

**The Development of a**  
**Rapid Diagnostic Test for the Detection of**  
*Haemophilus ducreyi*

**Mona Pillay**

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## **List of Abbreviations**

<b>AIDS</b>	Acquired Immunodeficiency Syndrome
<b>BSA</b>	Bovine Serum Albumin
<b>CA</b>	Chocolate Agar
<b>CDC</b>	Centers for Disease Control and Prevention
<b>CFU</b>	Colony Forming Unit
<b>dH<sub>2</sub>O</b>	Distilled Water
<b>DNA</b>	De-oxy Ribonucleic Acid
<b>EDD</b>	Estimated Delivered Dose
<b>EIAs</b>	Enzyme Immuno Assays
<b>FCA</b>	Freund's Complete Adjuvant
<b>GUD</b>	Genital Ulcer Disease
<b>HIV</b>	Human Immuno Deficiency Virus
<b>HRP</b>	Horseradish Peroxidase
<b>HSP</b>	Heat Shock Protein
<b>HSV</b>	Herpes Simplex Virus
<b>IC</b>	Immuno-chromatography
<b>IF</b>	Immunofluorescent
<b>kDa</b>	Kilo Dalton
<b>LOS</b>	Lipo Oligosaccharide
<b>MAb</b>	Monoclonal Antibody

<b>MALDI/TOF-MS</b>	Matrix Assisted Laser Desorption/Ionization Time Of Flight Mass Spectrometry
<b>M-PCR</b>	Multiplex Polymerase Chain Reaction
<b>mRNA</b>	Messenger Ribonucleic Acid
<b>NAD</b>	Nicotinamide Adenine Dinucleotide
<b>PCR</b>	Polymerase Chain Reaction
<b>PMN</b>	Polymorphonuclear
<b>STD</b>	Sexually Transmitted Disease
<b>STI</b>	Sexually Transmitted Infection
<b>US</b>	United States
<b>WHO</b>	Who Health Organisation

## **Abstract**

**Aim:** To develop an antigen detection test that would quickly exclude *H. ducreyi* infection in individuals with genital ulcers.

**Materials and Methods:** *H. ducreyi* strains A54 and A68 were grown on Modified Bieling (MB) agar plates and in MB broth under microaerophilic conditions. The 58.5 kDa GroEL Heat Shock Protein (HSP) was extracted from *H. ducreyi* strain A54 by means of sonication. The purified HSP was used to raise antibodies in rabbits. HSP determination and separation was done on SDS PAGE gels and protein was eluted by means of a passive elution process. Antibody was purified by affinity chromatography and a fraction of the antibody was conjugated to a chromogen to be used as a detection antibody. An ELISA was developed to evaluate the antibody response to the HSP. A second ELISA was developed to evaluate test parameters.

**Results:** A good immune response was achieved with the crude serum of one of the three rabbits when tested against the antigen by means of ELISA. However, after purification of the IgG from the serum of the same rabbit no antigen-antibody binding was observed. Anti-rabbit IgG was able to recognise the antibodies.

**Discussion and Conclusion:** While the Fc portion of the purified IgG remained active, the Fab portion of the antibody had lost biological activity. This loss of biological activity of antibody can be attributed to the low pH of the elution buffers used during the purification steps. Alternative antibody purification systems need to be explored. The use of monoclonal antibodies also needs to be considered.

## **Chapter One: Introduction**

Chancroid was once a prevalent disease found in many parts of Africa, Asia and Latin America (Trees and Morse, 1995). At the onset of the 20<sup>th</sup> century the occurrence of chancroid began to decline (Steen, 2001). The true incidence of chancroid is not entirely known due to incomplete reporting of such infections as clinical differentiation of chancroid from other forms of genital ulcer disease is almost impossible (Morse, 1989). In addition, there is a “lack of availability of diagnostic tests, especially in the resource-poor countries in which chancroid is most prevalent” (Al-Tawfiq and Spinola, 2002).

Epidemics of the disease have been shown to be associated with prostitution (Steen, 2001; O’Farrell, 2000), despite the fact that the prevalence in men is much higher than in women with reported male: female ratios ranging from 3:1 and 25:1 (Morse, 1989).

As emphasised previously by Vuylsteke (2004), the development of simple, cheap and rapid diagnostic tests for STIs “may represent an important break-through for STI control in symptomatic and asymptomatic women in developing countries” (Vuylsteke, 2004). In addition, if results are obtained rapidly, treatment accordingly based on syndromic management guidelines can be replaced by targeted treatment. As in other African countries, in South Africa the prevalence of chancroid among patients with genital ulcer disease has over the last 10 years decreased to less than 1 %. Despite this, all patients diagnosed with this syndrome are treated for chancroid. Therefore, the most important contribution of a rapid

point-of-care test to patient management would be the avoidance of the use of anti-chancroid drugs in patients with a negative test.

This study focused on the 58.5 kDa GroEL Heat Shock Protein (HSP) which is produced in abundance by the causative agent of chancroid, the bacterium *Haemophilus ducreyi*. HSPs are highly conserved (Parsons *et al*, 1992) and are produced when bacteria encounter different types of stresses such as heat, anoxia, heavy metal ions and ethanol (Lindquist and Craig, 1988). Brown *et al* (1993) already has successfully developed a monoclonal antibody, BB11 to this HSP (Brown *et al*, 1993).

In this study, we aimed to develop a rapid test that would quickly exclude the possibility of *H. ducreyi* infection when used to diagnose individuals with genital ulcers.

## Chapter Two: Review of Literature

### **2.1 Historical Background:**

*Haemophilus ducreyi* is a Gram negative bacterium and is the causative agent of chancroid which is a genital ulcer disease (GUD) (Morse, 1989). *H. ducreyi* is a fastidious organism (Albritton, 1989; Morse, 1989) which is problematic to culture (Trees and Morse, 1995; Hammond *et al*, 1996; Steen, 2001), especially from clinical specimens (Hammond *et al*, 1978; Trees and Morse, 1995).

Chancroid is a venereal disease which is characterized by ulceration and is often accompanied by painful tender inguinal lymphadenopathy. In some cases the swollen lymph nodes develop into buboes (Morse, 1989). Chancroid or soft chancre was described in 1852 by Leon Bassereau who was the first to differentiate the infection from hard indurated chancre of primary syphilis (as cited by Morse, 1989; Hammond, 1996; Lewis<sup>a</sup>, 2000). Bassereau had demonstrated this by reinfesting soft chancre patients at different skin sites by autoinoculation with purulent material from the patient's own ulcers (as cited by Morse, 1989).

In 1889, Augusto Ducrey had reported his findings which had led to the identification of the causative agent for chancroid, hence the name *H. ducreyi* in recognition of Ducrey's findings (as cited by Lewis<sup>a</sup>, 2000). Ducrey had described *H. ducreyi* as being short streptobacillary rods with rounded ends (as cited by Morse, 1989).



Even though controversy exists over the first *in vitro* culture isolation of *Haemophilus ducreyi*, credit is generally given to Lenglet for successfully isolating *H. ducreyi* in 1898 (as cited by Morse, 1989; Lewis<sup>a</sup>, 2000) and Bezancon in the 1900s (as cited by Morse, 1989). Bezancon *et al* (1900) had fulfilled Kock's postulates by identifying *H. ducreyi* as the microbe responsible for chancroid. He demonstrated this by inoculating the forearms of human volunteers with purified culture of the suspected microbes. The volunteers developed ulcers which were characteristic of chancroid. He was able to reisolate *H. ducreyi* from the resulting lesions and thus fulfilled Kock's postulates (as cited by Lewis<sup>a</sup>, 2000).

## **2.2 Taxonomic Position**

“The genus *Haemophilus* was established with the preliminary report of the Committee of the Society of American Bacteriologists on Characterization of Bacterial Types” (Albritton, 1989).

Bergey's Manual of Systematic Bacteriology places bacteria in the genus *Haemophilus* according to its growth requirements for particular components. A gram-negative rod or coccobacillus would be placed within the genus *Haemophilus* if these microorganisms shared a requirement for either hemin (factor X), nicotinamide adenine dinucleotide (NAD) (factor V) or both. If these factors are not required for the microorganism's growth then the microorganism in question is excluded from the genus (Bergey's Manual of Systematic Bacteriology, 1984).

Bezancon's work in the 1900s has been credited for being the first to culture *H. ducreyi* on blood containing media. His work is said to be of significance because he was able to show that cultures grown on blood agar after serial passage were still able to produce ulcers when reinoculated into humans (as cited by Albritton, 1989). It was this requirement of blood for growth which led the genus *Haemophilus* to be included in the first edition of Bergey's Manual (Morse, 1989).

It has been reported that doubts may be raised as to the proper classification of a bacterium when belonging to the genus *Haemophilus* if its guanine-plus-cytosine (G+C) content lies outside the range of 37 to 44 mol % (midpoint temperature), even if the organism in question possesses typical growth requirements for factors X or V (Morse, 1989). In the case of *H. ducreyi*, all conditions regarding 'G+C content' and 'common growth requirements' seem to be satisfied, as *H. ducreyi* is a gram-negative rod or coccobacillus which has a G+C content of 38 mol % (midpoint temperature) and a growth requirement for hemin (factor X) (Morse, 1989).

The criteria used above which confines the genus *Haemophilus* by its growth factor requirements has since been challenged by DNA homology studies (Morse, 1989; Bergey's Manual of Systematic Bacteriology, 1984). An example of such a study includes findings that *H. ducreyi* have the isoprenoid structure type of demethylmenaquinone and menaquinone which signifies a physiological and chemotaxonomic difference between other *Haemophilus* species and *H. ducreyi*. Therefore this data fails to support *H. ducreyi*'s inclusion into the genus *Haemophilus* (Carlone *et al*, 1988). In addition, it has been documented that the

presence of menaquinones within the Pasteurellaceae family to which *Haemophilus* species belong is also an unusual occurrence (Albritton, 1989).

According to Albritton (1989), in terms of classification, *H. ducreyi* appears to be a monospecies genus, genetically distant but sharing numerous morphological, structural and metabolic characteristics with members of the family Pasteurellaceae (Albritton, 1989). In 2003, the full genome sequencing of the species was performed (Munson *et al*, 2003). This confirmed its distant relationship with other organisms in the genus *Haemophilus*.

### **2.3 Epidemiology**

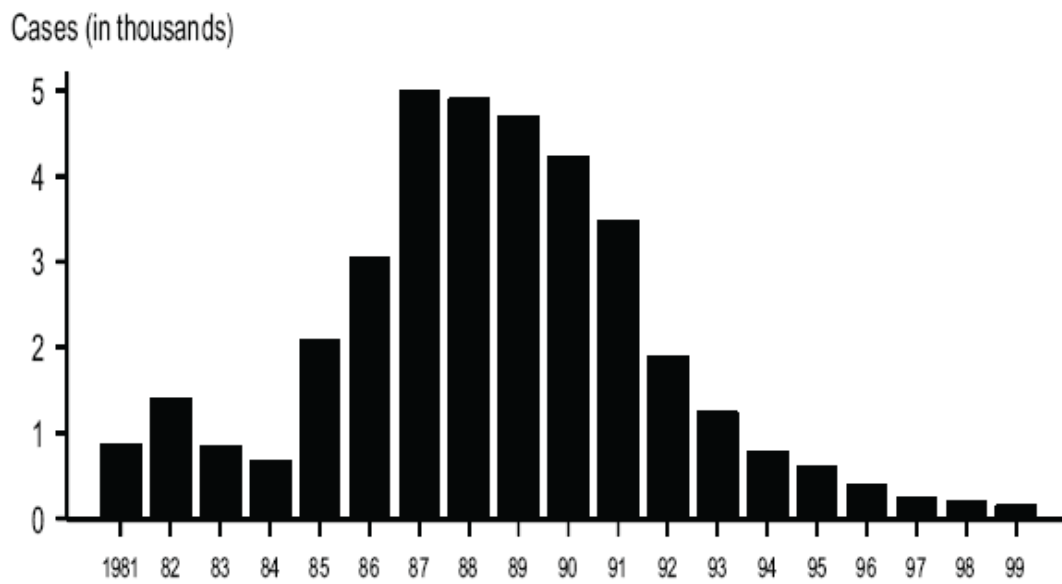
Chancroid (or soft chancre) is a sexually transmitted disease (STD) which was once common in Africa, Asia and Latin America (Trees and Morse, 1995; Lewis<sup>a</sup>, 2000). Although chancroid has been distributed throughout the world, it remains uncommon in the United States (Trees and Morse, 1995; Lewis<sup>a</sup>, 2000) and Western Europe (Lewis<sup>a</sup>, 2000). The true global incidence of chancroid is not known as specific diagnosis is difficult (Morse, 1989) due to the lack of available diagnostic tests, especially in poorer countries where the prevalence of chancroid is much higher (Al-Tawfiq and Spinola, 2002). Disease transmission may be associated with prostitution (Steen, 2001; O'Farrell, 2000; Mohammed and Olumide, 2008), drug abuse (O'Farrell, 2000, Mohammed and Olumide, 2008), alcohol (Mohammed and Olumide, 2008), syphilis (O'Farrell, 2000), an increased risk of HIV infection (O'Farrell, 2000) and poor hygiene; as topical hygiene was shown to be effective in reducing *H. ducreyi* transmission (Steen, 2001). Chancroid is more common in male as compared to female with reported male/female ratios ranging from 3:1 to 25:1 (Morse, 1989).

