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Determining The Presence, Genetic Variability, And Function Of Cytolethal Distending Toxin In Samoa Strains Of Haemophilus Ducreyi

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Determining the Presence, Genetic Variability, and Function of Cytolethal Distending Toxin in Samoa Strains of *Haemophilus Ducreyi*

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Biographical Sketch

Andrea Faye Anstead was born November 19, 1987 in Castalia, NC. She received her Bachelor of Science degree in Biology from Elizabeth City State University in 2010. Upon completion of her undergraduate career she taught sixth grade science at Henderson Middle School for two years before furthering her education at North Carolina Agricultural and Technical State University where she is a candidate for the Master of Science degree in Biology. She has also been an active member in the Undergraduate Student National Dental Association throughout her matriculation with aspirations of becoming a general dentist. Andrea previously conducted research in KwaZulu Natal, South Africa investigating the psychosocial effects of Type II diabetes under the supervision of Dr. Ephraim Gwebu and Dr. Malcolm Cort. She has been a certified pharmacy technician since 2009.

Dedication

This thesis is dedicated to my loving family and friends for all of their continual support, encouragement, and guidance as I transform my dreams into reality. Your actions have not gone unnoticed and I am forever indebted.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

CAP Chocolate agar plates

CDC Centers for Disease Control and Prevention

CDT Cytolethal distending toxin

DltA Ducreyi Lectin A

GUD Genital ulcerative disease

Hgb Hemoglobin Receptor

HIV Human Immunodeficiency Virus

HRBC Horse red blood cells

NHS Normal human serum

PCR Polymerase Chain Reaction

p.o. By mouth

STI Sexually Transmitted infection

WHO World Health Organization

Abstract

Haemophilus ducreyi is the causative agent of the genital ulcerative disease chancroid. Chancroid is an important facilitator for increased transmission of HIV amongst heterosexuals. Chancroid is endemic to Asia, Africa, and Latin America, but there have been several outbreaks within the United States. Chancroidal ulcers are categorized as soft, painful, bloody ulcers that may remain chronic if untreated. *H. ducreyi* produces and secretes a cytolethal distending toxin (CDT) that causes various cell types to undergo cell cycle arrest or apoptosis. The CDT holotoxin consists of three genes: *cdtA*, *cdtB*, and *cdtC*. In 2006, the first report of nonsexual transmission of *H. ducreyi* was discovered in Samoa. Studies indicate the three children visiting the island had chronic lower extremity ulceration from the gram negative bacteria (Ussher, Wilson, Campanella, Taylor, & Roberts, 2007). The objective of this study therefore was to determine the presence, genetic variability, and functionality of cytolethal distending toxin in Samoa strains of *H. ducreyi*. To examine the presence of CDT, strains SB 5755, SB 5756, SB 57575, BE 3145, and 35000HP were subjected to Polymerase Chain Reaction (PCR). This technique concluded that 100% of the strains produced *cdtA*, *cdtB*, and *cdtC*. Gel electrophoresis determined approximate sizes of DNA fragments to be 750, 950, and 700 bp respectively. Each gene was cloned independently and transformed into pCR2.1-TOPO plasmid vectors. These genes were successfully amplified into chemically competent Mach1-T1 *Escherichia coli* cells. Sequence analysis was performed to verify the presence of *cdtA, cdtB, and cdtC* and to examine genetic differences amongst the strains. The Samoa strains were tested on horse blood agar plates (HBAP) to determine its ability to cause DNAse damage and lyses horse red blood cells. *H. ducryei* strain 35000HP demonstrated large zones of lysis. The Samoa strains all exhibited clear zones of lysis, but were not as prominent as the wild- type. Increasing knowledge of CDT

activity solidifies it as a virulence factor and as a possible target for a novel toxoid vaccine against CDT producing bacteria.

CHAPTER 1

Introduction

The genital ulcerative disease chancroid, caused by the bacterium *Haemophilus ducreyi*, is one of the most prevalent sexually transmitted diseases and major causes of morbidity in the resource-poor countries of Asia, Africa, and Latin America (Steen). Chancroid is a co-factor that facilitates the transmission of human immunodeficiency virus (HIV) in areas that are endemic to chancroid. Chancroidal ulcers are accompanied by painful, tender, bloody lesions that aid in the spread of HIV (Telzak et al., 1993). Periodic outbreaks of chancroid in the United States make the disease a public health concern (Mutua, M'Imunya J, & Wiysonge, 2012)

The complete genomic sequence of *H. ducreyi* strain 35000HP has greatly expanded the scientific knowledge of the pathogenesis and biology of *H. ducreyi*. *H. ducreyi* exhibits several virulence factors responsible for the development of chancroid. The independent investigation of these virulence factors may help researchers combat this disease. Therefore, studies of toxin production by *H. ducreyi* may provide vital information concerning its survival in the human host (Mutua et al., 2012). Natural infection has not been detected in environmental or animal reservoirs (Mount, Townsend, & Bauer, 2007).

Haemophilus ducreyi, Campylobacter jejuni, Actinobacillus actinomycetemcomitans, and *Escherichia coli* are amongst several gram-negative species capable of expressing and secreting soluble cytolethal distending toxin (CDT) (Blazkova et al., 2010). CDT has been associated with G_2 cell cycle arrest, progressive cell distention, and apoptosis in various cell types. *H. ducreyi* CDT (*HdCDT*) is encoded by three genes *cdtA, cdtB,* and *cdtC* which make up the CDT holotoxin. Previous studies have proven that the functional subunit, *cdtB,* is capable of

cytotoxicity alone. Further studies are needed to determine if all three subunits must be present for maximum toxic activity to occur.

1.1 Statement of the Problem

In 2007, the first nonsexual chancroid transmission was reported on the lower extremities of three young, New Zealand children visiting Samoa (Ussher et al., 2007). Further examination of the ulceration amongst the children indicated the infection was not acquired by sexual transmission. Gram stain, culture, and isolation were performed on each of the swab specimens collected from the children. Sequence data from the samples concluded the specimens were 100% identical to *H. ducreyi* (Ussher et al., 2007). Traditional chancroid is known to be a sexually transmitted infection, but further studies are necessary to conclude variations amongst the wild type strain 35000HP and Samoa strains BE3145, SB5755, SB5756, and SB5757. The long term goal of this research is to identify virulence factors in these strains that will contribute to diagnostic and vaccine development. It is vital to understand the pathogenesis of *H. ducreyi* in order to differentiate chancroidal infections from other genital ulcerative diseases. Currently, there is a limited understanding of the pathogenicity and genetic composition of these *H. ducreyi* Samoa strains.

1.2 Statement of Purpose and Hypothesis Tested

Many studies have focused on the diagnosis and treatment of traditional strains such as 35000HP. This research is innovative because Samoa strains of *H. ducreyi* will be examined to determine if they are capable of fully expressing cytolethal distending toxins that are found in strain 35000HP. Further investigation is needed to determine the variability amongst these two classes of *H. ducreyi*. We expect to determine DNA sequence variations between the parent strain and the Samoa strains of *H. ducreyi*.

The *objectives* of this study were as follows:

1. To demonstrate the presence of *cdtA, cdtB,* and *cdtC* in Samoa strains of *H. ducreyi*

2. To determine DNA sequence variation amongst Samoa strains of *H. ducreyi*

3. To investigate the function of cytolethal distending toxin in Samoa strains of *H. ducreyi*

CHAPTER 2

Literature Review

2.1 The History of *Haemophilus ducreyi*

Chancroid is one of the five classical venereal diseases along with gonorrhea, granuloma inguinale, lymphogranuloma venereum, and syphilis. All of these sexually transmitted diseases, with the exception of gonorrhea and lymphogranuloma venereum, are categorized by genital ulcers (Leduc et al., 2008). In 1852, French scientist Bassereau and his colleagues, first distinguished a soft chancre and a hard chancre resulting from syphilis (Mao & DiRienzo, 2002). The first publication of the discovery of this soft chancre was made by Auguste Ducrey at the University of Naples in 1889. Ducrey's reports demonstrated that the inoculation of pus from ulcers could be used to re-infect patients at other skin sites (Trees & Morse, 1995).

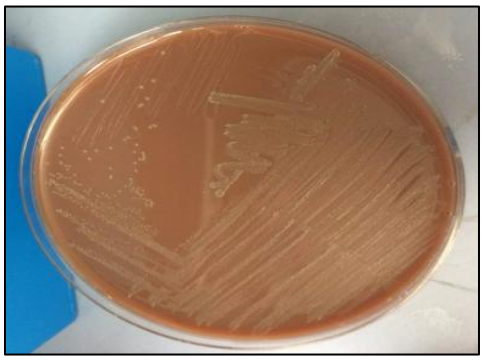


Figure 2.1 The Culture of *H. ducreyi Strain* BE3145.

Previous trials of cutaneous inoculations determined a single microorganism was found in each sample of the purulent material secreted from ulcers. The causative agent of these soft chancres was identified as a bacterium and not a virus as initially thought. The microorganism was observed in both the inside and outside of the neutrophils. Microscopic observations of the bacteria revealed the bacteria appeared as single cells in secretions and appeared as parallel chains within tissues that resemble a school of fish. Intracellular adhesion is responsible for

colonies to glide on solid media and remain intact. Ducrey's inability to culture the organism *in vitro* lead to serial inoculations to determine the infectious agent's specificity.

2.2 Characteristics of *Haemophilus ducreyi*

Organisms within the genus *Haemophilus* are fastidious, gram-negative coccobacillicus that require an X (hemin) or V (nicotinamide adenine dinucleotide) factor for optimal growth (Albritton W.L., 2000). *H. ducreyi* is located within the Pastuerellales family. The presence of quinines types demethylmenaquinone and menaquinone attribute to chemotaxonomic and physiological differences in *H. ducreyi* and other *Haemophilus* species (Magro et al., 1996). This characteristic of *H. ducreyi* does not support its inclusion in the genus *Haemophilus*. Unlike other *Haemophilus* species, *H. ducreyi* requires larger concentrations of heme to initiate growth.

Lee et al. determined that *H. ducreyi* could only grow in the presence of heme or heme containing proteins such as human hemoglobin, bovine hemoglobin, and bovine catalase, which serve as the primary source of iron (Telzak et al., 1993). The specific mechanisms which *H. ducreyi* acquires iron from hemoglobin and catalase are unknown. It has been speculated that *H. ducreyi* uses cell infiltration in cohorts with hemolysin to utilize intracellular sources of heme. Albumin is also essential for the growth of the bacterium but further studies are needed to determine if it is the source for acquired trace elements, serves as a nutritional supplement, or for the absorption of lethal metabolic byproducts (Albritton W.L, 1989).

H. ducreyi colonies can differ in appearance based on the media in which it is grown and the duration of incubation. Colonies are generally raised, compact, granular, grayish- yellow colonies. Colonies have been categorized as pinpoint size after 24 h and increase to up to 2 mm between 48 to 72 h of incubation (Piot et al., 1983). Intracellular adhesion is thought to be responsible for the *H. ducreyi* colonies ability to glide across media and remain intact. *H. ducreyi*

is only grown at 35ºC in the presence of 5% CO_2 on chocolate agar plates for 24 h. The clumping of *H. ducreyi* colonies during its culture on media makes it extremely difficult for quantification. Clinical specimens isolated on GC agar base containing 1 to 2% hemoglobin, 5% fetal bovine serum, and 3 μg of vancomycin per ml appears to have the highest sensitivity for the isolation of *H. ducreyi* (Blazkova et al., 2010) .

