cell line U-937 (Wood, 2001; Deng, Mock, Greenberg, van Oers & Hansen, 2008). It was observed through these studies that in the event of phagocytosis, *H. ducreyi* managed to survive and resist killing by the immune cells. Some of the virulence factors determined to contribute in the evasion of phagocytic killing are outer membrane proteins, such as DsrA and NcaA; *H. ducreyi* toxins such as, HdCDT; and lipoproteins, such as large supernatant lipoprotein A and B. Lipoproteins are secreted extracellular virulence factors produced by *H. ducreyi*, consisting of a lipid and protein complex. *lspA* and *lspB* encode for lipoproteins, large supernatant lipoprotein A and large supernatant lipoprotein B. These virulence factors are responsible for the inhibition of phagocytic activity. *In* vitro, they are required to work together to cause virulence in the host (Labandeira, Mock & Hansen, 2009).

2.5.1 *Haemophilus ducreyi* large supernatant lipoprotein A (LspA). The large supernatant lipoprotein, LspA, is cytoxic to immune cells and protects *H. ducreyi* from phagocytosis in a dual partner secretion system, discovered by Dr. Eric Hansen (Deng et al., 2008: Wood, 2001). The proteins LspA1 and LspA2 make up the phagocytic inhibitory factor that prohibits macrophages' cellular responsibility to the host (Deng et al., 2008). The *lspA* is 486 nucleotide base pairs in size (Figure 1.1) and has a predicted protein size of 161 kDa. Aside from the two-party system of LspA1 and LspA2, LspA must also work in conjunction with LspB to create full virulence in the human and animal models of chancroid (Labandeira, Mock & Hansen, 2009). *lspA* is located significantly further upstream in the genome than *lspB* and the genes are controlled by different regulatory facts.

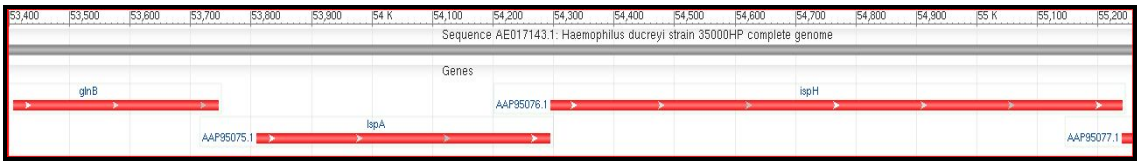

Figure 1.1. *lspA* Location in the 35000HP Strain (GeneBank)

2.5.2 *Haemophilus ducreyi* large supernatant lipoprotein B (LspB). The large supernatant lipoprotein, LspB, is also cytoxic to immune cells and protects *H. ducreyi* from phagocytosis in a dual partner secretion system. LspB was discovered by Dr. Eric Hansen (Deng et al., 2008: Wood, 2001). *lspB* is located on the same operon as *lspA*; however it has its on regulatory control factor. Like LspA, this large supernatant lipoprotein is also secreated and assists in resistance to phagocytosis. However, LspB does not function when not in association with LspA. There is not much known about *lspB*, therefore there are ongoing studies to understand its nature and character (Labandeira, Mock, Hansen, 2009). *lspB* is 1770 nucleotide base pairs in size (Figure 1.2) and has a predicted protein of 589 amino acids.

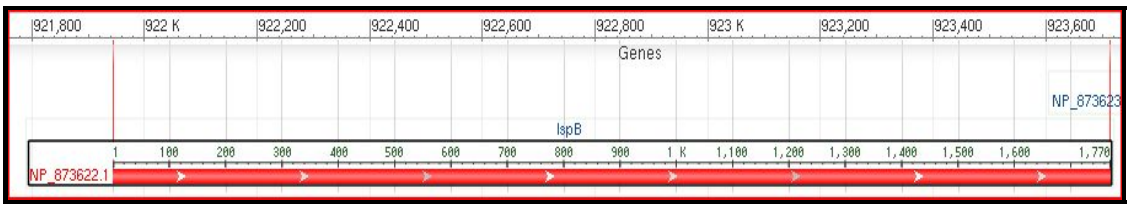

Figure 1.2. *lspB* Location in the 35000HP Strain (GeneBank)

2.6 *Haemophilus ducreyi* Class Divisions

H. ducreyi strains are classified according to differences in nucleotide and protein sequences of several virulence-associated factors (White, Leduc, Olsen, Jeter, Harris & Elkins, 2005). Strains have been classified into two categories, Class I (CI) and Class II (CII). Thus far, most genes encoding virulence-associated factors located in CI

H. ducreyi strains differ from genes in CII strains mainly at the N- terminus of the nucleotide sequences. The C- terminus is highly conserved in both classes, possibly due to a lack of selective pressure imposed upon the gene. Thus far, the only strain to have its entire genome sequenced and published is the common laboratory strain 35000HP. 35000HP is considered the CI "parent strain". Thus far, several CII strains have been identified; however their entire genome sequences have not been determined and published. Some of the CII strains include CIP 542 ATCC, DMC111, and HMC112, a CII parent strain (White et al., 2005).

2.7 Novel *Haemophilus ducreyi* Strains

Recently, studies have been formed around unknown strains of *H. ducreyi* that have detected in the Western Pacific. Thus far, there has been no published data of these strains being sequenced or characterized. These strains originated specifically from the Samoan islands, and were isolated from children that did not obtain the bacterium through sexual transmission (Ussher, 2007). Novel strains of *H. ducreyi* were detected on the non-genital lower limbs of

children visiting the island chain of Samoa. Due to the unusual acquisition of chancroid in these young children, we felt that it was important to characterize these diverse strains. There were four strains isolated from the youth for investigative purposes; SB5755, SB5756, SB5756, and BE3145.

There were three case studies performed with the children to treat and resolve the ulcerations. The first and second patients were treated with flucloxacillin and had ineffective results. They were later referred to a hospital that treated them with zithromax antibiotics resulting in a resolution of the ulcerations. This lead to a rapid treatment of the third patient with zithromax initially, and which resulted in positive recovery (Ussher, 2007). Previous studies showed that penicillin worked as an effective drug against *H. ducreyi*; however this was not the case for the novel strains.