According to the Centers for Disease Control and Prevention (CDC, Sexually transmitted disease treatment guidelines, 2006), chancroid in the United States (US) usually presents as discrete outbreaks but in some areas it may still be endemic. Approximately 10% of persons who are infected with chancroid in the United States are said to be co infected with HSV or *T. pallidum*. As compared to outside the US, the above percentage was reported to be higher in persons who were infected with chancroid (CDC, Sexually Transmitted Disease Treatment Guidelines, 2006). In the last several years leading up to the early 21<sup>st</sup> century, the reported number of cases within the United Kingdom has not reached epidemic proportions (O'Farrell, 2000).

*H. ducreyi* infectivity occurs within a short time frame and therefore can spread within a population by means of frequent person to person contact as a result of sexual activity. Since *H. ducreyi* is a human pathogen, their survival would depend on an adequate turnover of sex partners (around 15 to 20 sex partners per year) within sub-groups of the population (Steen, 2001).

In the 19<sup>th</sup> and early 20<sup>th</sup> centuries, chancroid which was once a prevalent disease had suddenly taken a different course. It was during the 20<sup>th</sup> century that evidence began to show signs of the disease declining (Steen, 2001). In 1908, the United States (US) army reported that chancroid which was once more prevalent than syphilis, began to decline at a faster rate than syphilis between 1908 and 1930. Furthermore, between 1947 and 1997, chancroid in the US showed a decrease by more than 80-fold (Steen, 2001). This declining rate of chancroid continued and in 1999 (figure 2.1), the US reported a total of 143 cases of chancroid with only sixteen states and one outlying area reporting one or more chancroid cases. Three states

reported nearly 72% of the 143 cases (CDC, Sexually Transmitted Disease Surveillance Report, 1999). In the US, the decline of chancroid is evident with the more recent statistics with reports of only 25 chancroid cases nationwide in 2008 (CDC, Sexually Transmitted Disease Surveillance, 2008).



**Figure 2.1: Chancroid – reported cases in the United States from 1961 to 1999.**

A sentinel study done in South Africa had reported on a significant decline in the number of ulcers attributed to chancroid (Johnson *et al*, 2005). In Durban, data on the aetiology of genital ulcer disease have been collected since 1984. These showed a decline in chancroid prevalence from more than 50 % in 1995 to < 1 % in 2004 (personal communication: Prof A.W. Sturm).

In West Africa, chancroid was found to be less common but disease transmission could still be maintained by importation from other regions (Steen, 2001). A decline in chancroid

prevalence has also been observed in countries such as the Philippines, Senegal and Thailand, an occurrence that may aid in stabilizing the HIV epidemics in these countries (Steen, 2001).

It is likely that several factors may have played a role in wiping out chancroid as an endemic disease in developed western civilization. This scarcity of chancroid began to unfold in Europe and North America even earlier than the discovery of sulfa drugs and penicillin (Steen, 2001). Nevertheless, credit is given to several classes of antibiotics, some of which are administered in single-dose therapy which provides a rapid cure and may as well have contributed to the decline of chancroid infections (Steen, 2001).

Steen (2001), reports that the early decline of chancroid is likely to be accredited to the social changes and varying patterns of commercial sex work (Steen, 2001), which perhaps interrupted the conditions needed to maintain chancroid epidemics.

#### **2.4 Acquisition of *H. ducreyi* infection**

Humans are the natural host for *H. ducreyi* infection where these microorganisms infect genital and nongenital skin, stratified squamous epithelium, mucosal surfaces and regional lymph nodes, resulting in the development of painful ulcerations (Spinola *et al*, 2002; Bong *et al*, 2002). *H. ducreyi* is thought to gain entry into the skin by means of superficial micro abrasions that may occur during sexual intercourse (Spinola *et al*, 2002).

Not much is known about the initial stages of natural occurring infection as patients tend to seek medical treatment at the ulcerative stage of infection (Bauer and Spinola, 2000; Spinola

*et al*, 2002). It is for this reason that experimental infection of humans are carried out to provide information on the initial stages of infection (Bong *et al*, 2002).

Human models of *H. ducreyi* infection have been developed to better understand the pathogenesis of chancroid (Bauer and Spinola, 2000; Bauer *et al* 2001; Bong *et al*, 2002). The human model of *H. ducreyi* infection basically involves the inoculation of human volunteers in the upper arm, namely the dermis and epidermis (Bong *et al*, 2002; Spinola *et al*, 2002) with *H. ducreyi* via puncture wounds which are performed with an allergy-testing device (Bong *et al*, 2002). The human model incorporates characteristics of a low estimated delivered dose (EDD) of *H. ducreyi* and shows the dynamics of papule and pustule formation resembling the early stages of chancroid, accompanied by a cutaneous infiltrate of polymorphonuclear (PMN) and mononuclear cells that imitate the histopathology of natural ulcers (Bauer and Spinola, 2000). To ensure the safety of the volunteers involved, infection is terminated when a volunteer has a painful pustule or ulcer or until 14 days of infection (Bauer and Spinola, 2000; Spinola *et al*, 2002).

Bauer and Spinola (2000) developed an immunodetection assay which was used to localize *H. ducreyi* within biopsy specimens obtained from the human model of *H. ducreyi* infection. The bacteria were not found to be associated with keratinocytes. They found *H. ducreyi* to be associated with neutrophils during the pustular stage of disease (Bauer and Spinola, 2000). Bauer and Spinola (2000) attributed this work as being the first localization study of *H. ducreyi* in chancroid (Bauer and Spinola, 2000). In 2001, Bauer *et al* (2001) reported on the localization of *H. ducreyi* cells in tissue of human volunteers at 0, 24 and 48 hours after inoculation and at the clinical end point. By 48 hours of infection, *H. ducreyi* was reported to

be present in the pustules and dermis. The bacteria had co-localized primarily with macrophages, PMNs, fibrin and collagen. No association was observed with other eukaryotic components. These findings suggest that fibrin and collagen may be important targets of adherence *in vivo* and that the extracellular localization of *H. ducreyi* during the pustular stage of infection could possibly be a means to avoid phagocytic killing, thus ensuring their survival (Bauer *et al*, 2001). To further understand the pathogenesis of *H. ducreyi* and its interactions with tissue components, Bauer *et al* (2006), had extended their work by investigating the *in vivo* localization of *H. ducreyi* at the ulcerative stage of disease and compared the findings with experimental infection. They showed that *H. ducreyi* co-localized with neutrophils and fibrin during the ulcerative stage, and no *H. ducreyi* was found to be associated with keratinocytes, fibroblasts and collagen. This report was the first to localize *H. ducreyi* in chancroid acquired naturally (Bauer *et al*, 2006).



## **2.5 Colony Morphology**

Colonies of *H. ducreyi* on solid medium are 0.5 mm in size at 24 hours and can increase to 1 or 2 mm within 48 to 72 hours (Morse, 1989). According to Sturm and Zanen (1984), colonies have a polymorphic appearance (Sturm and Zanen, 1984). In general, they are small, non mucoid, with a yellowish or grey colour (Albritton, 1989; Morse, 1989), as well as compact and granular (Morse, 1989). They can be translucent, opaque and semiopaque (Morse, 1989). Colony characteristics may vary depending on the type of growth medium and incubation period (Morse, 1989).

A characteristic observation of *H. ducreyi* colonies is that, when pushed across the surface of solid medium with an inoculating loop, colonies should glide easily and remain intact (Sturm and Zanen, 1984; Morse, 1989). This characteristic can be explained by the intercellular adherence of cells which was observed when whole colonies were examined by an electron microscope (Morse, 1989). Within the colony there is substantial loss of cell viability. This makes single-cell colony isolations very difficult (Albritton, 1989).

## **2.6 Clinical Features**

*H. ducreyi* may gain passage into epidermal regions through micro superficial cuts (Morse, 1989) which may occur during sexual intercourse (Lewis<sup>a</sup>, 2000; Lewis, 2003). The incubation period is short and varies between 4 and 7 days (Morse, 1989; Rosen *et al*, 2009). The incubation period for *H. ducreyi* is longer in persons who are HIV infected (Mohammed and Olumide, 2008; Rosen *et al*, 2009).

A chancroid lesion begins as a small tender papule, surrounded by an erythematous zone. The papule rapidly develops into a pustule within 2 or 3 days and eventually erodes to form a painful ulcer with soft (in contrast to hard chancre of primary syphilis) ragged undermined edges (Morse, 1989, Lewis<sup>a</sup>, 2000). The infection may lead to the development of a varying number of ulcers which are often accompanied by suppurative lymphadenopathy (Spinola *et al*, 2002). Without antibiotic therapy, these ulcers may persist for weeks or months (Lewis<sup>a</sup>, 2000), however infection is said to resolve within 3 months (Rosen *et al*, 2009). The base of the ulcer has been described as being irregular with many projections and depressions resulting in a granular appearance (Morse, 1989). The lesion is usually filled with yellow-grey, foul smelling (Wu *et al*, 2004; Rosen *et al*, 2009) necrotic material (Morse, 1989). Chancroid ulcers are very vascular and such bleed easily (Morse, 1989).

Several chancroidal ulcers have been previously described (Morse, 1989), these may include:

1. Giant or serpiginous ulcers (> 2cm) which may develop when several smaller ulcers come together.
2. Follicular type ulcers which develop in a hair follicle (from 1 to > 30) and may resemble a folliculitis or pyogenic infection. These superficial ulcers may be common on the vulva, labia majora, and in hairy regions.
3. The so-called dwarf chancroid (0.1 to 0.5 cm) which may appear rounded and shallow. Such ulcers can be distinguished by their irregular base and sharp hemorrhagic borders.
4. Transient chancroid ulcers which may resolve in 4 to 6 days and proceed to acute regional lymphadenitis with suppuration in 10 to 20 days. These presentations may be difficult to distinguish from lymphogranuloma venereum.

5. Papular chancroid develops as a papule and progresses into an ulcer. The lesions are described as being raised, particularly around the edges and is said to resemble the condylomata latum of secondary syphilis.
6. Phagedenic chancroid is described as widespread necrotic ulceration with damage of genital or perigenital skin (Rosen *et al*, 2009).

In male patients, chancroid lesions are more likely to appear on the distal prepuce, the inner surface of the prepuce on the frenulum (Morse, 1989; Lewis<sup>a</sup>, 2000; Lewis, 2003) or in the coronal sulcus (Morse, 1989). Lesions are less likely seen on the glans, the shaft of the penis, or around the anus (Morse, 1989). As a result of local extension, ulcer development may occur on the perineum, thigh, scrotum, or lower abdomen (Morse, 1989).

In women, most lesions appear around the opening of the vagina, which may include the fourchette, labia, vestibule, and clitoris (Morse, 1989). However, ulcers are also found on the cervix and perianal region (Lewis<sup>a</sup>, 2000). Extra-genital chancroid lesions which are uncommon may be present on the inner thighs, breasts, and fingers (Lewis, 2003; Lewis<sup>a</sup>, 2000).

At least 50% of chancroid cases have shown the occurrence of painful, tender inguinal lymphadenitis and the progression of lymph nodes into buboes (Lewis, 2003). Lymphadenopathy is said to be seen more often in men than in women. If not drained by means of incision then buboes tend to rupture spontaneously (Lewis, 2003).

Existing chancroid ulcers may also lead to autoinoculation of adjacent skin which can result in multiple opposing ulcers, also known as ‘kissing ulcers’ (Mohammed and Olumide, 2008; Rosen *et al*, 2009).

## 2.7 Diagnosis

The diagnosis of chancroid is based on the detection of *Haemophilus ducreyi* in genital ulcerations or bubo aspirates while excluding the possibility of infection by other genital ulcer pathogens (Lewis<sup>a</sup>, 2000). The clinical presentation of genital ulcer infections with either *T. pallidum*, *H. ducreyi* and HSV is not a reliable means of diagnosis since clinical presentation of these infections can be very similar (Lewis<sup>b</sup>, 2000). Diagnosis based on physical examinations show low sensitivity and specificity for chancroid, primary syphilis, and genital herpes (Lewis, 2003). In relation to the primary isolation of *H. ducreyi* from clinical material, Sottnek *et al* (1980) had faced difficulties in distinguishing *H. ducreyi* colonies from other fastidious organisms such as some *Corynebacterium* sp, *Haemophilus parainfluenzae* and *Haemophilus vaginalis*. In this study, six isolates initially thought to be pure *H. ducreyi* culture were later found to be mixed with *H. vaginalis* (Sottnek *et al*, 1980). Studies like these highlight the difficulties faced and the inaccuracy of prevalence figures of chancroid based on diagnosis made by culture and clinical presentation, which was once used by older studies as the ‘gold standards’ (Lewis<sup>b</sup>, 2000). Therefore, these diagnostic modalities have been replaced by molecular techniques.