2.3 Clinical Features and Treatment of *H. ducreyi*

Chancroid is often misdiagnosed therefore, it is difficult to thoroughly account for the number of cases reported each year (Trees & Morse, 1995). Clinical diagnosis of chancroid is difficult for several reasons. Accurate diagnosis of chancroid is dependent upon whether the clinical presentation is typical or atypical or if the lesions are primarily caused by *H. ducreyi* or a combination of several bacteria that can cause genital ulceration. Populations that are endemic to chancroid such as, New York, Florida, and Texas are more likely to accurately identify and treat the disease (Trees & Morse, 1995).

H. ducreyi enters the skin through breaks in the epithelium during sexual intercourse. *H. ducreyi* infects lymph nodes, mucosal surfaces, both genital and non-genital skin. Initial infection begins with papule formation, which resembles a pimple. Papule formation triggers the host to respond to *H. ducreyi* infection with increased amounts of leukocytes and macrophages. The papule then develops into a pustule after approximately 2-3 days. A pustule is pus filled raised abrasion that eventually ruptures to form an ulcer after several weeks. The ulcers are generally painfully and can be excruciating depending on the site of infection. Little is known about the initial phases of infection because most patients do not seek medical treatment until the ulcer becomes too painful to bear any longer. If left untreated, ulcers can persist for several months and lead to secondary infections. The diagnosis of chancroid can be difficult since ulcers

often resemble those of syphilis or herpes. The distal prepuce is the most common site of infection for men (H.J. Ahmed, 2001). Lesions maybe found on the labia, forchutte, and clitoris of women (Lewis, 2014). Internal lesions are do not occur as often and tend to painless. The isolation of *H. ducreyi* from ulcers is frequently unsuccessful. Prior to the rapid utilization of antibiotics, chancroidal ulcers were documented to heal at significantly slower rates.

Single dose antibiotic regimens after the presentation of pustule formation is shown to decrease the risk of sexually transmitted infections (Schmid, 1986). Despite the effectiveness of single dose treatment it is extremely too costly for populations that are endemic to the disease. The Centers of Disease Control and Prevention (CDC) recommends the following regimens for the treatment of chancroid: azithromycin, 1 g orally in a single dose; ceftriaxone, 250 mg intramuscular in a single dose; trimethoprim-sulamethoxazole, two tablets for seven days, or erythromycin base, 500 mg,clavulanic acid, 125 mg, p.o. three times a day for three days, and ciprofloxacin, 500 mg p.o. two times a day for three days, are alternative regimens (http://www.cdc.gov/std/treatment/2010/genital-ulcers.htm). Expecting women should only be treated with erythromycin or ceftriaxone.

Studies have also determined that individuals diagnosed with HIV and chancroid are more likely to fail single dose antibiotic treatments (Mutua et al., 2012). However, increasing the duration of fleroxacin, 400 mg p.o. proves to be an effective form of treatment for concurrent HIV infection (Fast, W.L Albritton et al.;1983). Studies also demonstrate that uncircumcised men are more likely to fail single dose treatments of intramuscular ceftriaxone or flexrone orally The increased use of antibiotics over years is also causing a concern for antibiotic resistance (Mount et al., 2007). Studies of antimicrobial resistance are extensive in *H. ducreyi* because it will affect the choice treatment. The diverse and broad list of antimicrobial resistance in *H.*

ducreyi requires caution during the treatment of chancroid, unless clinical isolates are frequently monitored for resistance. Areas in which chancroid is prevalent have made minimal effort to monitor and document resistance. Observation of geographical and temporal differences in antimicrobial resistance of *H. ducreyi* has been attributed to the presence or the absence of resistance plasmids (Dencer H.G., W.L.Albritton,1982). Thus far, plasmid mediated antimicrobial resistance has been reported to include penicillins, tetracycline, chloramphenicol, sulfonamides, and amino glycosides (Lewis D.A., 2000) The emergence of a resistant strain of *H. ducreyi* in Rwanda has prompted the World Health Organization to forbid the use trimethoprim/sulfamethoxzole for the treatment of chancroid (Thelestam & Frisan, 2004).

2.4 Known *H. ducreyi* Virulence Factors

The regulation of the expression of virulence factors in *H. ducreyi* during infection is a major area of investigation. These virulence factors assist in the successful invasion of the host's immune system. Prior studies have shown that the infiltration of outer membrane proteins, lipoproteins, and toxins may attribute to the survival of the bacteria and its ability to cause infection. An *in vivo* study by Bauer et al. (Bauer, Fortney et al. 2008) indicated that 531 *H. ducreyi* genes were expressed in human infection in comparison to broth-grown bacteria. Specifically, the upregulation of *LspA* and *LspB* genes re cytotoxic lipoproteins that shield *H. ducreyi* from phagocytosis during secretion. LspA proteins have been shown to inhibit phagocytosis by interfering with two kinase pathways, which allow *H.ducreyi* to coexist with phagocytic cells in ulcers (Deng, 2008)

Like many other gram negative bacteria, *H. ducryei* relies upon resistance to bactericidal activity in normal human serum (NHS). Various studies have described the *H. ducreyi* serum resistance protein A (DsrA) as a possible virulence factor. This *H. ducreyi* outer membrane

protein is necessary for resistance to killing by NHS and anti-*ducreyi* antibodies within the serum (Leduc et al., 2008). *H. ducreyi* is resistant to high levels of NHS up to approximately 50%. *H. ducreyi* is strictly dependent on the availability of both heme and iron from its host, since it is unable to synthesize it. (Pickett & Whitehouse, 1999). *H. ducreyi* can obtain heme from hemoglobin at the bacterial cell surface following adhesion of hemoglobin to the *H. ducreyi* hemoglobin receptor (*Hgb*). Hemoglobin receptor (*hgbA)* is present in the genome of wild type strain 35000HP and its expression is absolutely necessary to establish infection in human experimental models of chancroid.

In addition to acquiring hemoglobin, DrsA is also responsible for the attachment of extracellular matrix components fibronectin and vitronectin (Elkins, 2000). Cloned, sequenced, and mutagenized *drsA* from various *H. ducreyi* strains suggest the importance of outer membrane proteins in serum resistance. A second outer membrane protein, ducreyi lectin A (DltA), is a virulence factor that is required for the expression of full serum resistance in *H. ducryei* (Leduc, 2004). Thus far, isogenic mutants of the fimbria-like protein (*flp*) operon, the hemoglobin receptor (*hgbA*), the peptidoglycan-associated lipoprotein (*pal*), the *ducreyi* serum resistance protein (*dsrA*), large supernatant proteins (*lspA1* and *lspA2*), a collagen binding protein (*ncaA*), or a lectin (*dltA*) have each demonstrated naturally occurring chancroid infection in the human model.

H. ducryei produces two toxins, which include hemolysin and CDT. Hemolysin acts as a contact hemolysin for fibroblasts and other cell types. CDT has the ability to induce cell death on various types including keratinocytes and immune cells. Studies are currently underway to determine how these two toxins fully illicit cytolethal effects.

2.5 Cytolethal Distending Toxin

2.5.1 Structure and function. The *H. ducreyi* CDT was first described phenotypic ally by Purven and Lagergard as being chromosomally encoded by three adjacent genes, *cdtA, cdtB,* and *cdtC* (Purvén, Falsen, & Lagergård, 1995). The individual molecular masses of the proteins are 24 kDA, 31 kDA, and 20 kDA, respectively (Cope LD, Lumbley, 1997). These three genes are encoded by an operon that seems to be transcriptionally linked. Similar CDTs are also expressed by enteric organisms such as *Escherichia coli* (Ec-CDT), *Shigella* species, *Campylobacter* species (Cj-CDT), *Helicobacter* species, and *Actinobacillus actinomycetemcomitans* (Aa-CDT) (Haghloo and Galan, 2004; Thelestam and Frisan, 2004). HdCDT and *A. actinomyecetemcomitans* CdtABC share the highest sequence homology of 95% in comparison to the other enteric bacteria. In 1997, the first high resolution crystallographic analysis of HdCDT holotoxin was published as well as the individual subunits (Nesic D, 2005). A study conducted in 1987, by Johnson and Lior, using *E. coli* strain 0128 obtained from diarrhea, is attributed the study of cytolethal distending toxins in Chinese hamster ovary (CHO) activity. Sequence analysis from this study identified three open reading frames (ORF's). The designated open ORF's were identified as *cdtA, cdtB,* and *cdtC* (Scott and Kaper, 2004). ORF sequence analysis determined that there are 4 bp overlaps between *cdtA, cdtB,* and *cdtC*. CDT was first demonstrated as a genotoxin from Elwell et al. studies, who identified position specific homology between CdtB from EcCDT and CjCDT, mammalian DNAse I. HdCDT is classified as a AB_2 toxin, which CdtB functions as the A-subunit and elicits DNAse I like activity damaging DNA within the nuclei of various cell types (Stevens, Hassett, Radolf, & Hansen, 1996). The *cdtB* gene product of *H. ducreyi* is considered the active component of the CDT holotoxin and contains the most cytotoxic activity in comparison to *cdtA* or *cdtC.*

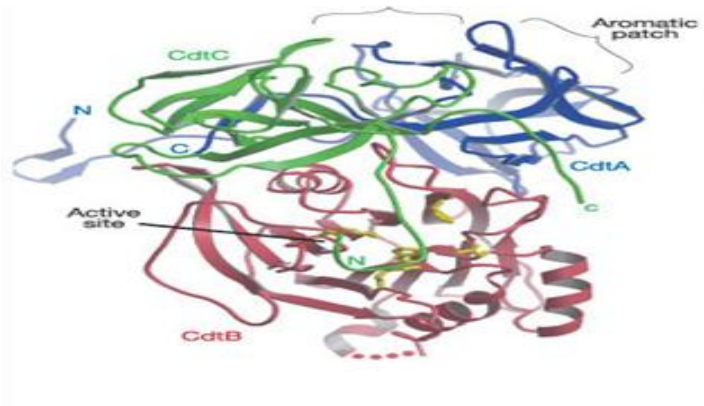


Figure 2.2 Crystal structure of the *H. ducreyi* CDT. The figure is shown as a ribbon cartoon tracing the three polypeptide chains. The active site and possible DNA contacting residues of CdtB are shown in yellow (Nesic, Hsu, & Stebbins, 2004).