This study was proposed to determine whether Samoan strains BE3145, SB5755, SB5756, and SB5757 from the children contained DNA of virulence factors *lspA* and *lspB* similar to CI or CII strains of *H. ducreyi*. The process of determining the class of these strains consisted of sequencing and comparing the DNA of the four strains with known virulence genes from the 35000HP parent strain. If the strains were determined to be Class I, they would be expected to have complementary sequences to the Class I genes. Five Class II strains have been identified thus far. If the sequences of the four novel strains were not significantly similar to the Class I genes, then further sequencing could be done with the Class II genes for comparison. With this information, a possible role for virulence and transmission of the novel strains may be deciphered.

It has been further hypothesized that the Samoan strains are neither Class I nor II, but that they belong to a novel class. The purpose of this research is to determine the characteristics of

the Samoan strains BE3145, SB5755, SB5756, and SB5757 and the virulence of their gene products. Ultimately the goal of this research is to assist in the development of a preventative drug for chancroid caused by *H. ducreyi*. This work will help to determine if the virulence-associated factors *lspA* and *lspB* are present in the four Samoa Strains. This work will also establish which class the genes belong to in each strain.

CHAPTER 3

Materials and Methods

3.1 Evaluation of *lspA* in *Haemophilus ducreyi* Strains in 35000HP, SB5755, SB5756, SB5757, BE3145, and HMC112

In an effort to determine whether *lspA* was present in four novel strains of *H. ducreyi*, Polymerase Chain Reaction (PCR) was used. This method would allow *lspA* to be amplified in these strains, followed by visualization through agarose gel electrophoresis. The *H. ducreyi* strains used in this research are listed in Table 1.

Table 1

Haemophilus ducreyi Experimental Strains

Strain Name	Strain Class	Location/ Year of Isolation	Source (Reference)
35000HP	Class I	Winnipeg (1975)	S. Spinola
HMC112	Class II	CDC (1984)	P. Totten (Totten and Stamm, 1994)
SB5755	Unknown	Samoa Island, South Pacific (2007)	Ussher (Ussher,2007)
SB5756	Unknown	Samoa Island, South Pacific (2007)	Ussher (Ussher,2007)
SB5757	Unknown	Samoa Island, South Pacific (2007)	Ussher (Ussher,2007)
BE3145	Unknown	Samoa Island, South Pacific (2007)	Ussher (Ussher,2007)

3.1.1 Amplification of *lspA* by PCR. Primers were designed to *lspA* based on the *H. ducreyi* 35000HP *lspA* sequence (accession number AE017143). Primers were intended to flank the ends of *lspA* in order to amplify and sequence the entire gene. The additional DNA for the primers was located 30 nucleotides up stream and downstream of *lspA*. The viability of the primers was verified using the free, online program Amplifx

(http://www.softpedia.com/get/Others/Home-Education/AmplifX.shtml). The forward and reverse primer sequences, 5'-cgg tgg tta att tta ata ata agg tac att- 3', and 5'-tcc tct tgg att agc taa taa aat att cat- 3', respectively, were submitted to Eurofins MWG Operon (http://www.operon.com/products/custom-oligos/order-custom-oligos.aspx?id=custom).

To prepare *H. ducreyi* samples for PCR, the bacteria were streaked for isolation on chocolate agar plates (CAP). The plates contained 1X GGC (0.1% glucose, 0.01% glutamine, and 0.026% cysteine) (Totten and Stamm, 1994), and 5% fetal bovine serum (Fetal Plex, Gemini Scientific, West Sacramento, CA). CI strains were incubated for 18-24 hours at 34.5^{o}C in 5% CO_2, and for 36-48 hours for CII strains. The bacteria was transferred to *H. ducreyi* freeze media (3g Trypticase, 25ml glycerol, and 75ml of H_2O) with 10% glycerol and stored at -80^{o}C.

Initially a 30 cycle PCR reaction to amplify *lspA* in strains 35000HP, SB5755, SB5756, SB5757, and BE3145 was performed. PCR working stocks consisting of *lspA* primers (*lspA* forward and reverse primer [100pmol each]), 35000HP bacteria sample, HMC112 bacteria sample, SB5755 bacteria sample, SB5756 bacteria sample, SB5757 bacteria sample, and BE3145 bacteria sample were created. The reactions contained LspAF (*lspA* forward primer) and LspAR (*lspA* reverse primer) at 100pmol each, Master Mix (1X Go-taq DNA polymerase, dNTPs [200µM each], 1.5 mM $MgCl_2$; Promega, Madison, WI), H_2O and whole *H. ducreyi* cells from isolation as previously stated. The following conditions were used: one cycle of 95°C for 5 minutes; one denaturing cycles at 95°C for 30 seconds; one annealing cycle at 50°C for 30 seconds; one elongation cycle at 72°C for 2 minutes; followed by a further extension cycle at 72°C for 7 minutes; ending in a holding cycle of completion at 4°C for infinity. Electrophoresis was performed on the resulting products at the end of the PCR cycle. Unfortunately, *lspA* was not amplified from the Samoa strains under these conditions.

Since original attempts to amplify *lspA* in the *H. ducreyi* novel strains were unsuccessful, a gradient PCR was performed. To perform gradient PCR, annealing temperatures that were 2°C – 5°C higher than the predicted annealing temperature for the primer with the lowest annealing temperature were used. The bacteria samples were prepared as described above. 3 sets of 5 PCR gradient tubes were constructed as listed above, to account for each temperature reaction. The conditions were set as follows for 40 cycles; jumpstart temperature at 94^{o}C for 2 minutes, denaturing temperature at 94^{o}C for 1 minute, annealing temperatures for 2 minutes: 1 set in row E - 55.1^{o}C, 1 set in row F - 53.7^{o}C, and 1 set in row H - 52.0^{o}C, elongation temperature at 72^{o}C for 2 minutes, further extension at 72^{o}C for 8 minutes, and held at 4^{o}C for infinity at the completion of the protocol. Electrophoresis yielded DNA fragments that were 536-546bp for *lspA* from strains 35000HP, SB5755, SB5756, SB5757, and BE3145.