The advent of AIDS poses new challenges. Not only is the transmission of the human immunodeficiency virus (HIV) facilitated by chancroid (Lewis<sup>a</sup>, 2000; Wu *et al*, 2004) but

concurrent HIV infection could modify the clinical presentation of chancroid (Lewis<sup>b</sup>, 2000).

### **2.7.1 Diagnostic techniques:**

#### **2.7.1.1 Microscopy**

The Gram Stain allows for the observation of microscopic features of *H. ducreyi*. It has been established that the average *H. ducreyi* is a gram negative rod (Morse, 1989) which is 1.2 to 1.5 µm in length and is approximately 0.5 µm in width with rounded ends (Albritton, 1989). Due to the polymicrobial flora found in most clinical smears of genital ulcers the direct examination of such smears by Gram stain microscopy is usually inconclusive (Lewis<sup>b</sup>, 2000). The streptobacillary forms of *H. ducreyi* have been described as “schools of fish”, “railroad tracks”, and “fingerprints” (Albritton, 1989). These morphological criteria can be used as a presumptive microscopic identification for *H. ducreyi* but its sensitivity and specificity has not been shown to be suitable for routine use (Albritton, 1989).

Electron microscopy biopsies were also suggested for diagnosis of chancroid but like the Gram stain, the sensitivity and specificity of the technique is unacceptable for routine use (Albritton, 1989).

#### **2.7.1.2 In vitro Culture**

Since *H. ducreyi* is a fastidious (Morse, 1989) microbe it is important that they maintain their viability after the collection of clinical specimens, until it is inoculated onto appropriate media at a diagnostic laboratory (Trees and Morse, 1995). This became possible with the development of a thioglycolate-hemin-based transport media which was shown to be a viable

method for the recovery of *H. ducreyi* after 4 days from specimens stored at 4°C (Dangor *et al*, 1993).

*In vitro* culture for *H. ducreyi* was once considered the gold standard for evaluating newer techniques of diagnosis (Lewis<sup>b</sup>, 2000). However, culture techniques for diagnosing chancroid have now been abandoned and replaced by DNA amplification techniques. Despite this, such a test is not commercially available (Al-Tawfiq and Spinola, 2002). The sensitivity of *H. ducreyi* culture relative to PCR is reported to be around 75% (Lewis<sup>b</sup>, 2000; Lewis, 2003).

The fastidious nature of *H. ducreyi* makes it a difficult microorganism to isolate from genital ulcer specimens (Trees and Morse, 1995). Therefore obtaining a pure culture of *H. ducreyi* from genital ulcers has proved to be difficult with isolation rates ranging from 0 to 25% (Hammond *et al*, 1978). A number of factors could be responsible for the different isolation rates but it has been pointed out that the type of growth factor supplement used, namely, IsoVitaleX [BBL], Vitox [Oxoid], and CVA [GIBCO] is unlikely to be responsible since the compositions of these supplements are similar. The only difference is that CVA enrichment contains 25.0 g of cysteine HCl per litre, whereas IsoVitaleX and Vitox contain 25.9 g of cysteine HCl per liter (Trees and Morse, 1995). The main factor influencing varying isolation rates is likely the differences in composition of the growth media and differences in nutritional requirements amongst the *H. ducreyi* strains (Trees and Morse, 1995).

The media of choice until 1978 were 30% rabbit blood agar and whole blood (Sturm and Zanen, 1984), and it was in this year that Hammond *et al* (1978) had introduced the use of a selective media which consisted of chocolate agar (CA) with IsoVitaleX for the isolation of

*H. ducreyi*. In this study the addition of 3 µg/ml of vancomycin to CA had resulted in the reduction of contamination with an increased amount of *H. ducreyi* (Hammond *et al*, 1978). However it has been reported that the addition of 3 µg/ml of vancomycin inhibits the growth of some clinical strains of *H. ducreyi* (Lewis<sup>a</sup> 2000; Lewis<sup>b</sup>, 2000). In 1981, Sturm described a modification of Bieling medium which had originally been developed for the isolation of *gonococci*. This medium containing lysed horse blood was successful in obtaining excellent growth with 48 strains of *H. ducreyi* (Sturm and Zanen, 1984).

The development of numerous selective artificial media for the growth of *H. ducreyi* has been summarized previously and reviewed by Morse in 1989 and again by Trees and Morse in 1995 (Morse, 1989; Trees and Morse, 1995). The combination of two media was proposed, namely Mueller-Hinton agar supplemented with 5% chocolate horse blood (MH-HB) and gonococcal agar supplemented with 2% bovine haemoglobin and 5% fetal calf serum (GC-HgS). This combination of media resulted in the isolation of *H. ducreyi* in 90% of chancroidal cases (Lewis<sup>b</sup>, 2000). A suitable cheaper option was also recommended which consisted of a media containing a gonococcal agar base supplemented with 5% Fildes' extract and unchocolated horse blood. This medium may serve as a cheaper option to GC-HgS or MH-HB and was successful in isolating *H. ducreyi* in 75% of chancroidal cases (Lewis<sup>b</sup>, 2000). Another affordable media was also described which consisted of 0.2% activated charcoal as a substitute for fetal calf serum (Lewis<sup>b</sup>, 2000).

Sturm and Zanen (1984) and Schmid *et al* (1995) had both found that most *H. ducreyi* strains grew best at 33°C for the cultivation of fresh *H. ducreyi* isolates and the primary isolation of *H. ducreyi* from ulcers, respectively (Sturm and Zanen, 1984; Schmid *et al*, 1995).

Furthermore, Sturm and Zanen (1984) had reported excellent growth of 29 *H. ducreyi* strains under campylobacter conditions. This was achieved by incubating plates cultured with *H. ducreyi* in a closed anaerobic jar consisting of two CO<sub>2</sub> and H<sub>2</sub> generating sachets with the absence of a catalyst (Sturm and Zanen, 1984).

#### 2.7.1.3 Polymerase chain reaction (PCR)

DNA amplification based techniques appear to offer a more sensitive and specific approach for the diagnosis of chancroid. It is for this reason that several PCR – based techniques have been developed and are used in clinical studies of genital ulcer disease. So far, the development and design of the different PCR assays have used specific primers to amplify sequences from either the *H. ducreyi* 16S ribosomal RNA gene, the rrs (16S) – rrl (23S) ribosomal intergenic spacer region, an anonymous fragment of cloned *H. ducreyi* DNA, or the *groEL* gene which encodes the *H. ducreyi* GroEL heat shock protein (Lewis<sup>b</sup>, 2000).

A number of different PCR assays designed previously were able to detect *H. ducreyi* in genital ulcer specimens with sensitivities constantly ranging from 80% to 100% as compared with culture (Parsons *et al*, 1995). However to an extent, some PCR assays performed on genital ulcer specimens were noted to be less sensitive as compared to samples prepared from *H. ducreyi* culture. The lessened sensitivity obtained from the genital ulcer specimens was attributed to *Taq* polymerase inhibitors present in the DNA preparations of the specimens. Sodium phosphate was said to be responsible for this observation which is present in the specimen transport medium (Lewis<sup>b</sup>, 2000).



Orle *et al* (1996) has developed a Multiplex PCR (M-PCR) assay that simultaneously amplifies DNA targets from *Treponema pallidum*, *Haemophilus ducreyi* and herpes simplex virus (HSV) types 1 and 2 (Orle *et al*, 1996). Orle *et al* (1996) reported that their M-PCR assay was found to be more sensitive than standard diagnostic tests for the simultaneous PCR detection of HSV, *H. ducreyi*, and *T. pallidum* from genital ulcers with sensitivities of 100, 98.4 and 91%, respectively (Orle *et al*, 1996). Their M-PCR has been developed by Roche Molecular Systems (National Guideline for the Management of Chancroid, 2001; Alfa, 2005) but has been used for research purposes only (National Guideline for the Management of Chancroid, 2001).

PCR or nucleic acid amplification is regarded as the gold-standard test for the diagnosis of chancroid (Al-Tawfiq and Spinola, 2002), but is not yet available commercially (Al-Tawfiq and Spinola, 2002; Lewis and Ison, 2006).

The use of PCR for diagnosis not only improved the sensitivity of laboratory diagnosis of chancroid but also made it possible to transport specimens over longer distances (Totten *et al*, 2000). However, one needs to take into account the time frame between collecting the specimen and laboratory result, mainly when the PCR assay is done off-site (Totten *et al*, 2000), as one needs to consider the viability of the specimen when testing is eventually performed.

However even though such DNA amplification based techniques prove to be sensitive, it is still not feasible for resource poor countries to implement these due to the high costs involved.

#### 2.7.1.4 Polyclonal/monoclonal antibodies and antigen detection

The role of immunofluorescence assays as a diagnostic tool for the direct detection of *H. ducreyi* in smears is limited. The reason for this is the low concentration of bacteria in ulcer specimens and the poor specificity of polyclonal anti-sera. The use of more specific monoclonal antibodies could circumvent this problem but these are not readily available (Trees and Morse, 1995).

An indirect immunofluorescence assay was developed for the detection of *H. ducreyi* which utilized antiserum raised in rabbits. In order to eliminate cross-reactivity the antiserum had to be extensively absorbed with *Haemophilus* species. However even after absorption the interpretation of a positive smear was often difficult because a degree of fluorescence was still observed with various *Haemophilus* sp, *Staphylococcus aureus* and *Bacteroides melaninogenicus*. Furthermore it was not possible to assess whether cross-reacting antibodies were present prior to immunization as pre-immunization sera was not obtained (Trees and Morse, 1995).

Finn *et al* (1990) had described the use of an indirect immunofluorescence assay for the detection of *H. ducreyi* which utilized rabbit antiserum and mouse monoclonal antibodies raised against a strain of *Haemophilus ducreyi*. The antiserum and the three monoclonal antibodies produced high immunofluorescence titres with all *H. ducreyi* strains tested. In the case of the rabbit antiserum, cross reaction was only observed with *Bordetella pertussis*. The monoclonal antibodies were shown to react with a single polypeptide band of molecular

weight 29 kDa (kilodalton) which was found to be located in the outer membrane fraction of *H. ducreyi* (Finn *et al*, 1990).

Another development was in the form of an immunofluorescence reagent (IF) where a monoclonal antibody (MAb) was raised against a 29 kDa outer membrane protein (OMP) in *H. ducreyi*. The sensitivity and specificity of the MAb as a IF reagent was evaluated by testing it on simulated *H. ducreyi* vaginal smears, smears from skin lesions of mice infected with *H. ducreyi* and genital ulcer smears from patients clinically diagnosed with chancroid. The IF test was able to identify more than 90% of culture positive cases of chancroid, as well as some of the culture negative cases. This data suggest that the 29 kDa OMP could potentially be a useful means of detecting *H. ducreyi* in clinical specimens (Karim *et al*, 1989).

Trees and Morse (1995) have summarized a number of studies involving MAbs that react with antigens of *H. ducreyi* (Trees and Morse, 1995). Some of these MAbs which have been described previously by other authors appear as promising reagents for the direct detection of *H. ducreyi* in genital ulcer smears (Trees and Morse, 1995).

Patterson *et al* (2002) had developed a rapid diagnostic immuno-chromatography (IC) test based on monoclonal antibodies to the hemoglobin receptor of *H. ducreyi*, HgbA. The sensitivity and specificity of the IC test was evaluated by using strains of *H. ducreyi* of different origin, other *Haemophilus strains*, and bacteria that cause super infection of genital ulcers. They reported specificity of 100% since all *H. ducreyi* strains tested were positive and all other bacteria tested were negative. With their test the least amount of colony forming units (CFU) of *H. ducreyi* detected was  $2 \times 10^6$  CFUs, and the lowest amount of purified HgbA

protein detected was 8.5 ng. They concluded that on a clinical setting this level of sensitivity may not likely be enough to detect *H. ducreyi* in all specimens, but additional work needs to be done in order to improve the sensitivity of the test thus making it a valuable bedside tool for the rapid diagnosis of *H. ducreyi* infection (Patterson *et al*, 2002).

#### 2.7.1.5 Nucleic acid probe technology

DNA-DNA hybridization techniques have been developed for the detection of *H. ducreyi* DNA by the use of labelled *H. ducreyi* derived probes (Lewis<sup>b</sup>, 2000). Parsons *et al* (1989) reported the development of three <sup>32</sup>P labelled DNA probes for *H. ducreyi*. The DNA probes were evaluated on their ability to hybridize with *H. ducreyi* DNA and were able to detect 10<sup>4</sup> CFUs of *H. ducreyi* in mixed and pure cultures. They reported that the DNA probes reacted strongly with 16 strains of *H. ducreyi* and no reactivity was observed with *Treponema pallidum*, *Neisseria gonorrhoea*, or HSV DNA (Parsons *et al*, 1989). However, Trees and Morse (1995) went on to report that DNA probes are not sensitive enough to detect *H. ducreyi* in clinical specimens (Trees and Morse, 1995). The sensitivity for the above test could be greatly increased in clinical specimens due to the possibility of initial amplification of *H. ducreyi* DNA in the specimen by directly growing the bacteria or by DNA amplifying procedures (Lewis<sup>b</sup>, 2000).