H. ducreyi CdtB has the ability to bind to the plasma membrane, enter the Golgi apparatus in a retrograde manner via endocytosis, and translocate across the endoplasmic reticulum to gain access to the nucleus of mammalian cells (Eshraghi A et al., 2014). Further studies of ER associated degradation (ERAD) pathways are needed to elucidate the translocation mechanism of CDT. CdtA and CdtC are the B-subunits which assist the toxin with cellular attachment and internalization. Researchers have suggested that post-translational solidifications of CdtA may play a vital role in the effective secretion of the holotoxin into culture supernatant (Deng. K, 2001). The CdtA and CdtC subunits are soluble lectin-type molecules that share structural homology with the B-chain repeats of the plant toxin ricin (Mao & DiRienzo, 2002). The CdtB subunit adopts the four-layered fold of the DNAse I family: a central 12-stranded β-sandwich packed between outer α-helices and loops on each side of the sandwich. Very little is known about the biology of CDT holotoxin. Studies indicate that all three subunits are essential for HdCDT to possess full cytotoxic activity on whole cells (Frisk A, Lebens, et al., 2001). Further CDT toxin studies are necessary to elucidate the survival mechanisms of *H. ducreyi* that cause these chronic ulcerative diseases. CDT is considered to be a virulence factor but its cytolethal activity depends primarily on the cell type rather than the origin of the actual toxin.

2.5.2 Role of CDT in disease. Wound healing is a critical process involving blood clotting, inflammation, new tissue formation, and tissue remodeling. Neutrophils normally arrive at the wound site within minutes of injury, to control bacterial infection and produce pro-inflammatory cytokines, which serve as some one of the earliest signals to activate immunocells such as fibroblasts and keratinocytes (H.J. Ahmed, 2001). Neutrophils are followed by the infiltration of macrophages, which are necessary for effective wound healing, since healing is severely impaired if macrophage infiltration is prevented. The regeneration of epithelial cells of the skin requires both migration and quick generation of keratinocytes, fibroblasts and epithelial cells (Smith & Bayles).

Extensive studies of CDT toxicity are mainly performed in vitro on eukaryotic cells such as, HEp-2, epithelial HeLa, human fibroblasts, keratinocytes, and human umbilical vein endothelial cells. Wiseing et al. demonstrated that myeloid cells and lymphocytes are more prone to CDT toxicity and quickly undergo apoptosis immediately after exposure to the toxin. About 90% of Jurkat T-cells or THO-1 monocytic cells underwent cellular death following 100 ng/mL HdCDT within 24-48 hours of intoxication. The same study demonstrated that only 30% of HaCaT epithelial cells or HeLa cells progressed to apoptosis following intoxication (Wising et al., 2005). Involvement of CDT in ulcer formation was shown in a rabbit model of chancroid, where intradermal inoculation of *H. ducreyi* administered with purified HdCDT resulted in significant accumulation of the bacteria-induced inflammatory lesions and in ulcer development (Svensson, Henning, & Lagergard, 2002). CDT can contribute to the delayed wound healing observed in chancroid. CDT activity was originally characterized as causing relatively slow morphological changes in cultured epithelial cells, including progressive cellular distention and apoptosis within 96 to 120 hours (Cortes-Bratti, Karlsson, Lagergard, Thelestam, & Frisan,

2001). It has been shown that HdCDT affects cell proliferation and survival of many cell types involved in wound healing. (Frisan, Cortes-Bratti, & Thelestam, 2001). HdCDT may interfere with angiogenesis, since it inhibits proliferation of normal human micro vascular endothelial cells from adult dermal tissue and human umbilical vein endothelial cells preventing new blood vessel formation in an *in vitro* angiogenesis model (Ando-Suguimoto et al., 2014). HdCDT affects effector cells of the innate and adaptive immune system: intoxication inhibits proliferation and IFN-γ secretion of T lymphocytes and induces apoptosis of B lymphocytes, and monocyte-derived DCs, the key activators of the adaptive immune responses (Guerra, Guidi, & Frisan, 2011).

2.5.2.1 Animal studies. There is minimal information about the pathogenesis of *H. ducreyi.* There have been no studies indicating the isolation of *H. ducreyi* from nonhuman reservoirs. For many years New Zealand white rabbits were used as the first animal models for studying *H. ducryei.* Rabbit models were injected with large doses of the bacteria and housed at room temperature. Rabbit models are not ideal models because the lesions that are formed from the bacteria do not fully resemble those of humans. Research indicated that *H.ducreyi* could not properly replicate at temperatures higher than 35ºC. *H. ducreyi* was successfully able to replicate with temperature dependent animal models housed at 15 to 17ºC (Wising, Molne, Jonsson, Ahlman, & Lagergard, 2005) . The decrease in temperature cooled the rabbit's skin and resulted in severe inflammatory response. The rabbit model has also served as a means to study toxicity and immunogenicity of purified CDT produced by *H. ducreyi.* Wising et al. determined that each individual gene product of the holotoxin is required for cytoxicity of cultured mammalian cells. Both control and immunized rabbit models experienced increased inflammatory response which

resulted in necrotic ulcers. There was significantly less pronounced ulceration in rabbit models inoculated with *H. influenza* than *H. ducreyi* .

H. ducryei cdtA, *cdtB*, and *cdtC* mutants have also been studied to determine virulence using the rabbit model as well. Lewis and colleagues confirmed that three isogenic mutants grew at similar rates to wild type 35000HP in both broth and the temperature dependent rabbit model (Bauer et al., 2008). In regard to lesion formation, all three *cdt* mutants were as virulent as the wild type parent strain. Viable *H. ducreyi* isolates were recovered from each of the lesions, which each contained similar amounts of colony forming units. This data suggests, *cdtABC* genes must be transcribed in the wild type parent cells growing in vivo in order to possess virulence *cdt* mutants.

A chambered mouse model was also developed to study the pathogenesis of chancroid. The mice were implanted with subcutaneous polyethylene tubes that contained various strains of *H. ducreyi* (Ricotta, Wang, Cutler, Lawrence, & Humphreys, 2011) . The bacteria were successfully able to grow in the tubes. Reports indicated there was variation among *H. ducreyi* proteins, which may have resulted in some of the mice being infected for periods up to 4 months.

Primate models have been implemented to research the pathogenesis of chancroid (Totten et al., 1994). In a study by Totten et al. adult macaques were inoculated with 10^7 to 10^8 CFU of *H. ducreyi.* The foreskin of male macaques and the labia of female macaques were chosen as inoculation sites. Chancroidal ulcers began to develop between 6 to 12 days following infection in male macaques. No visible ulcers developed on any of the female macaques that were inoculated with *H. ducreyi*. Researchers attributed the lack of ulceration among female macaques to the differences in the epithelium. Further serological tests would be needed to determine if

asymptomatic infection maybe occurring in female macaques. The primate model is now deemed beneficial for studying pathogenesis of chancroid in males.

The swine, rabbit, and mouse model have all demonstrated the ability to develop serum antibodies to *H. ducreyi* antigens within one to two weeks of exposure. On the other hand, there has been no documentation of serum antibody response to occur in the human infection model after two weeks. This also included those individuals who were inoculated twice. In patients who were infected naturally with chancroid, they were able to develop antibody response following three weeks of ulceration. Therefore, the serum antibody response in the animal model seems to be premature in relation to the delayed response in humans.

2.5.2.2 Human studies. Animal studies are a good indicator of infection of *H. ducreyi,* but there are many limitations to these models since it is naturally only found in human host. Under strict, carefully regulated conditions human infection of *H. ducreyi* is rather safe. The primary benefit of the human model is the use of human skin, which is the target of infection. Papule and pustule formation in the human model is highly similar to naturally occurring infection. The experimental lesions have been confirmed to be almost identical to natural lesions. However, the human model limited because the study can only be conducted for two weeks. Whereas in nature most patients do not treatment until after the infection has been present for three to six weeks. Other limitations of the human model include the artificially simulated route of infection, genital non-genital infection of the arm using an allergy device.

Between February 25, 1993 to December 31, 2007, 267 human volunteers with varying ethnicities and ages were infected at least once with *H. ducreyi.* This 15 year clinical trial is monumental because initial infections could be monitored prior to making observations after pustule formation. Adult volunteers were inoculated on the upper deltoid at various sites of

infection. After between two and five days following infection the volunteers saw papules begin to form. Subjects that were seropositive for HIV were excluded from this study. This 15 year clinical trial is monumental because initial infections could be monitored prior to making observations after pustule formation (Leduc et al., 2008; Palmer & Munson, 1995). In natural chancroid infection patients do not generally seek treatment until painful ulceration persists for 1 to 3 weeks. Adult volunteers were inoculated on the upper deltoid at various sites of infection. The site of infection did not seem to be limiting factor since *H. ducreyi* is capable of infecting non-genital skin. Volunteers began to see papules form 2 to 5 days after infection. Papule formation was equal among male and female volunteers, but male were two times more likely to have pustule formation. This data is consistent with natural chancroid infection because males show disease progression at a 3:1 ratio in comparison to their female counterparts. An increase in body temperature associated with menstruation maybe responsible for the lower rates of pustule formation in females since *H. ducreyi* is only viable at 35ºC. Some volunteers were able to develop protective immunity and clear infection without the disease progressing to its ulcerative state. On the other hand, those that developed ulcers were more susceptible to the reoccurrence of ulcers. The duration of infection for each volunteer was 14 days or less before being treated with a single dose of ciprofloxacin. The length of duration does not fully assess natural infection of the disease because progression following pustule formation was not observed. Untreated ulcers can persist for 1-3 months.

This study determined that the papule formation in males in females is almost identical, but the rate of pustule formation is dependent upon estimated delivered doses (EDDs) and gender. The typical EDDs used on the human model are 10^1 and 10^2 colony forming units (CFU).

Disease progression associated with gender differences may be responsible for the increased male to female ratios observed in naturally occurring chancroid.

There is limited knowledge of *H.ducreyi* infection disease progression in human models due to the termination of the study prior to the development of painful chancres. A study conducted by Spinola determined that *H. ducreyi* human infection of healthy individuals inoculated with the bacteria at multiple sites in the upper arm developed papule formation after 24 hours. The study determined that HdCDT may not be responsible for the initial papule formation in the acute stage, but the secretion of CDT on various cell types may be responsible for the enlargement of the chancroid lesions and progression of the disease. Slow wound healing of the chancroid ulcers and necrosis of endothelial cells as a result CDT secretion contribute to the prolonged infection. Apoptosis of T and B lymphocytes induced by CDT results in immunosuppression which delays or decreases immune function allowing *H. ducreyi* growth, and increasing tissue damage to the genitalia (Spinola, Wild, Apicella, Gaspari, & Campagnari, 1994). Decreased fibroblast activity and the limited infection to deeper tissues by CDT create shallow lesions which become a suitable environment for other sexually transmitted infections (STI), including HIV.

Chancroid has been identified as a co-factor for heterosexual acquisition and transmission of HIV. A study conducted by Magro et al. investigated the role of chancroid in relation to HIV transmission. This study demonstrated that men seropositive for HIV developed a greater number of ulcers than those who were HIV negative therefore, increasing shedding of the virus through chancroidal ulcers (Magro et al., 1996). Dermal abrasions are a route of entry and increase risk of HIV exposure. Studies have demonstrated that HIV can alter the clinical course and appearance of chancroid. Commercial sex workers have been implicated in chancroid

outbreaks occurring in the United States and Canada (Blackmore, Limpakarnjanarat, Rigau-Perez, Albritton, & Greenwood, 1985; Hammond et al., 1980).