Sample reactions for HMC112 were prepared and a PCR gradient was performed as stated above. The conditions were set as previously stated and electrophoresis yielded DNA fragment of 536-546bp for *lspA* in the HMC112 strain

3.1.2 Sequence analysis of *lspA* in *Haemophilus ducreyi* strains. Following PCR amplification of *lspA*, DNA sequencing was performed. To prepare the sequencing samples the PCR product was purified using a Qiagen Purification Kit (Qiagen, Valencia, CA). Sequencing of the purified amplified DNA would allow comparison of *lspA* to CI and CII parent strain sequences. Sequencing primer samples were created (2pmol/µl) from original primers, forward and reverse separately. Sequencing reactions were created with purified amplified DNA product (40ng/µl), sequencing primer (forward or reverse), and nuclease free water. The sequencing reactions were cataloged as follows: SB5755 forward sequence → Tube AE5835, SB5755 reverse sequence → Tube AE5826, SB5756 forward sequence → Tube AE5836, SB5756 reverse

sequence → Tube AE5828, SB5757 forward sequence → Tube AE5837, SB5757 reverse sequence → Tube AE5830, BE3145 forward sequence → Tube AE5838, BE3145 reverse sequence → Tube AE5834, HMC112-*lspA* forward sequence → Tube AE5865, HMC112-*lspA* reverse sequence → Tube AE5873. These reactions were submitted to Eurofins MWG Operon (http://www.operon.com/products/sequencing/) for sequence determination.

The chromatograph from the sequencing results were examined through FinchTV (www.finchtv.org). Through this program nucleotide sequences can be displayed and edited as determined by the user. In this study, the sequences were manually edited by examining the sequenced product and replacing the "n" values with the appropriate nucleotide that was determined based on the colored peaks which corresponded to A, T, G, or C. The sequences for each Samoa strain were submitted to NCBI BLAST and compared to the nucleotide blast results of *H. ducreyi* 35000HP to the results of the Samoa strains. They were then compared against the sequence results of HMC112.

3.2 Evaluation of *lspB* in *Haemophilus ducreyi* Strains in 35000HP, SB5755, SB5756, SB5757, BE3145

In an effort to determine whether *lspB* was present in four novel strains of *H. ducreyi*, PCR was once again used. This method would allow *lspB* to be displayed in the strains through electrophoresis. The same *H. ducreyi* strains listed in Table 1 above were used.

3.2.1 Amplification of *lspB* by PCR. Primers were designed to *lspB* based on the *H. ducreyi* 35000HP *lspB* sequence (accession number AE017143). Primers were intended to flank the edges of *lspB* in prospect of amplifying and sequencing the entire gene. The additional DNA for the primers were located 30 nucleotides up stream and downstream of *lspB*. The viability of the primers was verified using the free, online program Amplifx

(http://www.softpedia.com/get/Others/Home-Education/AmplifX.shtml). The forward and reverse primer sequences, 5'-ttg ttc atg gag cac ttt aac aaa tat gaa- 3', and 5'-gat ttt ttt aaa ttt gta att ttg gtt aaa- 3', respectively, were submitted to Eurofins MWG Operon (http://www.operon.com/products/custom-oligos/order-custom-oligos.aspx?id=custom).

The PCR bacteria samples that were created previously for the Samoa strains, 35000HP, and HMC112 were used. *lspB* PCR primer was additionally created. Previous knowledge from *lspA* prompted an immediate PCR gradient reaction attempt for *lspB*. To perform gradient PCR, annealing temperatures that were 2°C – 5°C higher than the predicted annealing temperature for the primer with the lowest annealing temperature were used. The bacteria samples were prepared as described above, with the exception of alternative primer usage (*lspB* PCR primer). Three sets of 5 PCR gradient tubes were constructed as listed above, to account for each temperature reaction. The conditions were set as previously stated for 40 cycles. Electrophoresis yield 1820-1830bp DNA fragments for *lspB* from strains 35000HP, SB5755, SB5756, SB5757, and BE3145. There was no amplified product of *lspB* for HMC112.

3.2.2 Sequence analysis of *lspB* in *Haemophilus ducreyi* strains. Following PCR amplification of *lspB*, DNA sequencing was performed. To prepare the sequencing samples the PCR product was purified using a Qiagen Purification Kit (Qiagen, Valencia, CA). Sequencing of the purified amplified DNA would allow comparison of *lspB* to CI sequences. Sequencing primer samples were created (2pmol/µl) from original *lspB* primers, forward and reverse separately. Sequencing reactions were created with purified amplified DNA product (60ng/µl), sequencing primer (forward or reverse), and nuclease free water. The sequencing reactions were cataloged as follows: SB5755 forward sequence → Tube AE5845, SB5755 reverse sequence → Tube AE5846, SB5756 forward sequence → Tube AE5849, SB5756 reverse sequence → Tube

AE5850, SB5757 forward sequence → Tube AE5851, SB5757 reverse sequence → Tube AE5852, BE3145 forward sequence → Tube AE5853, BE3145 reverse sequence → Tube AE5854. These reactions were submitted to Eurofins MWG Operon (http://www.operon.com/products/sequencing/) for sequence determination.

The chromatograph from the sequencing results were examined through FinchTV (www.finchtv.org). Through this program nucleotide sequences editing occurred. This included going through the sequenced product and replacing the "n" values with its appropriate nucleotide that its' colored peaked corresponded to. The sequences for each Samoa strain were submitted to NCBI BLAST and compared to the nucleotide blast results of *H. ducreyi* 35000HP to the results of the Samoa strains.