Specific rRNA-derived oligonucleotide probes have also been developed by Rossau *et al* (1991). These chemically synthesised probes were complementary to different regions in the 16S and 23S rRNA molecules of *H. ducreyi*. The testing of culture grown isolates with these probes by means of DNA-RNA hybridization techniques were reported to be very specific for

detecting *H. ducreyi*. However no data was given on the sensitivity of the technique (Rossau *et al*, 1991).

#### 2.7.1.6 Serological tests

Techniques such as enzyme immunoassays (EIAs), dot-immunobinding, agglutination, precipitation and complement fixation have been used to identify serological responses in humans and experimental animals acquiring chancroid (Lewis<sup>b</sup>, 2000). EIAs which have been developed either uses ultra sonicated whole-cell *H. ducreyi* antigens, purified *H. ducreyi* lipopoligosaccharide or outer membrane proteins as antigens, all of which have been assessed in the serological diagnosis of chancroid (Lewis<sup>a</sup>, 2000; Lewis<sup>b</sup>, 2000).

However, difficulties have been faced in interpreting results when using EIAs due to the presences of cross-reacting antibodies in serum specimens from control patients. This problem was overcome by adsorbing the serum to remove the cross-reacting antibodies (Elkins *et al*, 2000). An improvement in the specificity of EIA was observed when serum was adsorbed with other *Haemophilus sp* namely, *H. parahaemolyticus*, *H. influenzae*, *H. parainfluenzae* to remove cross-reacting antibodies prior to testing (Lewis<sup>a</sup>, 2000). Using PCR as the gold standard for diagnosing *H. ducreyi* infection with genital ulcer material, Chen *et al* (1997) was able to demonstrate that the adsorption EIA had a sensitivity of 53 % and a specificity of 71% whereas the LOS EIA was less sensitive (48%) but more specific (89%). When follow up sera results were included in the analysis, there was an increase in both the sensitivity (78%) and specificity (84%) of the adsorption EIA. This increased sensitivity and specificity of the adsorption EIA merely suggested that the proportion of patients testing positive for *H. ducreyi*

infection had increased as the infection advanced to ulcerative stage. Therefore the adsorption EIA is hindered as a diagnostic tool because of the lack of sensitivity observed during the first few weeks of infection and seemed to improve as infection progressed (Chen *et al*, 1997).

#### 2.7.1.7 Mass spectrometric methods

Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI/TOF – MS) has been found to be successful in profiling proteins from both intact and disrupted bacteria. Results are obtainable in 10 minutes thus enabling identification of bacteria (Lewis<sup>b</sup>, 2000).

## 2.8 Treatment

As far back as the 1970s, documentation shows that *H. ducreyi* acquires both plasmid and chromosomally mediated mechanisms of antimicrobial resistances which posed as a problem world-wide. Even though not much is known about chromosomally mediated antimicrobial resistance, reduced susceptibility has been documented for trimethoprim, penicillin, ciprofloxacin and ofloxacin in non plasmid – containing *H. ducreyi* strains (Lewis<sup>a</sup>, 2000). Most accessible information concerns plasmid-mediated resistance. Plasmids present in *H. ducreyi* strains encode for resistance to more than one class of antimicrobial agent. Furthermore, more than one resistance – encoding plasmid may be present in the same *H. ducreyi* strain (Lewis<sup>a</sup>, 2000). *H. ducreyi* strains have shown to possess plasmid-mediated resistance to tetracycline, chloramphenicol, sulphonamides, aminoglycosides, and  $\beta$ -lactam antimicrobial agents (Lewis<sup>a</sup>, 2000).

Treatment recommended by both the World Health Organisation (WHO, Guidelines for the Management of STIs, 2003) and the Centres for Disease Control and Prevention (CDC, Sexually Transmitted Disease Treatment Guidelines, 2006) are outlined in table 2.1. According to the CDC, ceftriaxone and azithromycin is an advantageous option in that it offers single dose therapy (CDC, Sexually Transmitted Disease Treatment Guidelines, 2006). This option avoids problems with compliance (Trees and Morse, 1995). Azithromycin (1g orally, once), ceftriaxone (250mg intramuscularly, once), or erythromycin (500mg orally, four times a day for 7 days) was reported to be successful treatment strategies for chancroid in the United States (Schmid, 1999).

**Table 2.1:** Recommended Regimens for the Treatment of Chancroid by the WHO and CDC

<b>Anti-Microbial Agent</b>	<b>World Health Organisation (WHO)</b>	<b>Centre for Disease Control and Prevention (CDC)</b>
<b>Azithromycin</b>	1 g orally, single dose	1 g orally, single done
<b>Ceftriaxone</b>	250 mg by intra-muscularly (IM) in a single dose	250 mg intra-muscularly (IM) in a single dose
<b>Ciprofloxacin*</b>	500 mg orally, twice daily for 3 days	500 mg orally, twice daily for 3 days
<b>Erythromycin Base</b>	500 mg orally, 4 times daily for 7 days	500 mg orally, 3 times daily for 7 days

\* Ciprofloxacin is contraindicated for pregnant and lactating women as stipulated by the CDC, Sexually Transmitted Disease Treatment Guidelines, 2006.

Uncircumcised or HIV positive patients may not respond well to treatment as compared to those patients who are circumcised or HIV-negative (CDC, Sexually Transmitted Disease Treatment Guidelines, 2006). Two studies reported on differences between circumcised and uncircumcised men. In one study the cure rate in the uncircumcised men was 82% compared with 93% in circumcised men; the second study showed cure rates of 59% and 94%, respectively (Schmid, 1999). Since a positive HIV status and a lack of circumcision has been associated with a less favourable response to therapy, it is recommended that for management purposes, all patients should be tested for HIV and if found to be HIV positive then treatment



in these patients should be followed more closely (Schmid, 1999; CDC, Sexually Transmitted Disease Treatment Guidelines, 2006). Longer treatment regime maybe required (Steen, 2001). The same should apply for uncircumcised men, where a close follow-up during treatment is also recommended if diagnosed with chancroid (Schmid, 1999).

According to the 2006 treatment guidelines of the CDC, at the time when chancroid is diagnosed, patients should be tested for HIV infection and then retested three months later for syphilis and HIV infection if these test results were initially negative. A follow-up examination is recommended where patients will be re-examined 3-7 days after initiation of therapy. If treatment is found to be successful within this time period after therapy, then ulcers would usually improve symptomatically. However if clinical improvement is unsatisfactory, the attending clinician needs to consider the following: 1) is the diagnosis correct, 2) is the patient co-infected with another STD, 3) is the patient infected with HIV, 4) is treatment used correctly, or 5) is the *H. ducreyi* strain which is responsible for infection resistant to the antimicrobial prescribed (CDC, Sexually Transmitted Disease Treatment Guidelines, 2006).

In regards to the treatment of buboes as a result of *H. ducreyi* infection, it is said that the drainage or the aspiration of fluctuant buboes results in symptomatic relief and may also prevent subsequent rupture (Lewis<sup>a</sup>, 2000; Lewis, 2003). A study done in New Orleans has addressed the issue concerning the re-accumulation of pus in the bubo site which may have required re-aspiration. They recommend the incision and drainage of buboes with subsequent packing as an effective method for treatment, thus avoiding re-aspiration (Ernst *et al*, 1995). This might not be an appropriate approach in developing countries where follow-up is usually inadequate (Ernst *et al*, 1995).

## 2.9 Chancroid and HIV transmission

Apart from the fact that chancroid is a painful genital ulcer disease (GUD), it is also an independent risk factor for the heterosexual transmission of the human immunodeficiency virus (HIV), making it an important public health problem (Elkins *et al*, 2000). *H. ducreyi* infections may influence HIV transmission by one of the following mechanisms, (Spinola *et al*, 2002):

1. The provision of an available portal of viral entry;
2. Viral shedding from the ulcer;
3. Increased viral load in blood and semen, and;
4. Recruitment of CD4 carrying cells into the skin.

In addition, the primary HIV-1 target cells, CD4 T cells have been isolated from within chancroid ulcers (Elkins *et al*, 2000). It is likely that the break in epithelial lining and the accumulation of CD4 carrying cells result in increased HIV transmission (Lewis<sup>a</sup>, 2000). Furthermore, mathematical models propose that the transmission of HIV and its relatedness to GUD has played an important part in amplifying the HIV epidemic in Sub-Saharan Africa (Spinola *et al*, 2002). Data from Kenya and Thailand suggest an increased risk of HIV transmission as much as 50-300 times per unprotected sex act as a result of genital ulcers. Therefore in countries where GUD is common, a large number of new HIV infections can be expected. Strong associations have also been reported between GUD and HIV seropositivity with odds and risk ratios being higher as compared to non ulcerative STDs (Steen, 2001).

Even though the incidence and prevalence of *H. ducreyi* infection differ by country and region, a close geographical association has been reported to exist between HIV infection and chancroid, which has been observed in African and Asian countries (Steen, 2001).

## **2.10 Heat shock proteins**

A group of proteins called Heat Shock Proteins (HSPs) are synthesized by all organisms in response to heat. In addition, HSPs are also induced by a variety of other stresses which may include heat, anoxia and exposure to ethanol and certain heavy metal ions. Such group of proteins are highly conserved, existing in both prokaryotic and eukaryotic organisms, and even though certain characteristics of the response may differ among organisms, many are said to be nearly universal or universal (Lindquist and Craig, 1988).

In addition, HSPs also exist in all organisms at normal temperature and are said to play essential roles in the normal functioning of the cell. It is assumed that the heat-shock response and its induction of HSPs synthesis may protect cells from the toxic effects of heat and other stresses that may have induced its production. The induction of the HSP response differs in each organism and occurs at very different temperatures; the organism in question is therefore expected to deal with such temperatures in their natural environment (Lindquist and Craig, 1988). Apart from their role in thermotolerance, HSPs have also been implicated in immunodominance and autoimmunity (Parsons *et al*, 1992). Antibodies and activated T cells have been shown to react with major HSPs of organisms like *Mycobacterium leprae*, *M. tuberculosis*, *Coxiella burnetti*, *Plasmodium falciparum*, *Schistosoma mansoni*, *Brugia malayii*, *Trypanosoma cruzi* and *Leishmania* (Lindquist and Craig, 1988).

Although not generally associated with the surface of the cell, many HSPs occur as immunodominant antigens. This strong immunogenicity can perhaps be explained by the abundance of these proteins allowing these to be processed and presented to lymphocytes following phagocytosis by antigen presenting cells (Lindquist and Craig, 1988). In addition, HSPs might be further induced due to the hostile environment in the macrophages in particular if the organism is a macrophage pathogen (Lindquist and Craig, 1988).

Two genes, *groEL* and *groES* described previously, consist of an operon under heat shock control positioned at 93.5 minutes on the *Escherichia coli* chromosome. These genes encode for the abundantly produced proteins, *groEL* a 65 kd Mr protein and *groES* a 15 kd Mr protein, which is said to be vital for cell growth at all temperatures (Lindquist and Craig, 1988). In addition, GroES and GroEL HSPs are essential for *E. coli* to maintain its cell viability at all temperatures (Parsons *et al*, 1992). GroE HSPs increase in abundance when cells are stressed and protect essential proteins from denaturation (Parsons *et al*, 1992).

Parsons *et al* (1992) had identified two adjacent open reading frames (ORFs) in which *H. ducreyi* encodes for a GroES HSP and GroEL HSP, of 10.3 kDa and 57.8 kDa, respectively. The *H. ducreyi* HSPs genes were found to show homology with the *groE* genes, and thus with the GroES and GroEL heat shock proteins from a number of other bacterial pathogens, as well as the conserved eukaryotic 60 kDa HSP (Parsons *et al*, 1992). Parsons *et al* (1992) had noticed high levels of *groE* mRNA from exponentially growing *H. ducreyi* in hemin broth when grown optimally at 33°C. Furthermore a heat-inducible, immunoreactive protein of about 60 kDa (GroEL) was found to be the pre-dominant protein produced by the exponentially growing *H. ducreyi* (Parsons *et al*, 1992).

Brown *et al* (1993) had reported on an increased synthesis of a 58.5 kDa HSP of *H. ducreyi* when grown between 30°C and 33°C, which had also appeared to be the dominant protein in the cell lysate. Due to its molecular weight and its heat shock inducibility, Brown *et al* (1993) suggested that it was the GroEL homologue and thus a member of the HSP60 family (Brown *et al*, 1993).

Induced heat shock with an increase from 33°C (optimal growth temperature) to 37°C had improved the thermostability of *H. ducreyi*, making stressed cells capable of withstanding the effects of rapid chilling on ice. Such an effect is attributed to the expression of other heat shock proteins which are necessary to maintain thermostability when stressed *H. ducreyi* cells were subjected to heat shock. In contrast, the expression of the relatively high levels of the *groE* operon seen in exponentially growing *H. ducreyi* prior to heat shock was insufficient to protect the cells from death by rapid chilling (Parsons *et al*, 1992).