CHAPTER 3

Methodology

3.1 Bacterial Strains and Culture Conditions

The *H. ducreyi* type strain 35000HP was obtained from Stanley Spinola at Indiana University. The Samoa strains BE3145, SB5755, SB5756, and SB5757 were provided by Dr. Isabella Leduc at the University of North Carolina at Chapel Hill. For optimal growth, all strains were maintained on chocolate agar plates (CAP) consisting of gonococcal media base (GCB; Difco), 1% IsoVitaleX (Becton Dickinson, Cockeysville, MD), and 5% fetal bovine serum (FBS) (Sigma, St. Louis, MO) and grown at 35°C in 5% CO_2 atmosphere in 92% humidity. Each strain was cultured for 24 h to form single colony isolates. A single isolate was selected and used to grow a full lawn of bacteria. Finally, the bacteria from each plate was swabbed into 100 µl of skim milk containing 10% glycerol and stored frozen at -80°C in a 1.5 µl cryogen tube. All *H. ducreyi* strains were recovered from frozen stocks on CAP 24 h before each experiment.

Table 3.1

Haemophilus ducreyi Strains Used in This Study

Strain Name	Location/ Year of Isolation	Source (Reference)
35000HP	Winnipeg (1975)	S. Spinola
BE3145	Samoa Island, South Pacific (2007)	Ussher (Ussher, 2007)
SB5755	Samoa Island, South Pacific (2007)	Ussher (Ussher, 2007)
SB5756	Samoa Island, South Pacific (2007)	Ussher (Ussher, 2007)
SB5757	Samoa Island, South Pacific (2007)	Ussher (Ussher, 2007)

The Mach1-T1 *E. coli* strain was grown on Luria Bertani (LB) media and frozen stocks stored at -80°C in LB containing 10% glycerol. When required, Mach1-T1 *E. coli* hosting pCR™-4TOPO® vectors were grown on LB media supplemented with 50 µg/ml ampicillin or kanamycin.

3.2 Amplification of *cdtA*, *cdtB* and *cdtC* in *H. ducreyi* Strains.

Polymerase Chain Reaction (PCR) was used to amplify *cdtA*, *cdtB* and *cdtC* in each strain of *H. ducreyi*. Primers used in this study are listed in Table 3.2. Primers NcdtA forward and NctA reverse, NcdtB forward and NctB reverse, and NcdtC forward and NcdtC reverse were designed using the *cdtA*, *cdtB* and *cdtC* sequences in strain 35000HP, respectively.

Table 3.2

Primers used to amplify cdtA, cdtB, and cdtC in this study

Primers	Sequence
NcdtA forward	TGCGAAGAACTTGTCCT
NcdtA reverse	CACAGAAAACCACATTAACTGC
NcdtB forward	GAAGCAACAGCGGTTAAT
NcdtB reverse	TGACTCGCCAAAGCCAATATAC
NcdtC forward	CGTGATCGCTAAGGAGGATATT
NcdtC reverse	TCTTCGCCCCACTAAGGATCTTG
M13 forward	GTAAAACGACGGCCAG
M13 reverse	CAGGAAACAGCTATGAC

Four nucleotides were added to the end of each primer to provide restriction sites for EcoRI digestion. PCR was performed using the following reaction mixtures with each primer pair: *cdtA*, *cdtB* or *cdtC*-specific primers at 100 pmol each, Promega Green Go-Taq Master Mix (1X

Go-Taq DNA polymerase, dNTPs [200 µM each]) according to manufacturer's instruction (Promega, Madison, WI), and *H. ducreyi* whole cells as DNA template. The PCR was performed under the following conditions: a single denaturation at 95°C for 5 min and 40 cycles, each consisting of 1 min denaturation at 95°C, annealing at 50°C for 1 min, extension at 72°C for 2 min and a final extension of 10 min. Equal volumes of each sample was loaded onto a 0.8% agarose gel and subjected to electrophoresis at 100V for 30 minutes. An approximate 750 bp fragment of DNA was expected for all *H. ducreyi* strains.3.3. Cloning of *H. ducreyi cdtA, cdtB,* and *cdtC* into the pCR™4-TOPO® vector

Following amplification of *cdtA*, *cdtB*, and *cdtC* from *H. ducreyi* strains SB5755, SB5756, SB5757, 35000HP, and BE3145, each gene was cloned into the multiple cloning site of the pCR™-4TOPO® cloning vector (Figure 3.1) using the TOPO® TA Cloning® Kit (Invitrogen; Cat #K4530-20). The *cdtA*, *cdtB*, and *cdtC* amplicons obtained from PCR reactions were used as the DNA template in the cloning procedure. Each cloning reaction contained the following: 1 µL PCR product, 3 µL H_2O, 1 µL salt solution, and 1 µL pCR™-4TOPO® vector. The reagents were incubated at room temperature for 5 minutes then placed on ice in preparation for transformation into One Shot Mach1-T1 *E. coli* . A positive control (750 bp amplicon) was included to verify success of the cloning reaction.

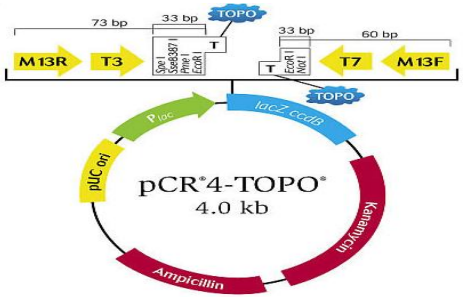


Figure 3.1 pCR4-TOPO-TA cloning vector map (Invitrogen).

3.3 Transformation of *E. coli* One Shot Mach1-T1 Competent Cells

Transformation of One Shot Mach1-T1 chemically competent *E.coli* was performed according to the manufacturer's instructions (Invitrogen; Cat #K4530-20). The Mach1-T1 *E. coli* strain was chosen to allow selection of positive transformants using blue/white selection 8 h after plating transformants on selective LB media containing 50 µg/ml ampicillin or kanamycin. Prior to performing the transformation One Shot Mach1-T1 *E. coli* cells were thawed on ice and LB ampicillin or kanamycin agar plates were warmed at 37°C until ready for use. Next 2 µL of each TOPO Cloning Reaction was placed into pre-labeled vials of Mach1-T1 cells, mixed gently and incubated on ice for 30 min. Heat shock was performed for 30 seconds at 42°C using a water bath. The vials were immediately transferred to ice. Next 250 µL of room temperature S.O.C medium was added to each vial of cells. The vials were then capped tightly and shaken horizontally (200 rpm) at 37°C for 1 hr. Finally, 50 µL of each transformation mixture was spread onto pre-warmed LB plates containing 50 µg/mL kanamycin or ampicillin and 40 µL of 40 µg/mL X-Gal. LB agar supplemented with ampicillin were prepared the day prior to cloning and used for all experimental *H. ducreyi* strains. The *E. coli* positive control, containing a pUC19 plasmid, was also grown on LB agar supplemented with ampicillin. To ensure even colony distribution, 20 µL of S.O.C. medium was added to each plate before it was incubated overnight at 37°C.

3.4 Analysis and Verification of *E. coli* Transformants

Following transformation, several hundred blue and white colonies were obtained from each transformation reaction. To identify colonies that contained the pCR™-4TOPO® vector with the *cdtA, cdtB* or *cdtC* insert, approximately 10 white colonies from each transformation were suspended in 1 mL of deionized water. A PCR reaction was performed under the following

conditions: 7.5 µl of DNA template, 1µl M13 (1 µg/µl) Forward primer (0.1 µg/µl), 1 µl M13 Reverse primer (0.1 µg/µl), 3 µl deionized water, and 12.5 µL Promega Master Mix to equal a 25 µl reaction. The following conditions were employed: a single denaturation at 95°C for 5 min and 40 cycles, each consisting of 1 min denaturation at 95°C, annealing at 50°C for 1 min, extension at 72°C for 2 min, and a final extension at 72°C for 10 min. Finally, 5 µl of each sample was then loaded onto an 0.8% agarose gel and subjected to electrophoresis at 100V for 30 minutes.

3.5 Gel Extraction and Purification of *cdtA*, *cdtB* and *cdtC* Amplicons

The *cdtA*, *cdtB* and *cdtC* DNA fragments, amplified by PCR as described above, were isolated from a 0.8% agarose gel using the QIAquick Gel Extraction Spin Kit (QIAgen; Quick Spin Handbook: Nov 2006). Each DNA fragment was excised from the agarose gel with a clean blade. The gels were then weighed in colorless tubes. Three volumes of Buffer QG were added to 1 volume of gel (100mg = 100 µl). Each sample was incubated at 50°C for 10 min and vortexes for 2 min to easily dissolve the gel. After the gel was dissolved completely, 1 volume of isopropanol was added to the sample and mixed. A QIAquick spin column was placed into its corresponding collection tube. Each sample was then applied to its QIAquick column and centrifuged for 1 min (13,000 rpm) to allow DNA binding. The flow-through for each collection tube was discarded and the columns were placed back into the corresponding collection tube. In order to remove all traces of the agarose 0.5 ml of Buffer QG was added to each QIAquick column and centrifuged (13,000 rpm) for 1 min.

Finally, 0.75 ml of Buffer PE was added to each spin column. The columns were allowed to stand for 5 min before centrifuging for 1 min. The flow-through was discarded and the column

was centrifuged for an additional 1 min (13,000 rpm) to remove any residual ethanol. The QIAquick column was then placed into a clean1.5 ml microcentrifuge tube.

To elute the DNA, 50 µl of warm Buffer EB was added to the center of the QIAquick membrane and centrifuged for 1 min. In some cases, to increase the DNA concentration 30 µl of elution buffer was added to the center of the QIAquick membrane, incubated for 1 min, and centrifuged for 1 min at 13,000 rpm.

3.6 QIAprep Spin Miniprep of pCRTM-4TOPO® Vector Containing *cdt* Inserts

The QIAprep Spin Miniprep Kit was used to purify high-copy plasmid DNA from 5ml overnight *E. coli* cultures grown in LB media with ampicillin (50ug/ml). Bacterial cells were re-suspended in 250 µl Buffer P1and transferred to a microcentrifuge tube. To each tube 250µl Buffer P2 were added and mixed by inverting the tube 6 times. The addition of 350 µl Buffer N3 and mixing resulted in the solution turning colorless. Each tube was then centrifuged for 10 minutes at 13,000 rpm in a tabletop centrifuge. The supernatant from each tube was then applied to the QIAprep spin column by pipetting. Each sample was centrifuged foe 60 seconds and the flow through was discarded. The samples in the QIAprep spin columns were washed by adding 0.5 ml Buffer PB and centrifuged for 60 seconds. The flow through from each column was discarded. The final wash was performed by adding 0.75 ml Buffer PE and centrifuging each sample for 60 seconds. The flow through was discarded and each sample was centrifuged again for 1 minute to remove any of the additional residual wash buffer. The QIAprep column was placed into a new 1.5 ml tube and the DNA was eluted with 30 µl of Buffer EB.