CHAPTER 4

Results

Previous studies have shown that *lspA* and *lspB* of *H. ducreyi* is present in the 35000HP CI strain (Vakevainen, Greenberg, Hansen, 2003). However, there have not been many reported cases of either gene in any CII strains. Furthermore, *lspA* and *lspB* has never been identified in the novel Samoa strains. In this study, to characterize strains SB5755, SB5756, SB5757, and BE3145 as CI or CII, the nucleotide sequences of *lspA* and *lspB* were examined.

4.1 Amplification of *lspA* in *Haemophilus ducreyi* Strains 35000HP, SB5755, SB5756, SB5757, BE3145, HMC112

Previous studies by Hansen et al. (2003) demonstrated that *lspA* is prominent virulence factor in known *H. ducreyi* strains. Investigation of *lspA* in the novel Samoa strains included successful amplification resulting in 536-546bp fragments. The optimum temperatures for analyzing *lspA* in the Samoa strains were 52.0^{o}C for SB5755, SB5756, SB5757, and BE3145 (Figure 2.1 a, b, and c). The optimal temperatures for analyzing *lspA* in the Class II HMC112 were 55.1^{o}C. (Figure 2.2). Following purification of the PCR product, the concentrations of the products were collected as follows: approximately 39ng/µl for SB5755, 150ng/µl for SB5756, 125ng/µl for SB5757, 62ng/µl for BE3145, and 28.5ng/µl for HMC112. 40ng/µl sequence reactions were created for each sample in their own catalog vials.

4.2 Sequence Analysis of *lspA* in *Haemophilus ducreyi* Strains

The *lspA* sequences, retrieved from Eurofins MWG Operon, in the SB5755, SB5757, and BE3145 strain were most similar to the Class I, 35000HP parent strain holding a 99% confidence level of similarity. The SB5756 had a 95% identity comparison to 35000HP however; it was the only Samoa strain that varied in nucleotide sequence to 35000HP (Figure 2.3).

a. Annealing temp 52.0°C

b. Annealing temp 53.7°C

c. Annealing temp 55.1°C

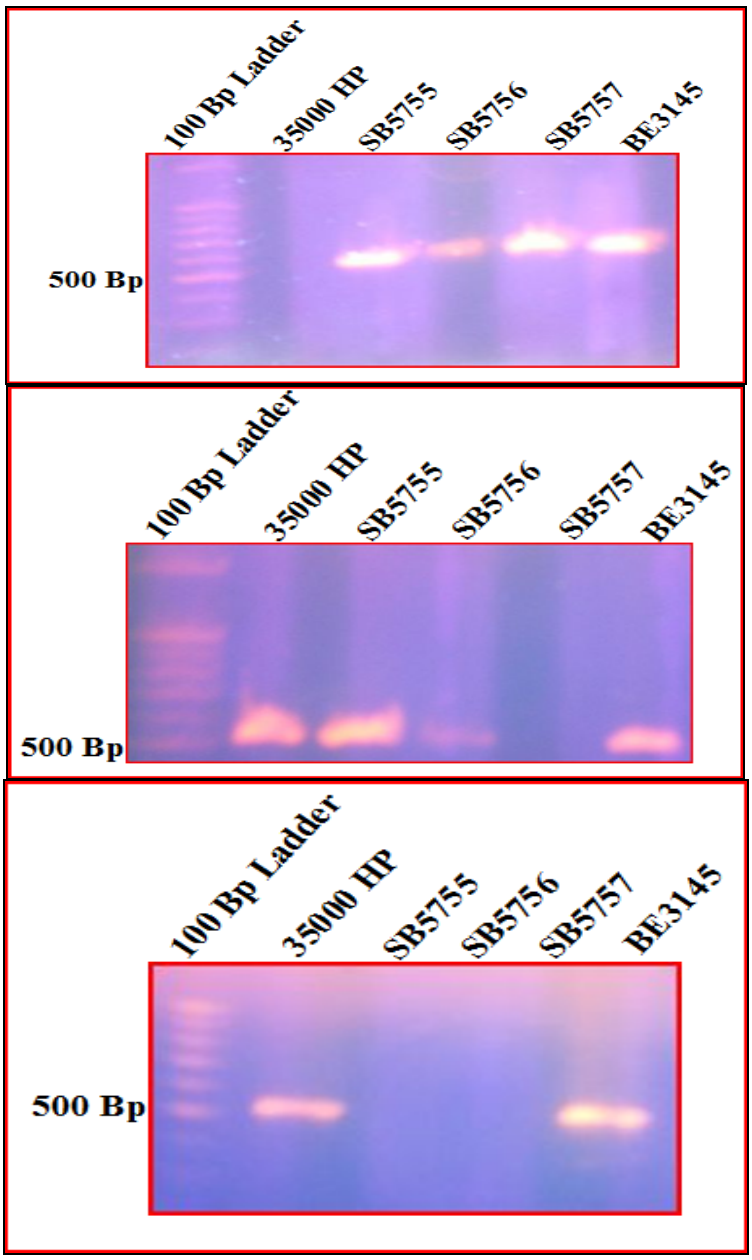

Figure 2.1. *lspA* in the Four Samoa Strains at Various PCR Annealing Temperatures

Sequence analysis shows at approximately two nucleotide parings there is a switch in complementary nucleotides between the SB5756 and 35000HP, indicated by the yellow color within the body of the sequence data. The beginning and the end of the data also is displayed in yellow, attributed to where the primers actually began annealing and elongating.