By investigating the response to heat shock in *H. ducreyi*, Brown *et al* (1993) had identified four HSPs of molecular weights, <14, 58.5, 74 and 78 kDa. They achieved this by labelling newly synthesized proteins of *H. ducreyi* with a pulse of <sup>35</sup>S-methionine and measured its production at temperatures ranging between 30°C – 40°C. By means of Western blot, the protein profiles were also probed with serum from an immunized rabbit, serum from a rabbit infected intradermally with *H. ducreyi*, mouse monoclonal antibodies (MAb) and a pool of human sera from patients with chancroid. The sera from the rabbits, the pooled human serum and the MAb, BB11 had reacted with epitopes on the 58.5 kDa HSP. The serum of the immunized rabbit and another MAb, CC11 had reacted with epitopes on the 78 kDa HSP. Their findings had showed that the 58.5 kDa HSP was immunogenic in the human and rabbit

model, and therefore plays a role in the immune response and the pathogenicity of *H. ducreyi* (Brown *et al*, 1993).

Frisk *et al* (1998) had demonstrated that the GroEL homolog of *H. ducreyi*, the 58.5 kDa HSP is not only produced in response to heat but also under *in vitro* growth conditions. The GroEL HSP is normally considered to be an intracellular located protein; however it was shown that in *H. ducreyi*, the 58.5 kDa HSP is also present on the cell surface (Frisk *et al*, 1998). Therefore by demonstrating the attachment of the 58.5 kDa HSP to eukaryotic cells, it implies the possibility of the HSP being involved in the attachment of bacteria to host cells (Frisk *et al*, 1998). In addition, Pantzar *et al* (2006) had demonstrated the attachment of the 58.5 kDa GroEL HSP of *H. ducreyi* to several glycosphingolipids and this was reported to signify a potential bacterial adhesion mechanism (Pantzar *et al*, 2006).

### **2.11 Control strategies for chancroid**

The diagnosis of chancroid is difficult and unreliable when based on culture (Sottnek *et al*, 1980; Steen, 2001) and clinical presentation (Lewis<sup>b</sup>, 2000, Steen, 2001). Furthermore these developing and resource poor countries may lack the laboratories and experienced medical staff for the etiological diagnosis of STIs. It is for these reasons that emphasis has been placed on the development of ‘Syndromic Management’ of STIs by the World Health Organisation. This strategy contributes to the effective control and possible eradication of STIs. “The syndromic management approach is based on the identification of consistent groups of symptoms and easily recognized signs (syndromes), and the provision of treatment that will

deal with the majority of, or the most serious, organisms responsible for producing a syndrome” (WHO, Guidelines for the Management of STIs, 2003).

South Africa’s Department of Health developed its own guidelines (figure 2.2) for syndromic management of STIs in 1995 and has adjusted these guidelines regularly based on changing prevalence in the aetiology of the syndromes and susceptibility patterns of the organisms (Data provided by Professor A.W. Sturm, Department of Medical Microbiology, Nelson R. Mandela School of Medicine, Durban, South Africa).

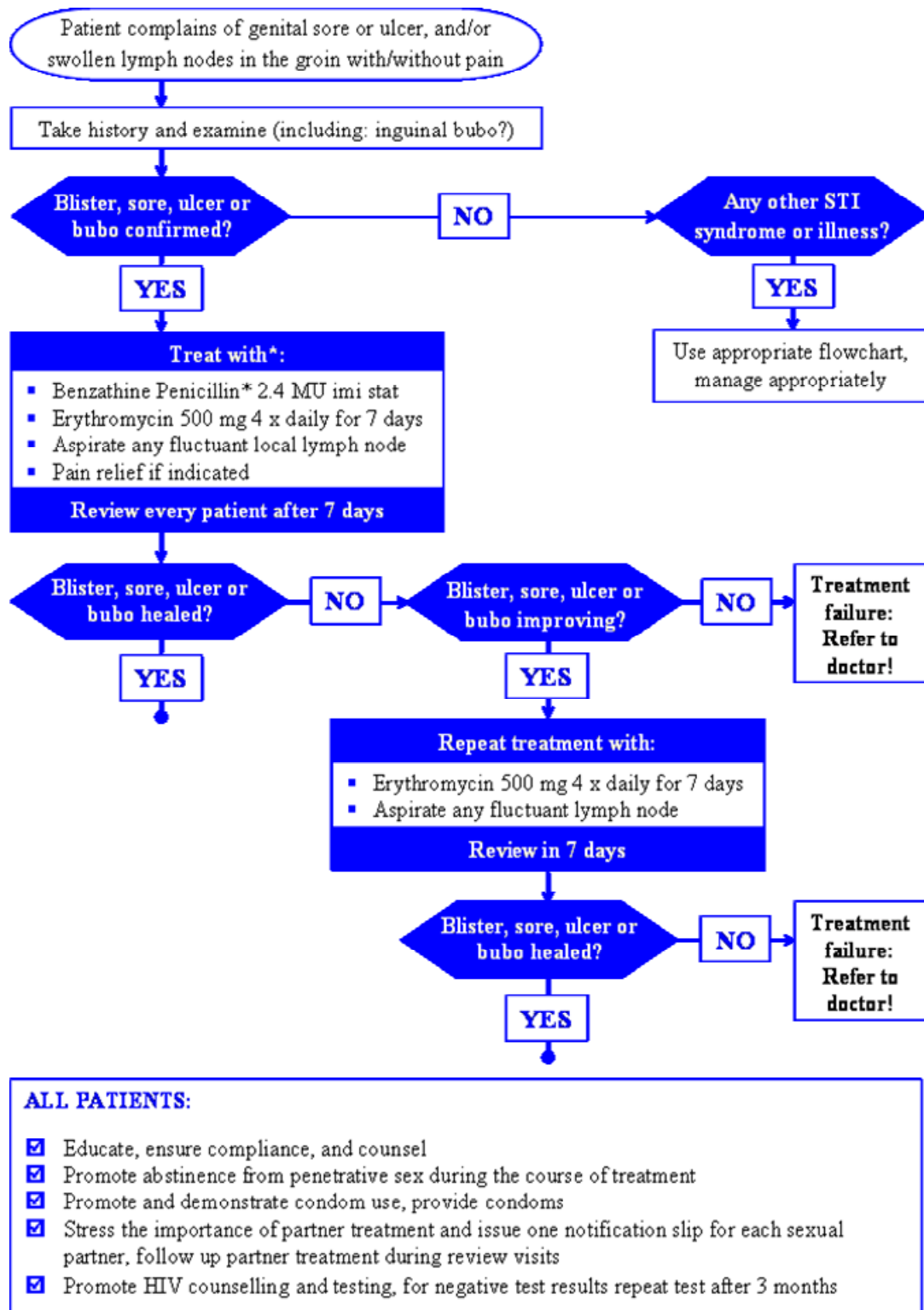


Figure 2.2: South African guidelines for Syndromic Management of genital ulcers



Hawkes *et al* (1995), had report on a small group of women (2%) in Gambia, namely commercial sex workers who had shown positive results when tested for chancroid by means of PCR, however these women possessed no signs or symptoms of the disease. These findings have important implications on syndromic management and control programmes for STDs as these infected asymptomatic women are likely to spread infection to their partners (Hawkes *et al*, 1995). It is for this reason that “persons who have had sexual contact with a patient who has chancroid within the 10 days before onset of the patient’s symptoms should be examined and treated even in the absence of symptoms” (UK National Guideline for the Management of Chancroid, 2007). However, testing or treating asymptomatic individuals for chancroid is not generally recommended (Lewis and Ison, 2006).

Bosu (1999) had outlined the advantages and disadvantages (table 2.2) concerning the approach to syndromic management. He concluded that even though “the approach is both rational and scientific” (Bosu, 1999), and that the advantages may seem to overshadow its disadvantages, focus should lie on each specific setting adopting the approach as one needs to take into account the differences in STD epidemiology, operational issues, acceptability and cost (Bosu, 1999).

**Table 2.2:** Advantages and disadvantages of Syndromic case management (Bosu, 1999)

<b>Criteria</b>	<b>Advantages</b>	<b>Disadvantages</b>
<b>Algorithm</b>	Simple Problem-orientated; based on presenting symptoms	Low sensitivity and specificity for cervical gonococcal and chlamydial infections in women. Risk scores are not highly sensitive or specific.
<b>Initiation of therapy</b>	Rapid. Patients are treated at first visit without need for laboratory confirmation.	Not easily accepted by doctors.
<b>Treatment outcome</b>	High rates of cure	Requires surveillance of antibiotics susceptibility profiles. Some algorithms do not provide for 2 <sup>nd</sup> or 3 <sup>rd</sup> line treatment.
<b>Cost</b>	Saves money on laboratory tests. Cost-effective.	Leads to over treatment which may be costly
<b>Coverage</b>	Can achieve high coverage of population	Does not detect asymptomatic cases
<b>Implementation</b>	Can be easily integrated into the PHC system. Can rely on non medical staff to manage cases.	Needs to be adapted to specific settings
<b>Management support systems</b>	Simplifies reporting, supervision and ordering of drugs. Facilitates data collection for surveillance and planning.	May have to set up a parallel information system in order to monitor trends in traditional aetiological STDs.

The CDC contributes to the management of STDs by calling upon STD/HIV prevention counselling which relies on the “effective delivery of prevention messages which requires that providers integrate communication of general risk reduction messages that are relevant to the client (i.e., client-centered counseling)” (CDC, Sexually Transmitted Disease Treatment Guidelines, 2006). These counselling sessions aim to educate clients about reducing the risks of STD/HIV infection and include topics such as abstinence, condom use, having fewer sex partners, changing sexual behaviours and vaccination (CDC, Sexually Transmitted Disease Treatment Guidelines, 2006).

According to Steen (2001), chancroid is a suitable STI for eradication because it meets three important criteria:

1. The infection can be identified, both syndromically and aetiologically. Treatment is rapid and accuracy of disease surveillance is improved;
2. Chancroid is a curable infection due the availability of a number of effective single dose antimicrobial treatments, one which is known to provide broad post treatment prophylaxis, and finally;
3. *H. ducreyi* does not have a non-human reservoir and can only be sustainable within sub-groups of the population as a result of sexual networks and therefore somewhat restricted.

Therefore an effort to eliminate and eradicate chancroid from endemic areas remains a possible one (Steen, 2001).

Important control measures such as condom distribution, education on disease transmission, preventative behaviour or measures, the availability of special clinical services which allows for regular examinations and treatment for high risk individuals has been found to be effective in controlling chancroid infections in countries such as Kenya, South Africa and Thailand (Steen, 2001).

## **Chapter Three: Material and Methods**

### **3. Ethical Approval**

Ethics approval was obtained from the Animal Ethics Sub-Committee of the University Ethics Committee, University of KwaZulu-Natal. Reference: 021/07/Animal.

### **3.1 Bacterial Isolates**

*Haemophilus ducreyi* strains A54 and A68 were obtained from the Department of Medical Microbiology at the Nelson R. Mandela School of Medicine, University of KwaZulu Natal. These strains were clinical isolates collected during projects run by the Department of Medical Microbiology.

### **3.2 Growth and harvesting of *H. ducreyi***

Bacteria were revived from stock samples stored in cryovials at - 80°C. Bacteria was grown on Modified Bieling (MB) agar plates (appendix 1) as well as in MB broth (appendix 1), and incubated at 33°C for 48 hours under microaerophilic conditions. Gram stained smears (appendix 2) were done on culture obtained from MB agar plates and MB broth to confirm culture purity.

Initially, bacteria was cultured on solid medium (10 MB agar plates) which was then scraped off after 48 hours and grown in 30 ml of MB broth for a further 48 hours to increase the biomass. Inoculated broth was then centrifuged for 20 minutes at 3000 x g. The supernatant

was discarded and the bacterial pellet was washed two to three times with 2 ml phosphate buffered saline (PBS) (appendix 6) until pellet was clearly visible.

### **3.3 Lysis of bacterial cells**

The bacteria were sonicated with a Branson Sonifier 250, for 5 minutes at a duty cycle of 50 and a control output of 6. During the sonication process, the tube containing the bacteria was placed in ice to prevent heating of bacterial contents.

The sonicated sample was centrifuged for 15 minutes at approximately 4000 x g. The supernatant was filter sterilized through 0.2 micron filters (Millipore, USA) and the pellet was discarded. The resulting bacteria free filtrate was screened on SDS PAGE gels (appendix 3 and 4) and the remainder was aliquoted into eppendorf tubes and stored at - 20°C until further use.

### **3.4 Separation of the proteins and estimation of molecular weight**

Separation of the proteins and estimation of its molecular weight was performed with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) (appendix 3 and 4), according to Bio-Rad's instruction manual. Large gel slabs were prepared with one large well and one small well, to which 400 µl of prepared bacteria free filtrate and protein Molecular Weight (MW) standard (Fermentas, USA) were loaded, respectively. Prior to loading the large well, 300 µl of bacteria free filtrate was mixed with 100 µl of sample reducing buffer (appendix 3). The final 400 µl of prepared bacteria free filtrate sample was transferred into the large well in the sodium dodecyl sulphate polyacrylamide gel. The small well received 10 µl

of the protein standard. The loaded gel was allowed to run at a voltage of 100 volts and a current of 50 amps for approximately 6 hours.