3.7 Sequence Analysis and Multiple Sequence Alignment of *cdtA*, *cdtB* and *cdtC* in *H. ducreyi* Samoa Strains

DNA concentrations were determined for each sample of purified DNA using a nanodrop. Samples were prepared for sequencing using two methods: (i) purified amplicons were prepared according to Simple Seq guidelines provided by Eurofins MWG Operon (http://www.operon.com/). DNA sequences were obtained electronically from Eurofins MWG Operon in an ABI format. (ii) Eton Bioscience. Purified amplicons were submitted in a 96 well plate in 5 µl quantities. To view, edit and perform Basic Local Alignment Search (BLAST) with all nucleotide sequences, FinchTV (www.geospiza.com/Products/finchtv/), a free web-based DNA sequencing chromatogram trace viewer was used in this study. Multiple DNA sequence alignments were performed using ClustalW1 or 2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and sequence comparison tables were produced using BoxShade (http://ch.embnet.org/software/BOX_form.html).

3.8 Hemolytic Activity of *H. ducreyi*

To examine the function of the CDT toxin in each *H. ducreyi* strain, horse blood agar plates (HBAPs) were used to perform hemolytic assays. Bilayer plates consisting of GC agar base (Difco), 1% XV factor supplement, and 5% horse blood were obtained from Fisher Scientific (cat # 50176862). *H. ducreyi* strains were streaked for isolation on CAPs, directly from frozen stocks, and grown overnight at 35°C. Single colonies were streaked onto HBAP in triplicate and grown for 72 h at 35°C. CDT activity was determined by observing the zone of lysis.

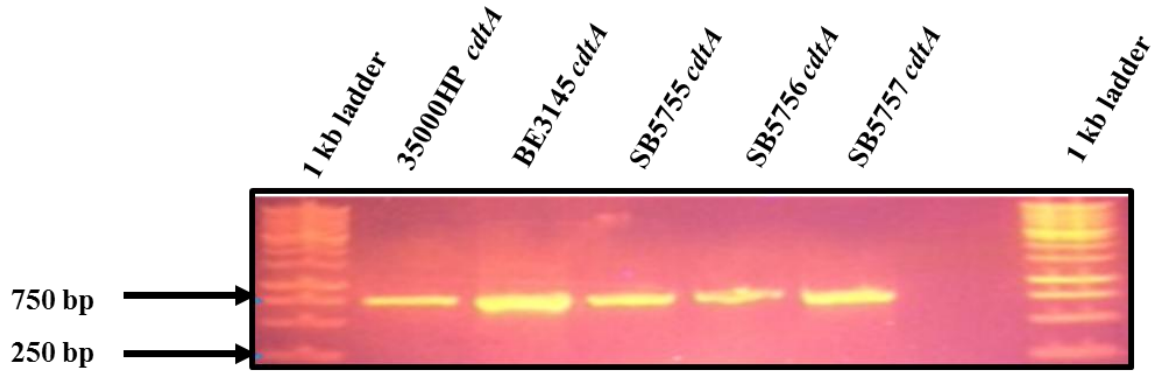
CHAPTER 4

Results

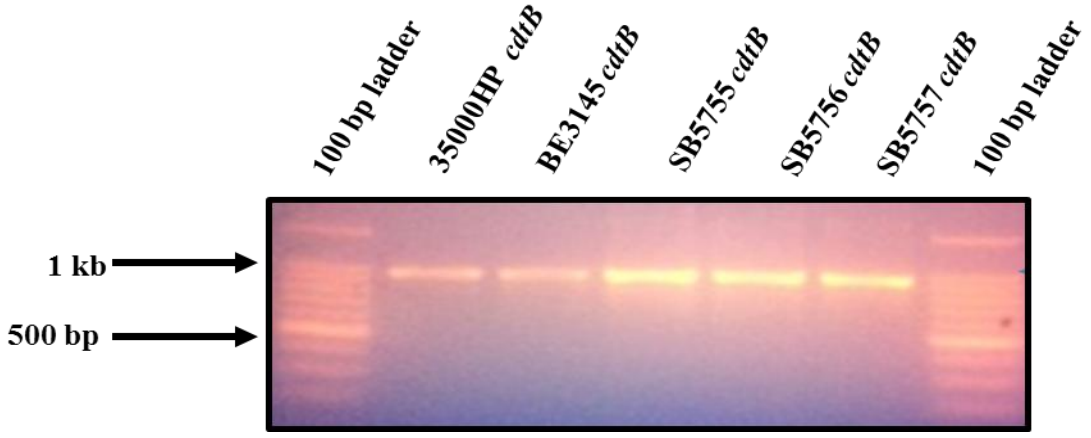
4.1 Amplification of *cdtA, cdtB, and cdtC* in Samoa Strains of *H. ducreyi*

Previous studies by Totten and Morse (2000) demonstrated that PCR was a more efficient and reliable method to diagnosis *H. ducreyi* than Gram stain and culturing the fastidious bacteria. In comparison to prior methods, PCR tests have demonstrated 95% or higher accuracy for the detection of the bacterium performed on the same samples. Previous studies also indicate that *cdtA* in *H. ducreyi* strain 35000HP could be amplified using PCR primers designed to *cdtA.* Therefore, to amplify *cdtA*, *cdtB,* and *cdtC* from Samoa strains *BE3145, SB5755, SB5756, and SB5757*, whole cells were subjected to PCR and agarose gel electrophoresis on 0.8% agarose gel. As predicted, an approximately 750bp DNA product containing *cdtA* was successfully amplified using the NcdtA PCR primers (Figure 4.1a). Additionally, an approximate 950 bp *cdtB* fragment and 700bp *cdtC* DNA product were obtained using NcdtB and NcdtC, respectively (Figures 4.1b and 4.1c). Therefore, these data indicate that *cdtA*, *cdtB,* and *cdtC* are all present in Samoa strains BE3145, SB5755, SB5756, and SB5757.

(A)



(B)



(C)

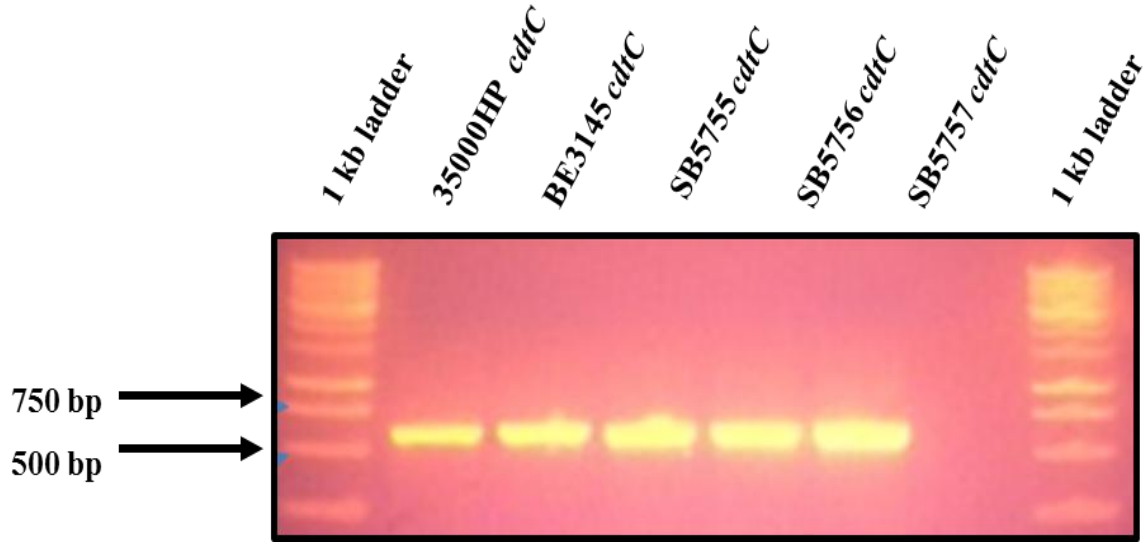


Figure 4.1 Amplification of *cdtA, cdtB, and cdtC in H. ducreyi* strains 35000HP, BE3145, SB5755, SB5756, and SB5757. All PCR samples were subjected to gel electrophoresis using a 0.8% agarose gel. Strain names are listed above. Molecular weight standards are indicated to the left of the gels.

4.2 Transformation of pSB56 *cdtA*, pSB57 *cdtA*, and pBE3145 *cdtA* into *E. coli* Mach1-T1 Cells

The blue-white screening technique demonstrated the successful transformation of pSB5756*cdtA*, pSB5757*cdtA*, and pBE3145*cdtA* into Mach1-T1 chemically competent *E.coli* cells using the TOPO TA Cloning Kit. Several hundred white bacterial colonies containing *cdtC* were produced 24 hrs after exposure to X-gal. There were significantly fewer blue colonies formed, indicating the absence of *cdtA* in the vector. To analyze the positive clones, 10 white colonies were picked from each LB media plate, subjected to PCR with NcdtAF/NcdtAR primers, and agarose gel electrophoresis.

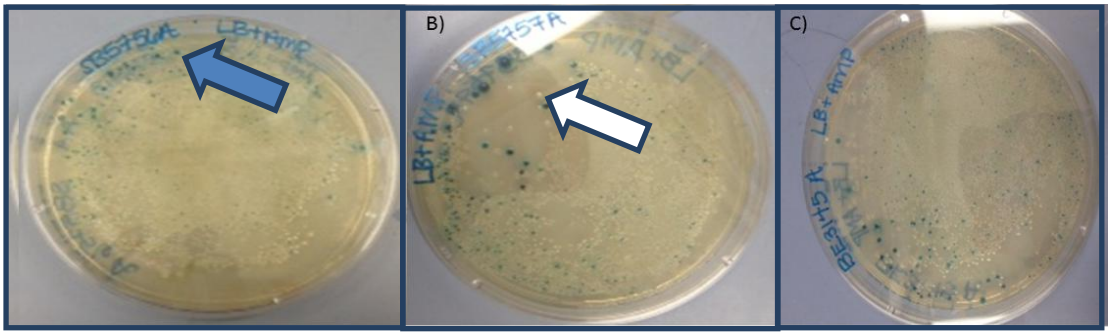


Figure 4.2 Blue and white colony assay to identify *E. coli* transformants containing (A) pSB5756*cdtA*, (B) pSB5757*cdtA*, and (C) pBE3145*cdtA*. *E. coli* transformants containing pSB5757*cdtA* is indicated by the white arrow, while a transformant containing a pCR™-4TOPO® vector lacking the SB5756 *cdtA* insert is indicated by the blue arrow.

4.3 Amplification of p35000 *cdtA*, pBE3145 *cdtA*, pSB5755 *cdtA*, pSB5756 *cdtA*, and pSB5757 *cdtA* into Mach1-T1 *E. coli* Transformants

To further verify the presence of *cdtA* in *E. coli* obtained from blue-white selection, white colonies grown on LB agar containing 50 µg/ml Ampicillin were subjected to PCR using the M13 forward and M13 reverse primers, specific to the multiple cloning site of the TOPO-TA vector (355-370 nt and 205-221 nt). The DNA products were visualized using a 0.8% agarose.

The *cdtA* from all strains transformed in this study were successfully cloned into the Topo-TA vector (Figure 4.3). A DNA fragment of approximately 250 bp was amplified instead of the expected 1kp amplicon, but was later successfully amplified.