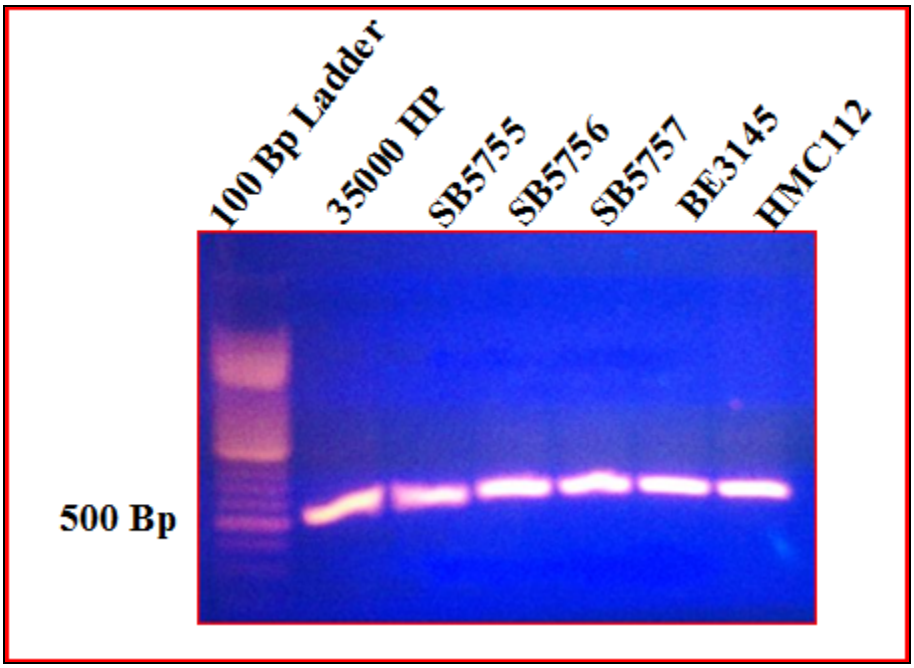

Figure 2.2. *lspA* in the 35000HP strain, the Four Samoa Strains, and the HMC112 Strain

None of the Samoa strains held sequences identical to HMC112. There were many purine and pyrimidine switches in the HMC112 sequence when compared to the Samoa strains, indicated by the blue colored nucleotide (Figure 2.4).

4.3 Amplification of *lspB* in *Haemophilus ducreyi* Strains 35000HP, SB5755, SB5756, SB5757, BE3145, HMC112

Previous studies by Hansen et al. (2009) demonstrated that *lspB* is not only a prominent virulence factor in known *H. ducreyi* strains, but is required on the same operon as *lspA* to emit full virulence. Investigation of *lspB* in the novel Samoa strains included successful amplification resulting in 1820-1830bp fragments. The optimal temperatures for analyzing *lspB* in the Samoa strains were 52.0^{o}C for SB5755. The optimum temperatures for analyzing *lspB* in SB5756, SB5757, and BE3145 were 55.1^{o}C (Figure 2.5 a, b, and c). Following purification of the PCR product, the concentrations of the products were collected as follows: approximately 84ng/µl for

SB5755, 59ng/µl for SB5756, 37ng/µl for SB5757, and 17ng/µl for BE3145. 60ng/µl sequence reactions were created for each sample in their own catalog vials.