### **3.5 Localisation of 58.5 kDa heat shock protein**

A method described previously was used (Nurhadi *et al*, 2003). After completion of the gel electrophoresis (3.4), two longitudinal strips, including the portion of gel used for the protein standard were cut from the sides of the gel and stained with Coomassie Blue solution (appendix 3) until protein bands were visible. The remainder of the gel was left to rest on a glass plate in the refrigerator. After the two strips of the gel were stained, the excess of Coomassie Blue dye was removed by destaining the gel strips with destaining solution (appendix 3). The two destained strips were then lined up along the edges of the unstained gel (from refrigerator) and used as a guide to locate the band of interest in the 58.5 kDa region in the unstained gel. This presumed 58.5 kDa band was cut out with a sterile blade and the pieces of gel were stored in sterile eppendorf tubes in a refrigerator until further use.

### **3.6 Protein elution from gel matrix**

The elution process was adopted from Nurhadi *et al* (2003) with some modification (Nurhadi *et al*, 2003). The pieces of gel containing the band of interest were minced with a sterile blade. The minced gel pieces were washed three times with 1 ml of sterile distilled water. A maximum of 500 µl of protein elution buffer (appendix 5) was then added. The suspension was vortexed and allowed to stand overnight at room temperature. The next day, the mixture was centrifuged at 21 000 x g for 10 minutes, after which the supernatant was collected into a clean eppendorf tube. A further 250 µl (maximum) of the protein elution buffer was added to

the gel pieces, vortexed and centrifuged. The resulting supernatant was combined with the first 500 µl of eluted protein. An aliquot of the supernatant was then tested for the presence of the 58.5 kDa protein by means of SDS-PAGE.

### **3.7 Purification of proteins**

Proteins were purified by means of a protein purification kit, the ProteoSpin detergent clean-up kit (Norgen Biotek Corp., Canada) and protein was quantified by the Qubit™ Quantitation Platform (Invitrogen Corp., USA), according to the manufacturer's instructions. Purified proteins were aliquoted into 100 µl volumes in sterile tubes and stored at - 20°C until further use.

### **3.8 Production of polyclonal antibody**

Antibodies to the *H. ducreyi* 58.5 kDa GroEL heat shock protein (antigen) were raised in rabbits at the Biomedical Resource Unit, Westville campus, University of KwaZulu Natal. Three New Zealand white rabbits were used for this procedure.

#### **3.8.1 Bleeding of rabbit**

The rabbit was placed in a restrainer. The ears were shaved using a soapy liquid and surgical blade. The ear was rinsed, dried and disinfected with ethanol (or other antiseptic). A 20G needle was inserted into the ear vein and blood was collected in a 5 ml blood tube. The needle was removed and pressure was applied using cotton wool to prevent further bleeding. The collected blood was allowed to clot and the serum collected. The baseline antibody level against the 58.5 kDa *H. ducreyi* protein was measured using an ELISA.



### 3.8.2 Rabbit immunisation

On day 0 a 1 ml purified antigen (50 µg/ml protein) mixed with equal volume of Freund's Complete Adjuvant (FCA) was prepared using 2 interconnecting glass syringes. Of this, 0.5 ml was injected intra-muscularly into each hind leg. The rabbits received Booster injections of 0.25 ml (50 µg/ml) antigen solution without FCA in each hind leg on days 10 and 20. On day 28, 5 ml of blood was collected as described before (3.8.1) to test for immune response.

When a good immune response was achieved, the rabbit was sedated using a combination of xylazine (0.6 ml) and ketamine (0.6 ml) administered intra-muscularly, and was bled by cardio-puncture. The blood was collected in 5 ml blood tubes and kept at room temperature until a clot was formed. Serum was collected after centrifugation, aliquoted and stored at -20°C. The rabbit was eventually terminated by intravenous sodium pentobarbitone injection. The carcass was incinerated at the Biomedical Resource Unit.

## **3.9 Purification of IgG**

Polyclonal antibodies against the 58.5 kDa heat shock protein were purified by means of ProSep A chromatography media (Millipore, USA).

### 3.9.1 Column preparation

A 10 ml pre-packed ProSep-A column was used to purify the antibody. The ProSep-A media within the column was poured out into a 250 ml glass beaker containing 50 ml phosphate buffered saline (PBS), pH 7.4 (appendix 6). The empty column was then half filled with PBS, pH 7.4. The ProSep-A/PBS mixture was then poured back into the 10 ml column with the aid

of a glass rod. As this was done the column was continuously vibrated by hand to ensure the proper settling of ProSep-A media within the column. Used columns were regenerated with phosphoric acid, pH 1.5 and flushed with PBS, pH 7.4, for the next use.

### 3.9.2 Purification of the IgG fraction

Serum was prepared as described previously (Harkins, 2001) with slight modifications. Lipids and other cellular debris were removed from the serum by means of centrifugation at 4°C for 20 000 x g for 30 minutes. The supernatant was then dialysed overnight against PBS, pH 7.4 (appendix 6). The dialysate was filtered through a 0.45 µm filter (Millipore, USA) before loading onto the prepared ProSep-A column (3.9.1).

The column was flushed with PBS, pH 7.4, and the fluid was collected in 1 ml aliquots. Absorbance values of these aliquots were obtained by means of spectrophotometry. When an absorbance of 0.05 was reached, the PBS was replaced by IgG elution buffer, pH 3 (appendix 5). When an absorbance value of just greater than 3 was reached, the IgG fraction was collected in 1 ml aliquots and dialysed against PBS, pH 7.4, overnight.

### 3.9.3 Process of dialysis for purified IgG

A piece of dialysis tubing (Sigma-Aldrich, USA) compatible with the volume of fluid to be dialysed was closed at one end with a piece of string. The tube was filled with the IgG fraction obtained from column chromatography (3.9.2) and closed. The tube was then suspended into a clean glass beaker containing PBS, pH 7.4 (appendix 6). Dialysis was allowed to occur at 4°C, overnight. The PBS was replaced 3 times. The following day, the IgG was removed from the

dialysis tube and its purity was checked on a SDS PAGE gel. The purified IgG fraction was aliquoted into 100 µl volumes. These were stored at - 20°C until further use.

### **3.10 Enzyme linked-immunosorbent assay – ELISA**

Enzyme linked immunosorbent assays (ELISAs) combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to enzyme where activity can be measured quantitatively. ELISAs are therefore used to measure antigen or antibody concentrations.

#### **3.10.1 Determination of optimal coating concentration of antigen**

Sterile 96 well microtitre plates were used. To determine the optimal concentration of the 58.5 kDa HSP for use as coating antigen, different concentrations were tested. Eight serial 2-fold dilutions of the purified antigen were made using coating buffer (appendix 6). The dilutions for each concentration was done in 2 ml eppendorf tubes and then transferred into the appropriate wells of the microtitre plate. The resulting concentrations were 9 µg/ml, 4.5 µg/ml, 2.25 µg/ml, 1.12 µg/ml, 0.56 µg/ml, 0.28 µg/ml, 0.14 µg/ml, and 0.07 µg/ml.

One hundred microlitres of antigen with the above respective concentrations were added to the wells. The microtitre plate was then covered and placed in a sealed humidified container overnight at room temperature. The following day, the plate was washed three times with 200 µl of wash buffer (appendix 6) per well. The fluid was removed by flicking the plate over a sink. The remaining drops were removed by patting the plate on a paper towel. The remaining protein binding sites in the coated wells were then blocked by adding 100 µl of blocking

buffer (appendix 6). The plate was then covered with a lid and incubated in a sealed humidified container for 1 hour at room temperature. The excess blocking buffer was washed off with wash buffer and the plate was blotted dry. Fifty microlitre of serum from an immunized rabbit was added to each well and the plate was incubated for 1 hour at room temperature. The excess serum was removed by washing three times with wash buffer and the plate was then blotted dry. Thereafter 100 µl of detection antibody, goat-anti-rabbit-IgG-horseradish peroxidase (Sigma-Aldrich, USA) (1:10 000 with 3% skimmed milk powder in phosphate buffered saline and 0.01% tween 20 (PBST)) (appendix 6) was added to each well. This was followed by incubation in a sealed humidified container for 1 hour at room temperature. The excess detection antibody was washed off with wash buffer and the plate was blotted dry. To each well, 200 µl of chromogenic substrate (o-phenylenediamine dihydrochloride) (Sigma-Aldrich, USA) was added and colour was allowed to develop at room temperature. The reaction was stopped by adding 50 µl of 3 M HCl to each well. Colour intensity was measured by an ELISA plate reader at 492 nm. This procedure was carried out for each rabbit in triplicate.

### 3.10.2 Construction of a standard curve

To determine the optimal antigen concentration, the average of the 3 ELISA absorbance readings (3.10.1) per dilution was used to construct a standard curve for each rabbit. The average absorbance reading was plotted on the standard curve and the optimal concentration of antigen was then extrapolated from the curve.

### **3.11 Evaluation of immune response by means of ELISA**

To coat the wells of a 96 well microtitre plate, an antigen solution containing 5 µg/ml of antigen was made up with coating buffer (appendix 6). One hundred microlitres of the antigen solution was added to each well and the plate was covered with a lid. This was followed by overnight incubation in a sealed humidified container at room temperature. The next day, the remaining antigen solution was discarded and the wells were washed 3 times with 200 µl of wash buffer (appendix 6). The plate was then blotted dry. The remaining protein-binding sites were blocked by adding 100 µl of blocking buffer (appendix 6) per well. The lid was replaced on the plate which was then left to incubate in a sealed humidified container for 1 hour at room temperature. Unbound protein was removed by washing the plate three times with wash buffer. Rabbit serum was serially diluted two-fold from a 1:5 starting dilution which was made up in 2 ml eppendorf tubes. Fifty microlitres of each serum dilution was transferred from the eppendorf tubes to the respective wells. The plate was then covered with a lid and incubated in a sealed humidified container for 1 hour at room temperature. The excess serum was removed by washing (3x) and blotting dry. Thereafter 100 µl of detection antibody, goat-anti-rabbit-IgG-horseradish peroxidase (Sigma-Aldrich, USA) (1:10 000 with 3% skimmed milk powder in phosphate buffered saline and 0.01% tween 20 (PBST)) (appendix 6) was added to each well. The plate was then covered with a lid and incubated in a sealed humidified container for 1 hour at room temperature. The excess detection antibody was washed off (3x) with wash buffer and plates were blotted dry. To each well, 200 µl of chromogenic substrate (o-phenylenediamine dihydrochloride) (Sigma-Aldrich, USA) was added and colour was allowed to develop at room temperature. The reaction was stopped by adding 50 µl of 3 M HCl to each well. Colour intensity was measured by an ELISA plate reader at 492 nm.

This ELISA procedure was carried out with serum of each of the three rabbits in triplicate. Pre-immunisation serum from each rabbit was used as a negative control. For each rabbit, positive/negative (P/N) ratios were calculated for each dilution series by taking the average of the three absorbance readings obtained with post-immunisation serum and dividing this by the average of the three absorbance readings obtained with pre-immunisation serum. The P/N ratios were used to choose the rabbit with the optimal immune response. IgG from the serum of the rabbit with the optimal response was purified and conjugated to horseradish peroxidase following a protocol (appendix 7) provided by Dr. M. Bubb, Director of National Institute of Bioproducts, Pinetown, South Africa.

### **3.12 Determination of optimal concentration of antibodies**

To determine the optimal concentration of capture antibody, a checkerboard titration was performed in a 96 well microtitre plate. Five rows of wells were coated with 100  $\mu$ l capture antibody (purified) solution in coating buffer (appendix 6) with the following concentrations: 100  $\mu$ g/ml, 80  $\mu$ g/ml, 60  $\mu$ g/ml, 40  $\mu$ g/ml, 20  $\mu$ g/ml, 10  $\mu$ g/ml, 5  $\mu$ g/ml and 1  $\mu$ g/ml. The plate was covered and incubated overnight in a sealed humidified container at room temperature. The following day, coating buffer was removed by washing three times with 200  $\mu$ l of wash buffer (appendix 6). The remaining protein-binding sites in the coated wells were then blocked by adding 100  $\mu$ l of blocking buffer (appendix 6). The plate was again incubated in a sealed humidified container for 1 hour at room temperature and the wash step was repeated three times. To each well, 100  $\mu$ l of a 5  $\mu$ g/ml antigen in PBS, pH 7.4 (appendix 6) was added and for the negative control, PBS without antigen was used. The plate was incubated in a sealed humidified container for 1 hour at room temperature. After incubation, the wells were washed

(3x) to remove excess antigen. Thereafter 100 µl of detection antibody, horseradish peroxidase-rabbit-IgG-anti-HSP (1:50 with 3% skimmed milk powder in phosphate buffered saline and 0.01% tween 20 (PBST)) (appendix 6) was added as a two-fold serial dilution. The plate was incubated in a sealed humidified container for 1 hour at room temperature. The excess detection antibody was washed off three times with wash buffer and plate was blotted dry. To each well, 200 µl of chromogenic substrate (o-phenylenediamine dihydrochloride) was added and colour was allowed to develop at room temperature. The reaction was stopped by adding 50 µl of 3 M HCl to each well. Colour intensity was measured by an ELISA plate reader at 492 nm.