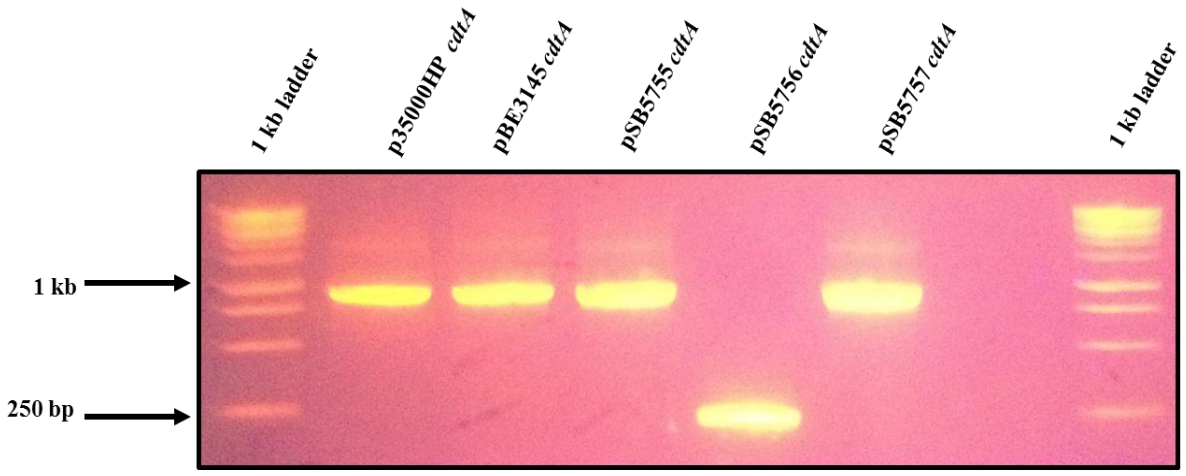


Figure 4.3 Amplification of *p35000 cdtA, pBE3145 cdtA, pSB5755 cdtA, pSB5756 cdtA,* and *pSB5757 cdtA* into Mach1-T1 *E. coli transformants.* Lanes 2-6 are *E. coli* transformants containing p35000*cdtA*, pBE3145*cdtA*, pSB5755*cdtA*, pSB5756*cdtA*, and pSB5757*cdtA.* M13 forward and M13 reverse primers were used to amplify the specific region of interest.

4.4 Amplification of p35000 *cdtB*, pBE3145 *cdtB*, pSB5755 *cdtB*, pSB5756 *cdtB*, and pSB5757 *cdtB* into Mach1-T1 *E. coli* Transformants.

Previous studies demonstrated that *cdtB* could be cloned and transformed into *E. coli* to express a gene of interest using specific M13 primers. The *H. ducreyi* genomic DNA could be amplified using PCR primers, designed to *cdtB*. Therefore, to amplify *cdtB* from *E.coli* whole cells were subjected to PCR and agarose gel electrophoresis. As predicted, an approximately 1 kb DNA product containing *cdtB* was successfully amplified in Mach1-T1 chemically competent cells Figure 4.4. Double bands produced by BE3145 *cdtB* may indicate non-specific binding of the M13 forward and M13 reverse PCR primers to the DNA template.

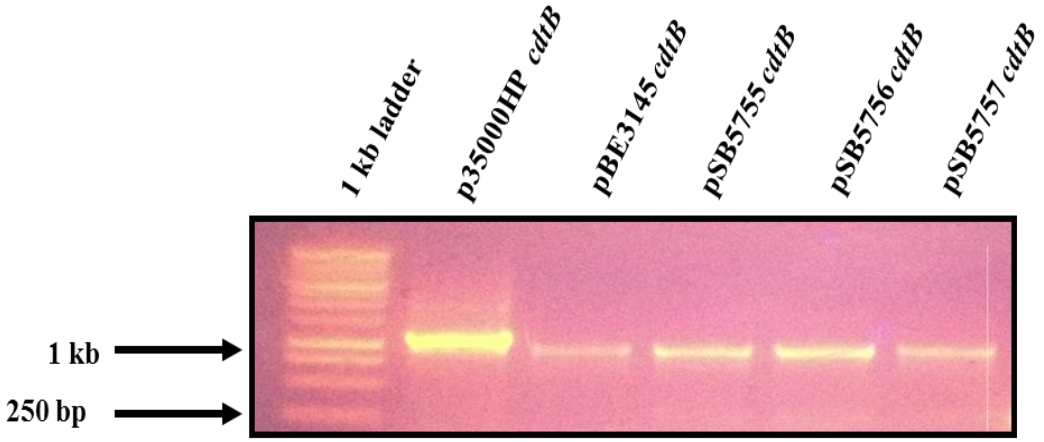


Figure 4.4 Amplification of p35000 cdtB, pBE3145 cdtB, pSB5755 cdtB, pSB5756 cdtB, and pSB5757 cdtB into Mach1-T1 E. coli transformants. Lanes 2-6 are *E. coli* transformants containing p35000 *cdtB,* pSB5756 *cdtB,* pSB5757 *cdtB,* and pBE3145 *cdtB.* All transformants were successful in the amplification of *cdtB*. Each *cdtB* demonstrated the expected fragment size of 1 kb. M13 forward and M13 reverse primers were used to amplify the specific region of interest. Lane 1 is the 1kb DNA molecular marker.

4.5 Amplification of p35000 *cdtC*, pBE3145 *cdtC*, pSB5755 *cdtC*, pSB5756 *cdtC*, and pSB5757 *cdtC* into Mach1-T1 *E. coli* Transformants

Previous studies by Bauer et al. demonstrated that the *H. ducreyi* genomic DNA could be amplified using PCR M13 primers, designed to *cdtC* (Bauer et al., 2008). Therefore, to amplify *cdtC* from *E. coli* genomic DNA, colonies produced from blue- white screening were subjected to PCR and agarose gel electrophoresis. As predicted, an approximately 1 kb DNA product containing *cdtC* was successfully amplified (Figure 4.5)

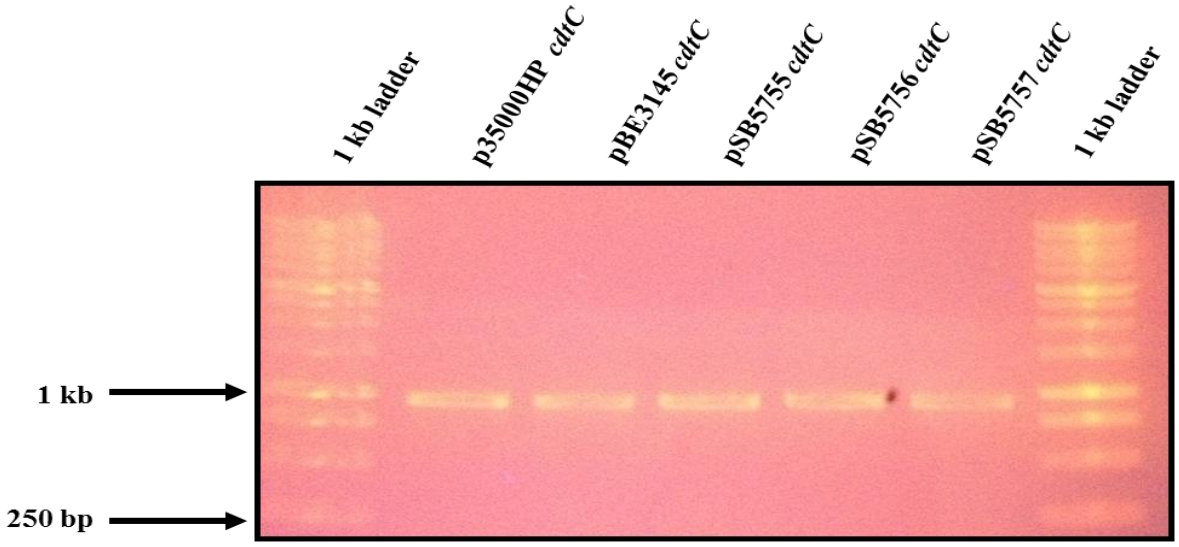


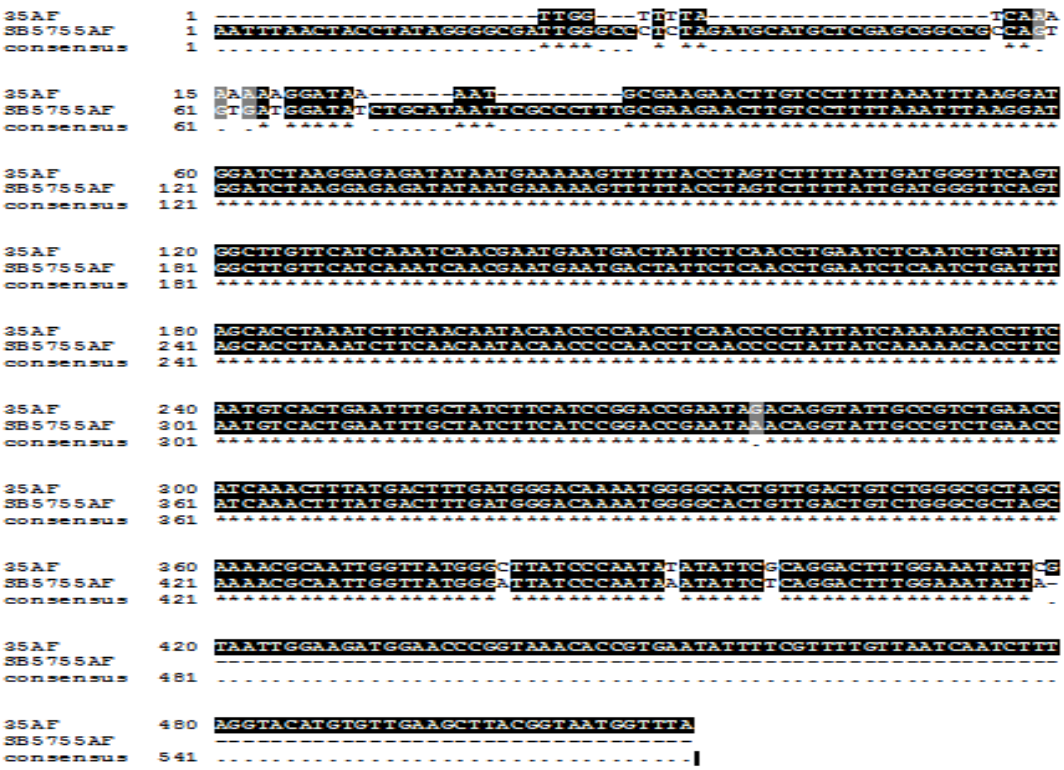
Figure 4.5 Amplification of p35000 *cdtC,* pBE3145 *cdtC,* pSB5755 *cdtC,* pSB5756 *cdtC, and* pSB5757 *cdtC into Mach1-T1 E. coli* transformants. Lane 1 is the 1kb DNA molecular marker. The expected size DNA fragments, 1000 bp, were amplified from the Mach1-T1 genomic DNA. Lanes 2-6 are *E. coli* transformants containing p35000 *cdtC,* pBE3145 *cdtC,* pSB5755 *cdtC,* pSB5756 *cdtC,* and pSB5757 *cdtC*. Lane 7 is the 1kb DNA molecular marker.