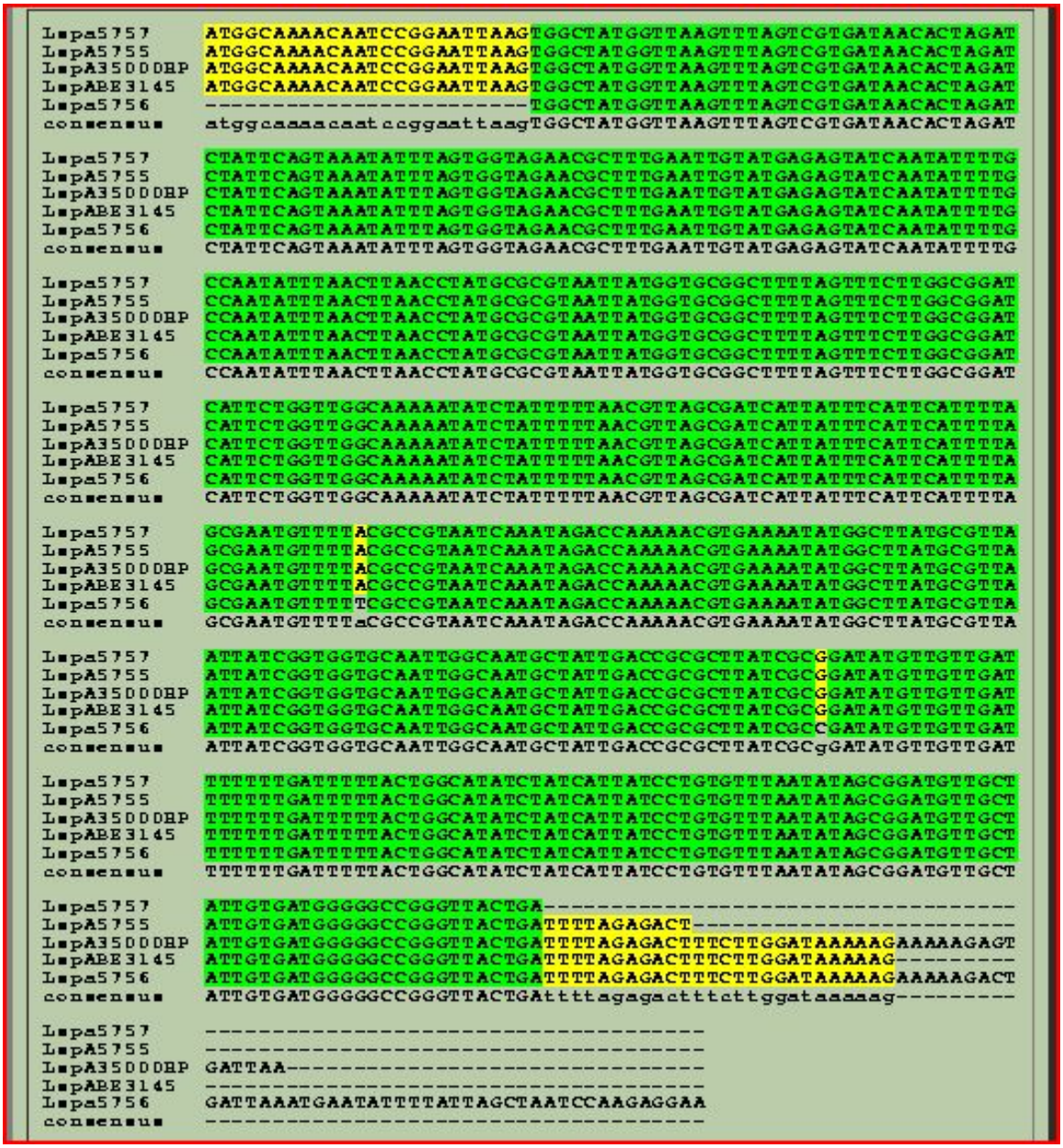

Figure 2.3. lspA Consensus Sequence between 35000HP and the Samoa Strains

4.4 Sequence Analysis of *lspB* in *Haemophilus ducreyi* Strains

The *lspB* sequences, retrieved from Eurofins MWG Operon, in the SB5755, and SB5756 strains were most similar to the Class I, 35000HP parent strain holding a 99% confidence level of similarity. The *lspB* sequences in the SB5757 strain were 97% identical to the 35000HP strain,