### **3.13 Purification of vervet-anti-rabbit-IgG**

Vervet serum was purified by column ProSep A chromatography media (Millipore, USA) as described in 3.9, 'Purification of IgG.'

### **3.14 Purification of goat-anti-rabbit-IgG**

Goat serum was purified as described previously (Kendall, 1938). A saturated ammonium sulphate ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) solution was prepared by dissolving 19 g of ammonium sulphate into 25 ml of sterile distilled water. One and a half millilitre of the saturated ammonium sulphate solution was added to 3 ml of rabbit serum. This was followed by centrifugation for approximately 5 minutes at 5000 x g at 4°C. The supernatant was discarded and the precipitated proteins were dissolved in 1.5 ml of sterile distilled water to which 750 µl of saturated ammonium sulphate solution was added. This was then centrifuged (5000 x g, 4°C, 5min) and the supernatant was discarded. This process was repeated five times. The pellet was

then dissolved in approximately 1.5 to 3 ml of PBS, pH 7.4 (appendix 6), depending on the size of the pellet, and the residual ammonium sulphate was removed by dialysis against PBS buffer, pH 7.4, overnight at 4°C. The following day, the contents of the tube was aliquoted into 100 µl volumes and stored at - 20°C until further use.

### **3.15 Evaluation of the biological activity of purified antibody**

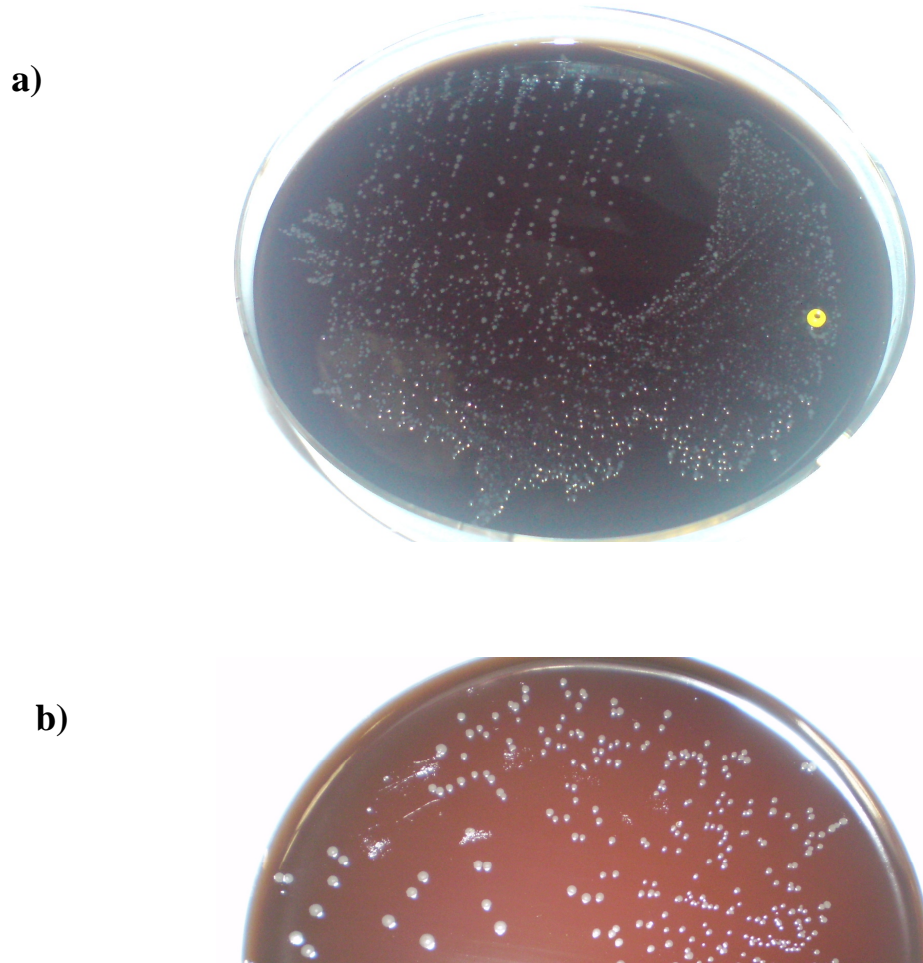
The biological activity of the purified IgG was measured as follows. Three wells of a microtitre plate were coated with purified vervet anti-rabbit antibodies (3.13) and purified goat anti-rabbit antibodies (3.14). The purified rabbit anti-HSP IgG was coated on three separate wells. The same rabbit IgG was conjugated with horseradish peroxidase and added to the first set of wells coated with vervet anti-rabbit antibodies and goat anti-rabbit antibodies. A commercially available conjugated goat anti-rabbit IgG ((Sigma-Aldrich, USA) was added to the rabbit anti-HSP coated wells. Following this, substrate was added to all of the coated wells and the reaction was stopped. ELISA procedure (blocking and washing) and incubation times used were the same as described in 3.10.1. Bovine serum albumin (BSA) was used as a negative control and was subjected to the same conditions as described above.



## Chapter Four: Results

### 4.1 Culture characteristics of *Haemophilus ducreyi* on solid media

The colony morphology of *H. ducreyi* grown on Modified Bieling agar is shown in figure 4.1.

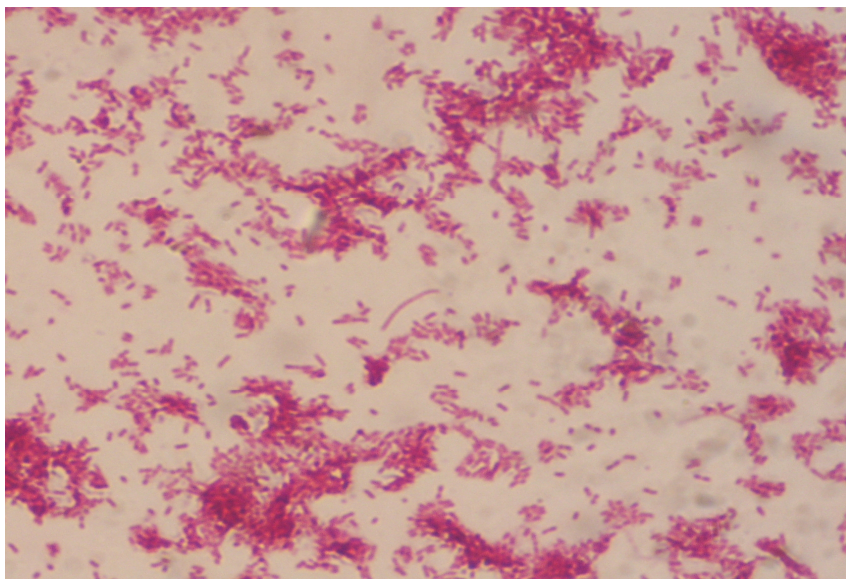


**Figure 4.1 a and b: Colony formation of *H. ducreyi* strain A54 on Modified Bieling (MB) agar at 48 hours.**

Colonies appeared to be small, non mucoid and grey in colour. These colonies are polymorphic in nature with some almost translucent in appearance and others opaque. When touched with a loop the colonies glide over the surface of the agar.

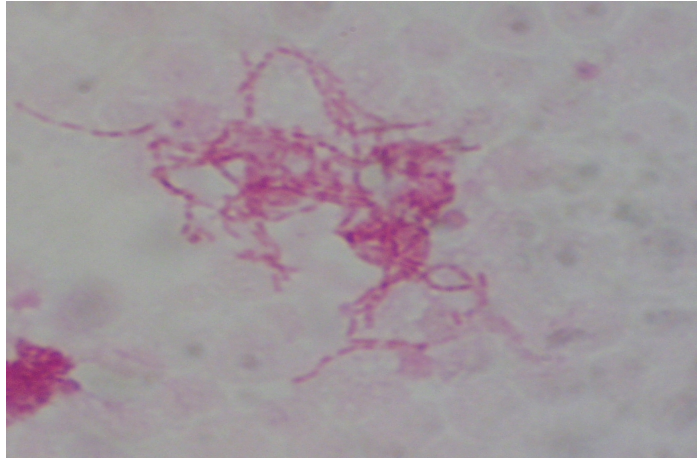
## 4.2 Gram stain

Bacteria were Gram negative and appeared mainly as short rods, usually in clumps or in short chains. Figure 4.2 shows the characteristics of *H. ducreyi* commonly seen on solid agar which are described as whorls. This is in contrast to the streptobacillary form and the characteristic parallel chains described as ‘railroad tracks’ which are seen in Gram stains of liquid culture as shown in figure 4.3 a, b and c.

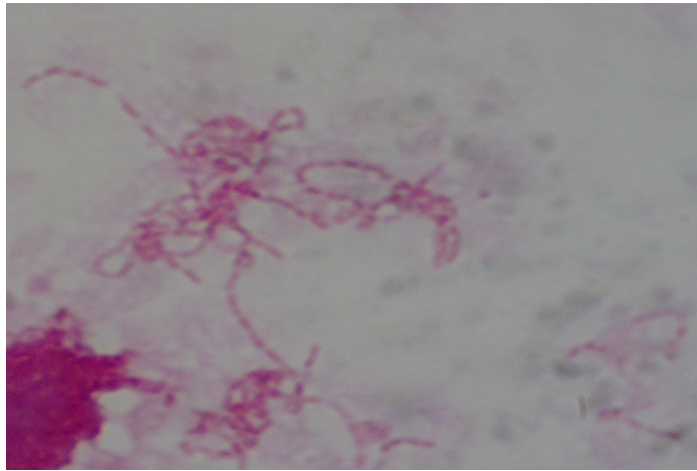


**Figure 4.2: Gram stain smear of *H. ducreyi* from culture on Modified Bieling agar plates.**

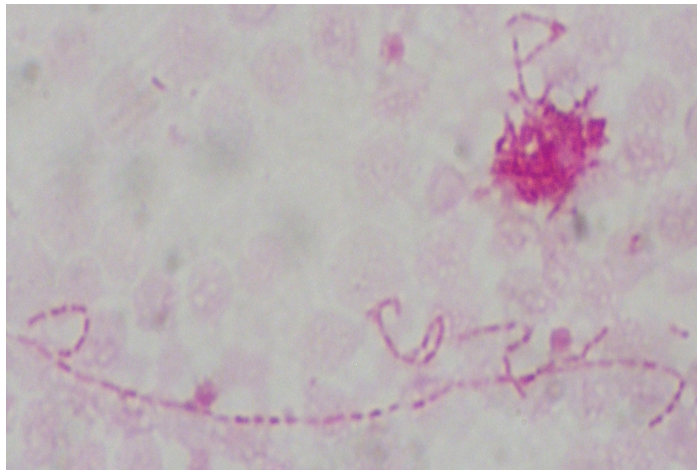
(a)



(b)