4.6 Sequencing of pSB5755 *cdtA* and pSB5757 *cdtA* from Mach1-T1 *E. coli* Transformants

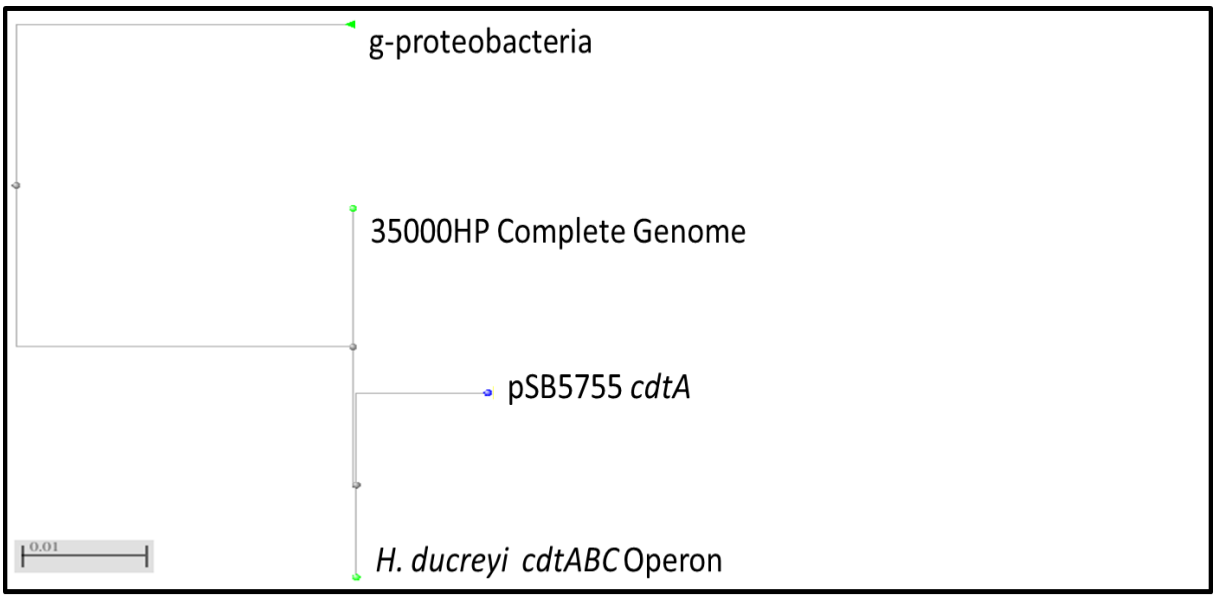
Despite, obtaining partial nucleotide reads for transformants we were still able to proceed with DNA sequencing. DNA sequence analysis was performed on pSB5755 *cdtA* and pSB5757 *cdtA* from Mach1-T1 *E. coli* transformants to verify plasmids inserts were indeed *cdtA* from *H. ducryei* strains. Sequencing results were obtained from Eurofins MWG operon and ETON Bioscience. Examination of the deduced nucleotide sequences of the pSB5755 *cdtA* and pSB5757 *cdtA* with FinchTV revealed that the *cdtA* was isolated from *H. ducreyi* (Figure 4.6 and 4.7).

4.6.1 Nucleotide sequence comparison of 35000HP and pSB5755 *cdtA*. Purified nucleotide sequences of SB5755 *cdtA* and 35000HP *cdtA* were translated into amino acid sequences and subjected to amino acid sequence comparison using Finchtv. The *cdtA* from 35000HP and SB5755 were determined to be 99% identical to the 35000HP amino acid sequence obtained from the STDGEN Database (http://stdgen.northwestern.edu/), as shown in Figure 4.6a. Phylogenetic analysis of wild-type 35000HP and Samoa strain SB5755 *cdtA* was performed in NCBI (using the neighbor-joining method. The value of each branch is the estimated confidence level (expressed as a percent) for the position of the branch as determined by boot-strap analysis. The comparison determined that there is not a high degree of variability between 35000HP and SB5755 *cdtA*. Taken together, these results indicate that *cdtA* is present in strain SB5755 and it is closely related to the *cdtA* of strain 35000HP.

(A)



(B)



(C)

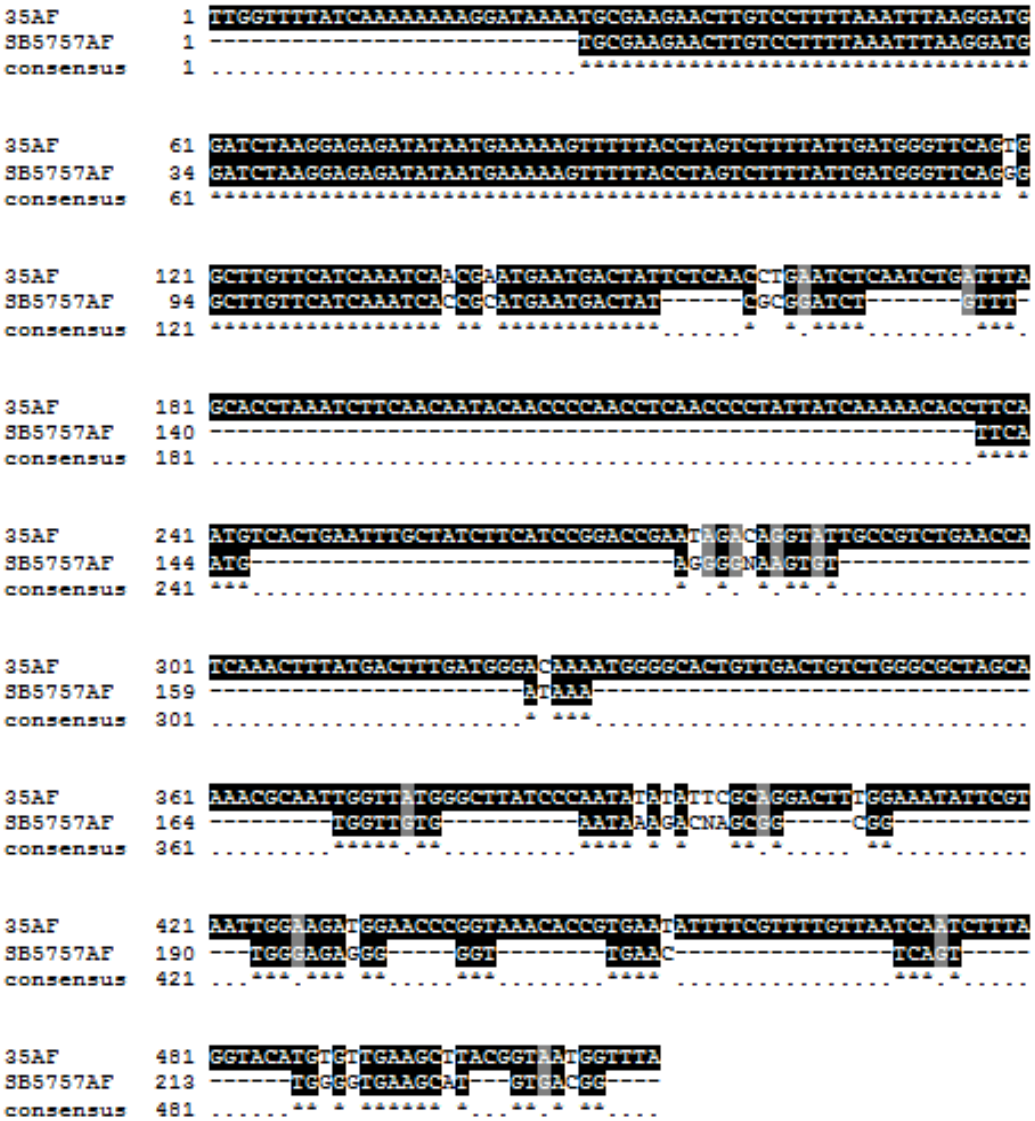
Description	**% Identity**	**Accession Number**
***Haemophilus ducreyi* 35000HP genome**	**99%**	**AE017143.1**
***Haemophilus ducreyi* cytolethal distending protein (cdtA, cdtB, and cdtC)**	**99%**	**U53215.1**
Aggregatibacter actinomycetemcomitans cdtA, cdtB,* and *cdtC	**93%**	**AB017807.1**

Figure 4.6 Sequencing results of pSB5755 *cdtA.* (A) pSB5755 and 35000HP *cdtA* nucleotide sequence comparison table produced using BoxShade. Strain names are listed to the left. Black, shaded regions indicate identity. Gray boxes demonstrate similar residues that were classified as either purines or pyrimidines. Numbers to the left of the figure indicate the nucleotide position within the sequence. (B) Phylogenetic tree analysis of pSB5755 *cdtA* and 35000HP *cdtA.* The scale bar represents differences in the nucleotide sequences. (C) Sequences producing significant alignments

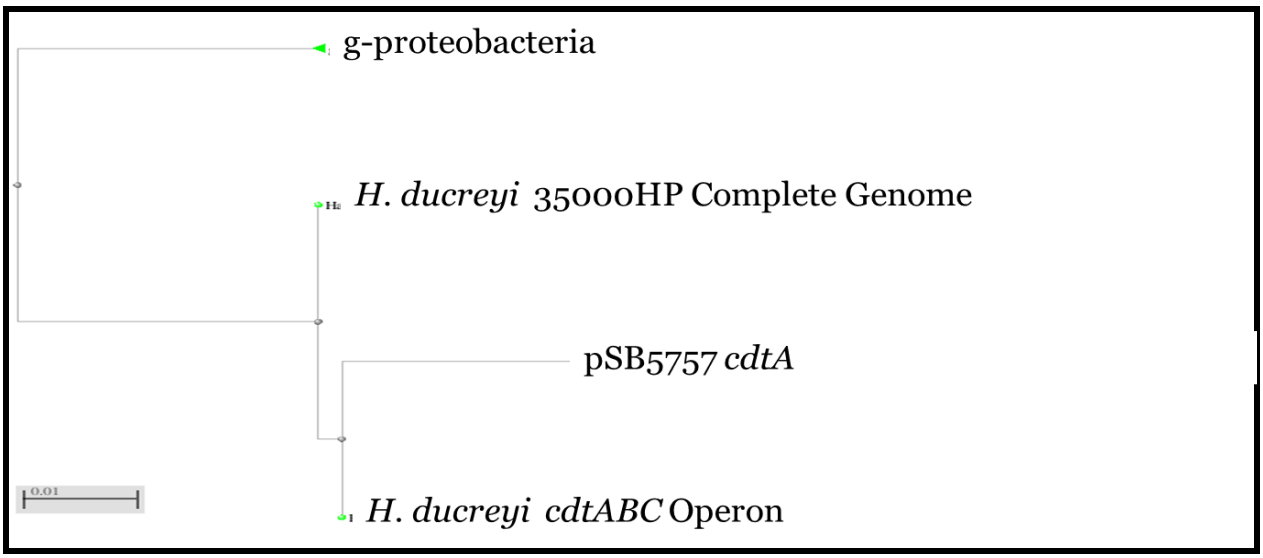
4.6.2 Sequence analysis of 35000HP and pSB5757 *cdtA*. Nucleotide sequence results were gathered from FinchTV of SB5755 *cdtA.* A BLAST (Basic Local Alignment Search Tool) of the amino acid sequence determined that SB5757 *cdtA* was 98% identical to the 35000HP

amino acid sequence obtained from STDGEN Database (http://stdgen.northwestern.edu/), as shown in Figure 4.6.2c. In this study, several attempts to determine the sequence of *cdtA*, *cdtB*, and *cdtC* from Samoa strains BE3145, SB5755, SB5756, and SB5757 were unsuccessful. Due to the poor quality of the sequence data, characterization of those strains could not fully be determined.