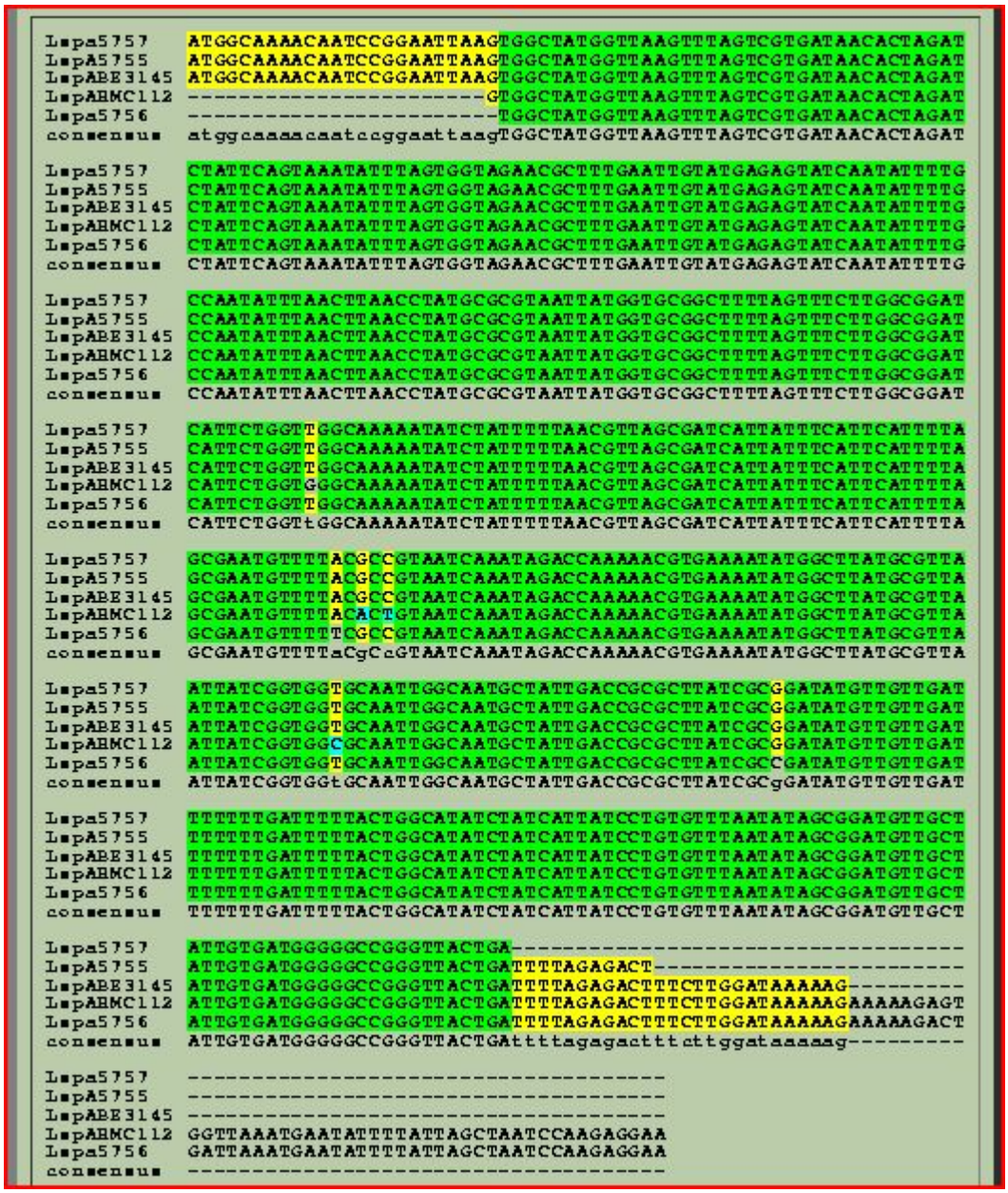

Figure 2.4. *lspA* Consensus Sequences between HMC112 and the Samoa Strains

a. Annealing temp 52.0°C

b. Annealing temp 53.7°C

c. Annealing temp 55.1°C

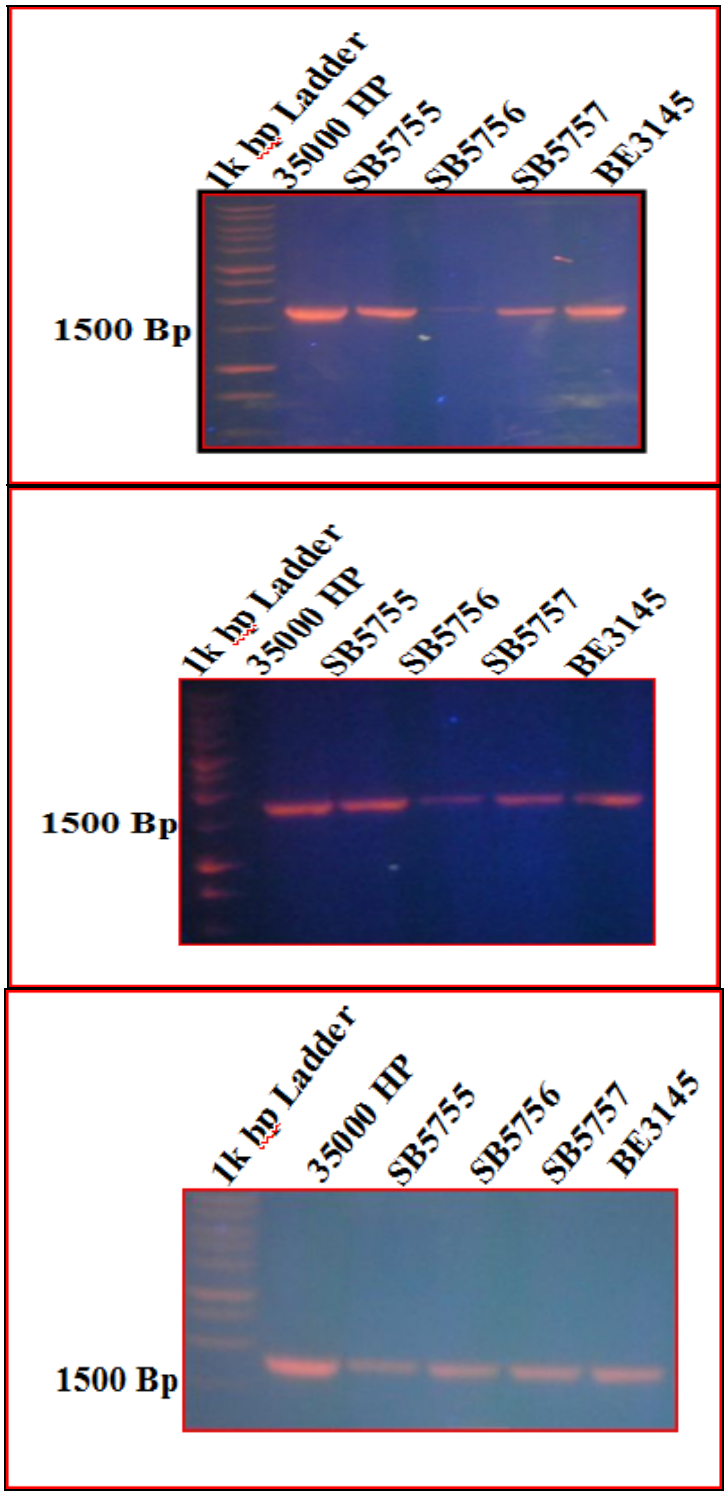

Figure 2.5. *lspB* in the Four Samoa Strains at Various PCR Annealing Temperatures

35000HP strain was the BE3145 strain, showing that even though there were some instances of like sequences between the two it was not directly identical to the CI strain (Figure 2.6).

Figure 2.6. lspB Consensus Sequence between 35000HP and the Samoa Strains

4.5 Phylogeny Analysis

In order to prove an identity comparison of *lspA* and *lspB* in the novel strains, the Class I parent strain, and the Class II parent Strain a phylogenic tree was constructed. This assisted in

determining which strain had like sequences to either parent strain. According to the analysis SB5755, SB5757, and BE3145 contained like nucleotide sequences of *lspA* identical to those in 35000HP (Figure 2.7). The *lspA* sequences in SB5756 was not as identical to 35000HP as its peers, however it was not identical to HMC112 either (Figure 2.8).

The *lspB* sequences in SB5756, SB5757 were identical to those in 35000HP. SB5755 contained a slight variation in nucleotide identity than the previous two strains, however it remained closely comparable to 35000HP. BE3145 was the least identical to 35000HP and we were unable to compare it to HMC112 due to failed attempts during PCR amplification (Figure 2.9).

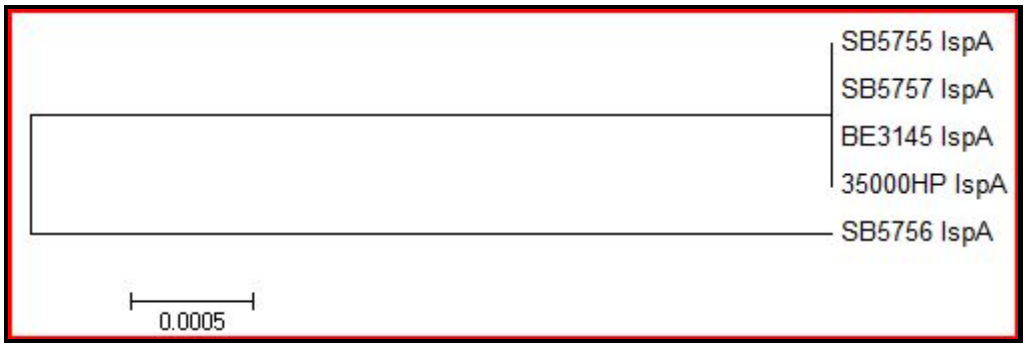

Figure 2.7 lspA Phylogenic Tree Sequence Comparison with 35000HP and the Samoa Strains