(A)



(B)



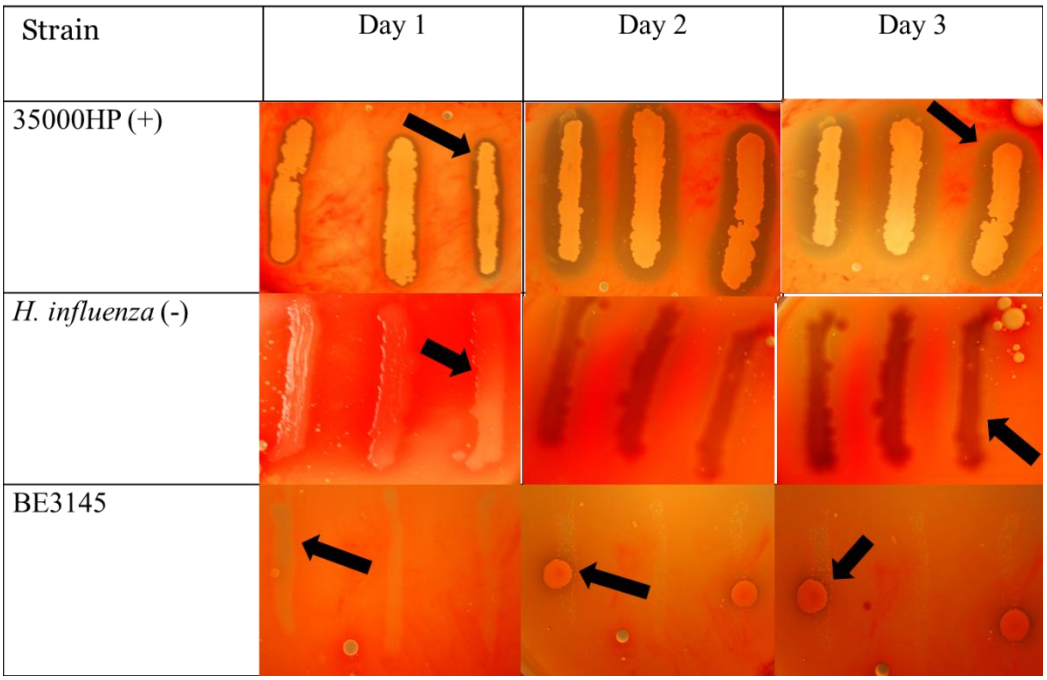
(C)

Description	**% Identity**	**Accession Number**
***Haemophilus ducreyi* 35000HP genome**	**98%**	**AE017143.1**
***Haemophilus ducreyi* cytolethal distending protein (cdtA, cdtB, and cdtC)**	**98%**	**U53215.1**
***Aggregatibacter actinomycetemcomitans* complete genome**	**90%**	**CP007502.1**

Figure 4.7 Sequencing results of pSB5757 *cdtA*. (A) Nucleotide sequence comparison of the *H. ducreyi* amplicon pSB5757 *cdtA* and wild type 35000HP. Shaded regions indicate similarities between the strains. Numbers to the left of the figure indicate amino acid number within the strains. (B) Phylogenetic tree of pSB5757 *cdtA*. (C) Production of significant sequence alignments

4.7 Hemolytic Assay

(A)



(B)

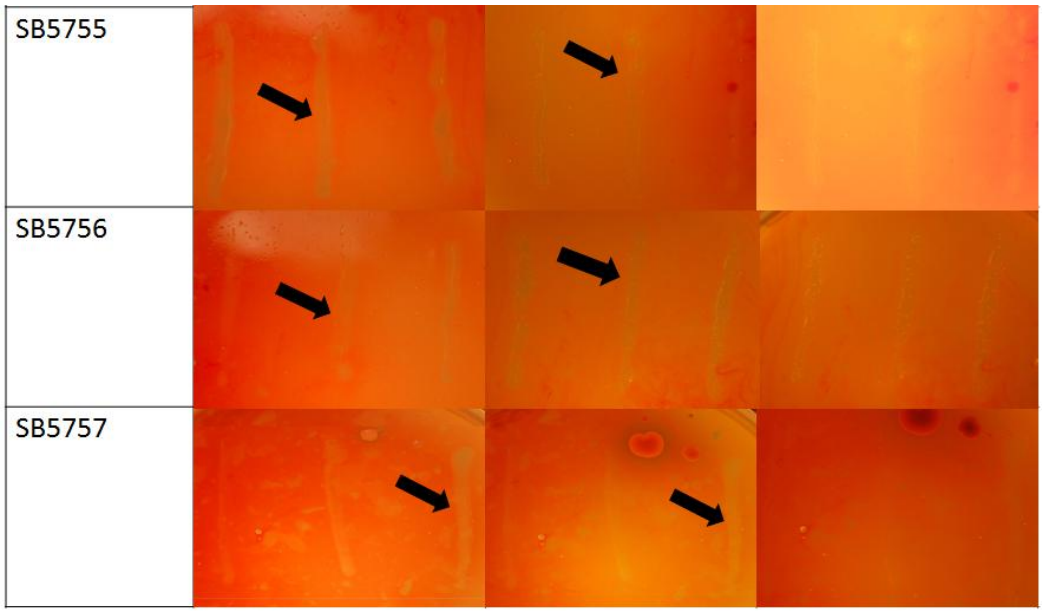


Figure 4.8 Hemolytic phenotypes of *H.ducreyi* 35000HP and Samoa strains on HBAPs. (A) Hemolysis of strain 35000HP on HBAP after three days of incubation produced large colonies showing clear zones of hemolysis. *H. influenza* was non-hemolytic and served as a negative control. (B) After three days of incubation Samoa strains BE3145, SB5755, SB5756, and SB5757 produce smaller clear zones of lysis in varying amount.

As expected, clear zones of lysis were seen with strain 35000HP on bilayer plates containing 7.5% horse red blood (HBAPs). Wild-type 35000HP developed larger single colonies in comparison to the Samoa strains as observed in *Figure 4.7*. Samoa strains SB5755, SB5756, SB5757, and BE3145 were also incubated for three days at 35°C in the presence of 5% $CO_{2,}$ but less hemolytic activity was observed. While each strain produced varying amount of hemolysis *Haemophilus influenza*, serving as a negative control, did not exhibit any lysis. Despite prominent growth of *H. influenza,* the absence of lysis zones verified that only the secretion of CDT caused the lysis of the horse red blood cells in 35000HP and the Samoa strains of *H. ducryei.*

CHAPTER 5

Discussion and Future Research

Haemophilus ducreyi is a fastidious, gram negative coccobacillus that is the etiological agent of the sexually transmitted infection (STI) known as chancroid. Chancroid facilitates the heterosexual transmission of HIV and is commonly detected in Africa, Asia, and Latin America. Successful management of chancroid should greatly impact the dynamics of the HIV epidemic and furthermore, contribute to alleviation of the occurrence of chronic skin ulceration in these countries. The complete genomic sequence of *H. ducreyi* strain 35000HP has greatly expanded the scientific knowledge of the pathogenesis and biology of *H. ducreyi*. The independent investigation cytolethal distending toxin as a virulence factor may assist researchers combating this disease.

H. ducreyi is amongst several enteric species capable of expressing and secreting soluble cytolethal distending toxin (CDT) (Blazkova et al., 2010). CDT has been associated with G_2 cell cycle arrest, progressive cell distention, and apoptosis in various cell types. *H. ducreyi* CDT (*HdCDT*) is encoded by three genes *cdtA*, *cdtB*, and *cdtC* which make up the CDT holotoxin.

The first description of nonsexual chancroid transmission was reported in 2007, after three young children visiting the island of Samoa developed lower limb ulceration from nonsexual *H. ducreyi* infection. The study of *cdt* in these Samoan strains may provide insight into the chronic infection of the disease and its survival within its human host. We hypothesized that the Samoa strains of *H. ducreyi* would express *cdt* and function in a similar manner to wild-type 35000HP. The objective of this study was to determine the presence, variability, and function of cytolethal distending toxin in Samoa strains of *H. Ducreyi.*

In this report, we used PCR and gel electrophoresis to demonstrate that *H. ducreyi* strains SB 5755, SB 5756, SB 57575, BE 3145, and parent strain 35000HP all produced *cdtA, cdtB,* and *cdtC* genes. PCR DNA fragments were successfully amplified and had approximate sizes of 750, 950, and 700 bp respectively. Each gene was cloned independently and transformed into pCR2.1-TOPO plasmid vectors. These genes were successfully amplified into chemically competent Mach1-T1 *E. coli* cells.

Examination of genetic differences amongst the Samoa strains using sequence analysis proved to be extremely challenging. Thus far, 35000HP *cdtA* and SB5755 *cdtA* are the only strains to be successfully sequenced and analyzed. The other strains all produced extremely short reads with an ample amount of human contamination. Sequence analysis of the remaining Samoa strains are ongoing. The identification of variation or homology among the strains may play a vital role understanding the pathogenesis and the treatment of chancroid. Strains that have been isolated in various geographical regions have shown differentiation among antibiotic resistance, which affects therapeutic regimens. Amino acid sequence analysis of SB5755 and SB5757 *cdtA* have between 98-99% similar identities therefore, we can hypothesize that they function similarly to wild-type 35000HP. Further analysis obtaining full genomic reads will be beneficial in differentiation of the Samoa strains. Phylogenetic analysis of 35000HP and SB5755 *cdtA,* as shown in Figure indicates the two strains are closely related. The culture of *H. ducreyi* and isolation of CDT is vital in determining the hemolytic activity of the Samoa strains. The Samoa strains of *H. ducreyi* were tested on horse blood agar plates (HBAP) to determine its ability to cause DNAse damage and lyse red blood cells. As expected, the wild-type 35000HP was able to cause lysis of the HRBCs after three days of incubation in the presence of CO_2. Each of the Samoa strains was able to lyse cells, but their zone of lysis was not as prominent as the

wild-type. Increasing width of lysis zones indicate that CDT was continually secreted over a three day period, significantly in 35000HP. The small degree of genomic variation in each *cdt* may attribute to the various levels of lysis produced by each Samoa strain.

Ussher et al. (2007) reported the first case of nonsexual transmission of chancroid by novel strains of *H. ducreyi*. This study demonstrates that Samoa strains BE3145, SB5755, SB5756, and SB5757 each contain *cdtA, cdtB, and cdtC.* DNA sequence analysis verified the successful amplification of *cdt.* Evolutionary analysis determined that these nonsexual strains are common ancestors of wild-type 35000HP. CDT is functional in 35000HP, BE3145, SB5755, SB5756, and SB5757. Inserting *cdtA, cdtB,* and *cdtC* plasmids into an expression vector maybe the next step to differentiate the Samoa strains of *H. ducreyi.*

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