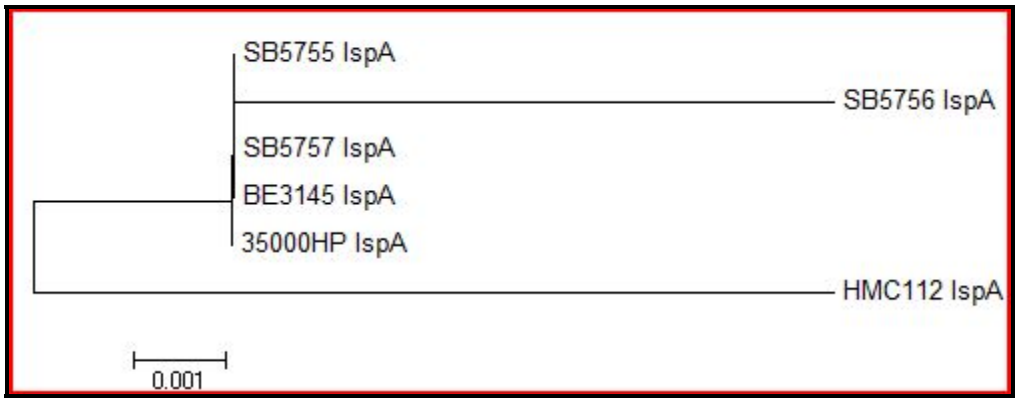

Figure 2.8. lspA Phylogenic Tree Sequence Comparison with 35000HP, the Samoa Strains, and HMC112 Strain

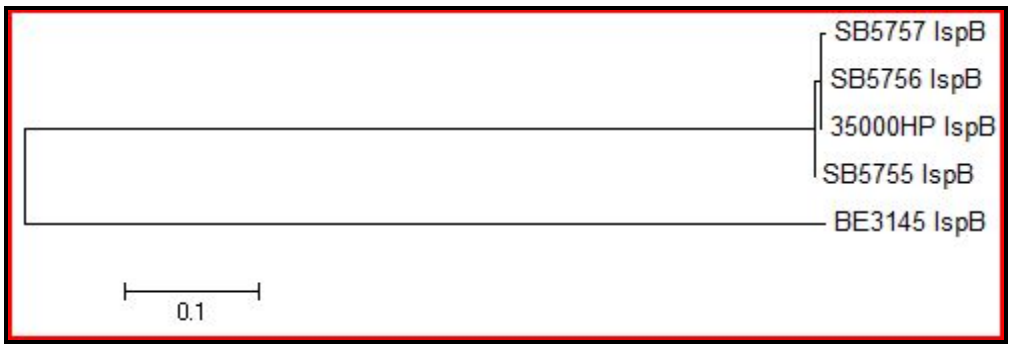

Figure 2.9. lspB Phylogenic Tree Sequence Comparison with 35000HP and the Samoa Strains

CHAPTER 5

Discussion

Haemophilus ducreyi is a gram negative, sexually transmitted bacterial pathogen. This strict human pathogen causes the genital ulcer disease chancroid, which results in the formation of deep, painful ulcers. This study examined the presence of two virulence factors, LspA and LspB, in four novel strains of *H. ducreyi*. Initial experimentation led us to investigate the possibility of the Samoa strains needing specialized temperature conditions for the annealing of primers. We determined that these strains were very temperature sensitive, and detection of virulence factors by PCR was challenging. Therefore, we decided to manipulate the annealing temperature via a PCR gradient of reactions to accommodate this observation. The optimal temperature for detecting *lspA* in the Samoa strains was determined to be 52.0^{o}C. The gene was able to be successfully amplified and identified within the strains at this temperature. The amplification of *lspA* was the only gene able to be amplified in the Class II strains. This could be due to *lspB* requiring more specific conditions in order to be detected in Class II strains. The amplification of *lspA* was not temperature specific for HMC112. Analysis via FinchTV proved that there were abundant DNA sequence similarities in the SB5755, SB5757, and BE3145 to the 35000HP parent strain. Majority of the sequenced product was identical to the parent strain, after blasting the Samoa strain against 35000HP (Table1). More of the reverse sequences were able to be identified in the parent strain than the forward. This displays the orientation in which the gene runs in the strains. There were no similarities between any of the Samoa strains and HMC112 strains containing *lspA*.

The optimal temperature for detecting *lspB* in the Samoa strains was determined to be 55.1^{o}C. *lspB* was able to be successfully amplified and identified within the strains at this

temperature. *lspB* produced strong amplified DNA possibly due to its large size. However, amplification of *lspB* in the CII HMC112 strain was unsuccessful. This could be due to the fact that CII strains need special requirements of cofactors to amplify genes at times, for example magnesium concentration variations. Analysis via FinchTV proved that all four Samoa strains contained a portion of similar sequences to the parent strain. Due to the inability to obtain any sequence data for *lspB* in the Class II strains, this leads us to further investigate the remaining sequences of the gene. It is possible that this gene contains a hypothesized novel class sequence. Even though the *lspB* Samoa sequences were approximately 97% identical to the parent strain (Table 2), according to the BLAST data, *lspB* was not entirely covered by the outside flanking primers for the Samoa strains. Inner primers must be made to receive full sequencing of the entire gene.

A phylogenic tree was composed to observe the overall sequence comparison and family relation between 35000HP, the Samoa strains, and the HMC112 strain. According to the analysis of *lspA* and the parent strain all Samoa strains except SB5756 were similar to the Parent strain. SB5756 was considered an outlier. When compared to HMC112 none of the Samoa strains were similar. BE3145 and SB5757 contained *lspA* sequences most similar to the parent strain. SB5755 contained *lspA* sequences less similar to the parent strain in comparison to its peers, however not outstandingly different. SB5756 was displayed as an outlier to the parent strain and but did not resemble sequences close to HMC112 either. Even though all of the Samoa strains appeared to posses actual *lspB* sequences similar to the parent strain, BE3145 was displayed as an outlier in comparison to its associates in regards to the parent strain. SB5755 was seen as vaguely diverse to the parent strain, leaving SB5756 and SB5757 the most closely similar strains to the Parent strains containing *lspB* sequences.

Table 2

Identity Comparison of Samoa Strains Containing lspA and lspB

Strain	Gene	Identity
SB5755	*lspA*	100% to CI 35000HP
	lspB	100% to CI 35000HP
SB5756	*lspA*	99% to CI 35000HP
	lspB	100% to CI 35000HP
SB5757	*lspA*	100% to CI 35000HP
	lspB	99% to CI 35000HP
BE3145	*lspA*	100% to CI 35000HP

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