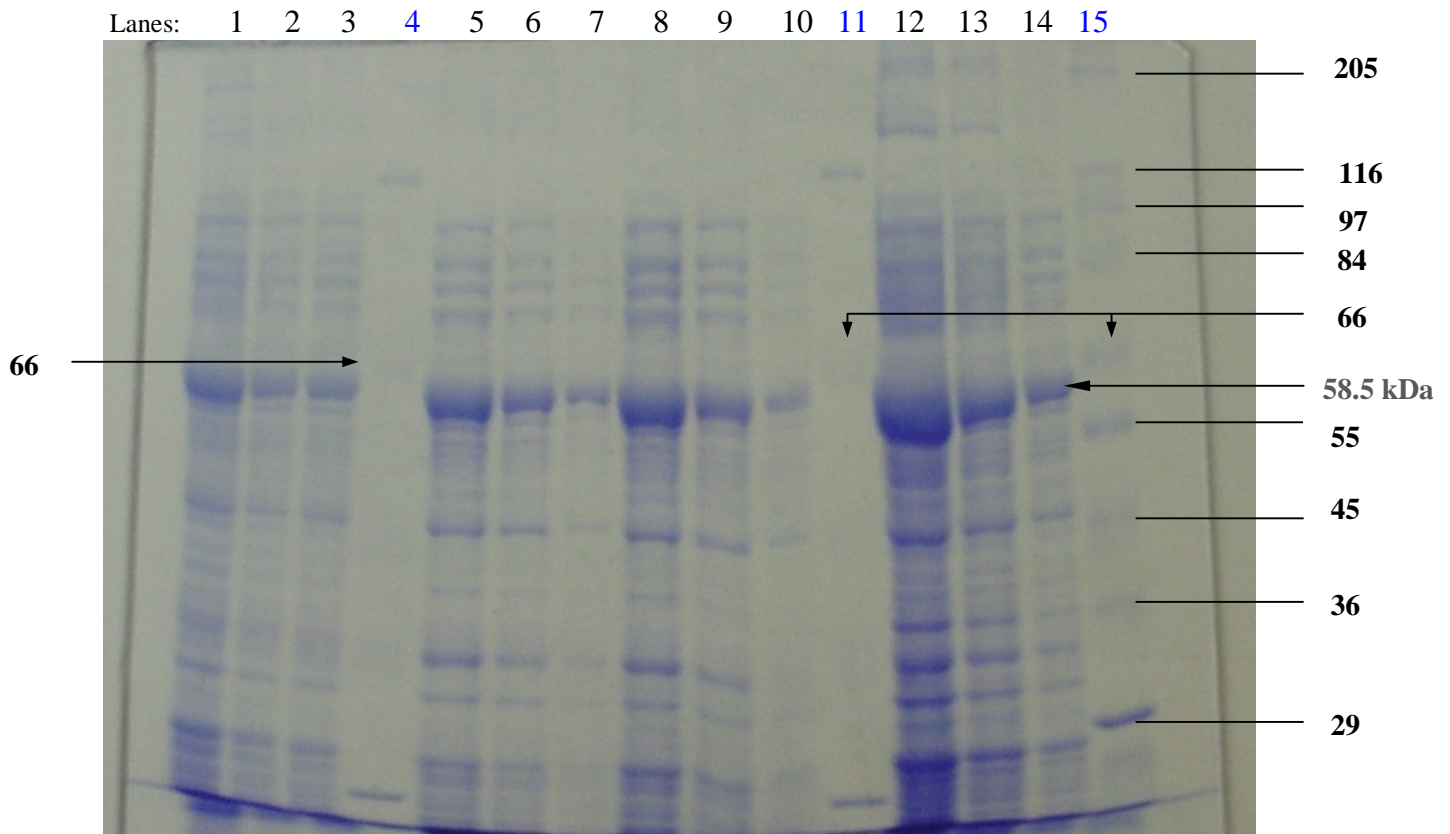
(c)



**Figure 4.3 a, b and c: Gram stains of *H. ducreyi* from culture grown in Modified Bieling broth.**

### 4.3 Extraction, separation and purification of bacterial proteins

*H. ducreyi* cells harvested from Bieling broth culture were lysed by sonication (3.3). Figure 4.4 shows the SDS-PAGE gel electrophoresis of the lysed strains A54 and A68.



**Figure 4.4: SDS PAGE gel showing location of the heat shock protein from sonicate samples of *H. ducreyi* strains A54 and A68.**

Lanes 1, 2, 3 - sonicate preparation 1 (strain A54) – 20 $\mu$ l, 10 $\mu$ l and 5 $\mu$ l, respectively;

Lane 5, 6, 7 - sonicate preparation 2 (strain A54) – 20 $\mu$ l, 10 $\mu$ l and 5  $\mu$ l, respectively;

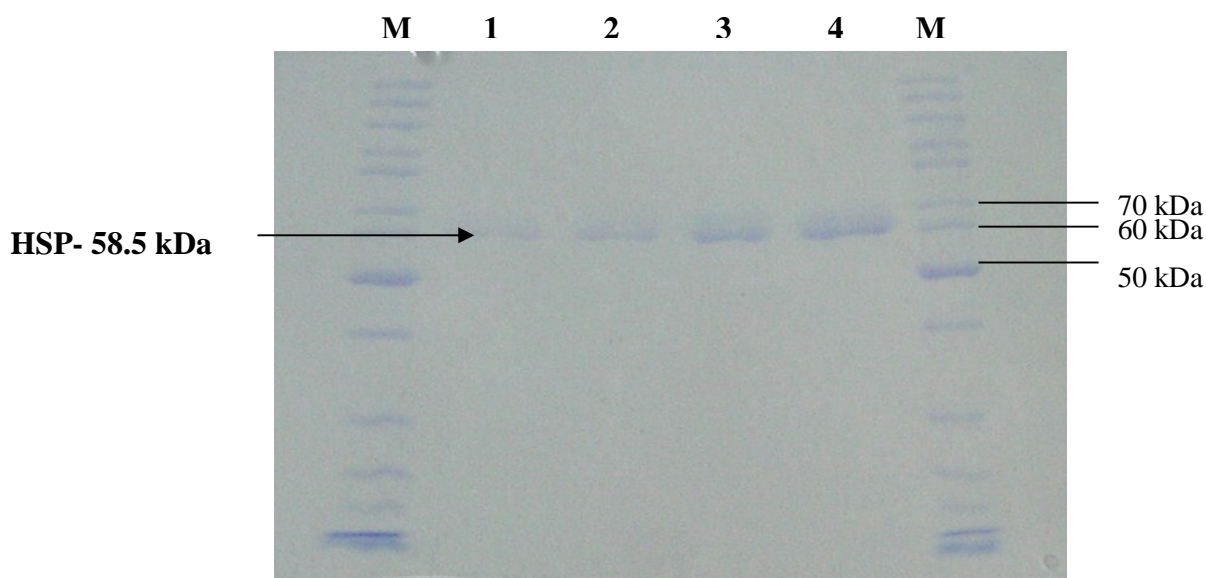
Lane 8, 9, 10 - sonicate preparation 3 (strain A54) – 20 $\mu$ l, 10 $\mu$ l and 5 $\mu$ l, respectively;

Lane 12, 13, 14 - sonicate preparation 4 (strain A68) – 20 $\mu$ l, 10 $\mu$ l and 5 $\mu$ l, respectively;

Lane 4; 11: Marker - 116 – 14, 4 kDa; Lane 15: Marker - 205 – 6, 5 kDa

Strain A54 was analysed in triplicate. The profiles obtained were identical. Furthermore the protein profile of the sonicate sample of *H. ducreyi* strain A68 was comparable to the protein profile of sonicate samples of *H. ducreyi* strain A54. A large band of an approximate molecular weight of 60 kilodaltons (kDa) was found in each of the sonicates. This band was likely to represent the 58.5 kDa GroEL heat shock protein.

The protein band of approximately 60 kDa was cut out of the gel and protein was eluted as described in 3.6. The protein solution obtained was again electrophoresed by means of SDS-PAGE. Figure 4.5 show that this contained only one protein with the expected MW of 58.5 kDa.

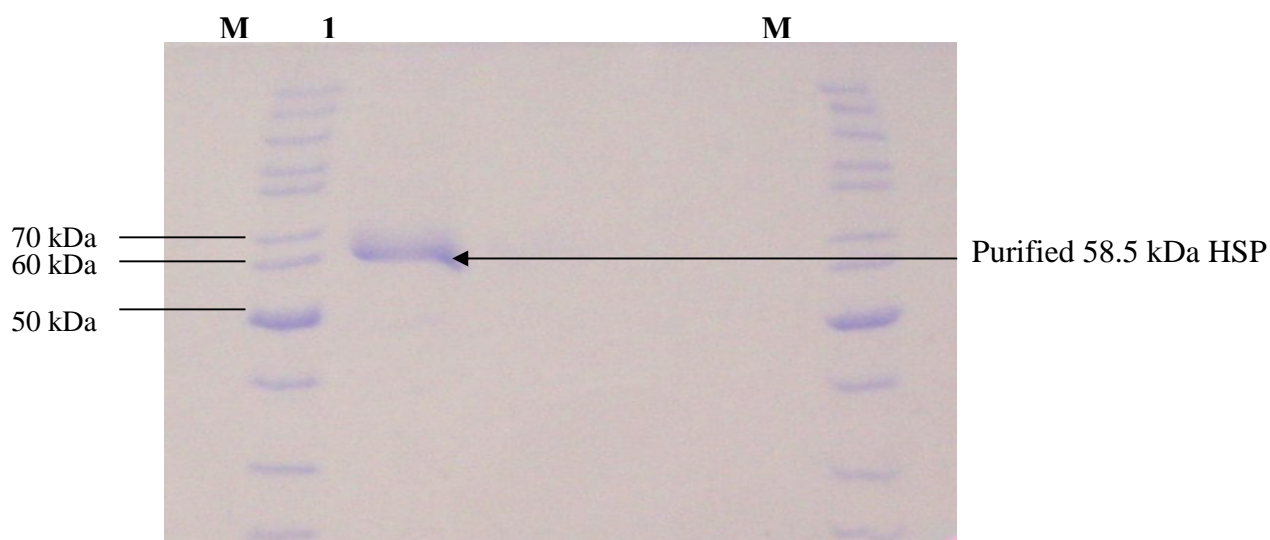


**Figure 4.5: SDS PAGE gel showing eluted 58.5 kDa GroEL heat shock protein by passive elution process.**

Lanes M: Protein marker, 20 kDa – 200 kDa;

Lanes 1, 2, 3, 4 - gel eluted heat shock protein – 5  $\mu$ l, 10  $\mu$ l, 15  $\mu$ l, 20  $\mu$ l, respectively.

The eluted protein was further purified by means of the ProteoSpin detergent clean-up kit and checked for purity by SDS-PAGE. The results are shown in figure 4.6.



**Figure 4.6: SDS PAGE gel showing the presence of the purified 58.5 kDa GroEL heat shock protein by means of the ProteoSpin kit.**

Lanes M: Protein marker, 20 kDa – 200 kDa;

Lane 1: Purified heat shock protein

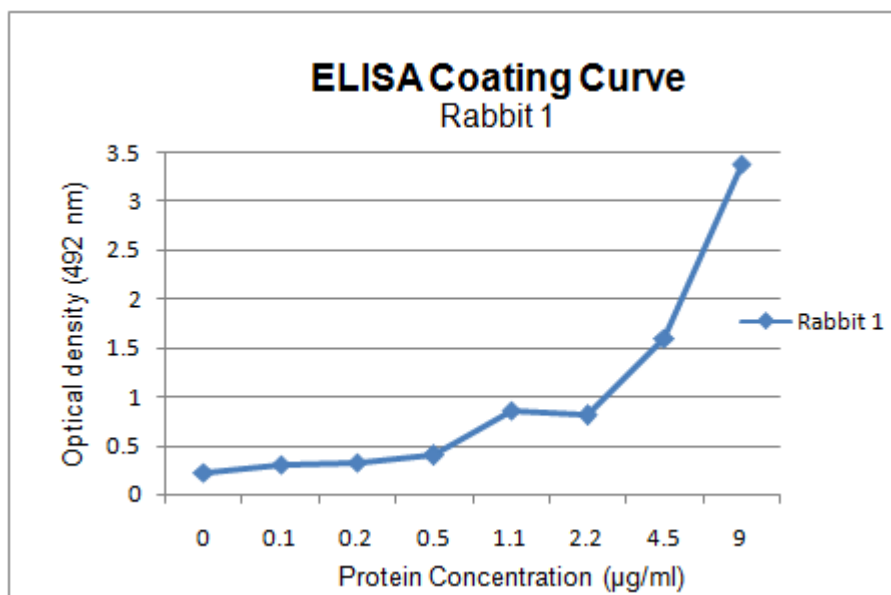
#### **4.4 Testing of rabbit serum for anti GroEL HSP antibodies**

The purified protein (3.7) was used to immunize the rabbits. Enzyme-linked immunosorbent assay (ELISA) methodology was used on rabbit sera to measure the results (3.10.1). A 96-well microtitre plate was coated with a serial dilution of the purified 58.5 kDa GroEL HSP providing the solid-phase antigen for the antibody detection system. Serum from each rabbit was added to the wells, followed by a goat anti-rabbit IgG conjugate. Following the enzymatic

reaction, optical density values were obtained at a wavelength of 492 nm (table 4.1, 4.2 and 4.3). The results were used to construct a curve for the determination of the optimal coating concentration of antigen. This was done by taking the average of the 3 optical density readings per antigen dilution (9 µg/ml, 4.5 µg/ml, 2.25 µg/ml, 1.12 µg/ml, 0.56 µg/ml, 0.28 µg/ml, 0.14 µg/ml, and 0.07 µg/ml). A curve was constructed for each rabbit by plotting the optical density versus the concentration (µg/ml) of protein (antigen) in the wells (figure 4.7, 4.8 and 4.9).

**Table 4.1:** Absorbance readings from ELISA for Rabbit 1

Coating Antigen Conc. ( $\mu\text{g/ml}$ )	Optical density (OD) readings (492 nm)			Average OD
<b>9</b>	3.838	2.521	3.781	<b>3.38</b>
<b>4.5</b>	2.071	1.911	0.793	<b>1.591</b>
<b>2.2</b>	0.733	0.718	0.967	<b>0.806</b>
<b>1.1</b>	1.058	0.694	0.840	<b>0.864</b>
<b>0.5</b>	0.138	0.514	0.547	<b>0.399</b>
<b>0.2</b>	0.136	0.322	0.497	<b>0.318</b>
<b>0.1</b>	0.150	0.261	0.513	<b>0.308</b>
<b>0.0</b>	0.241	0.365	0.073	<b>0.226</b>

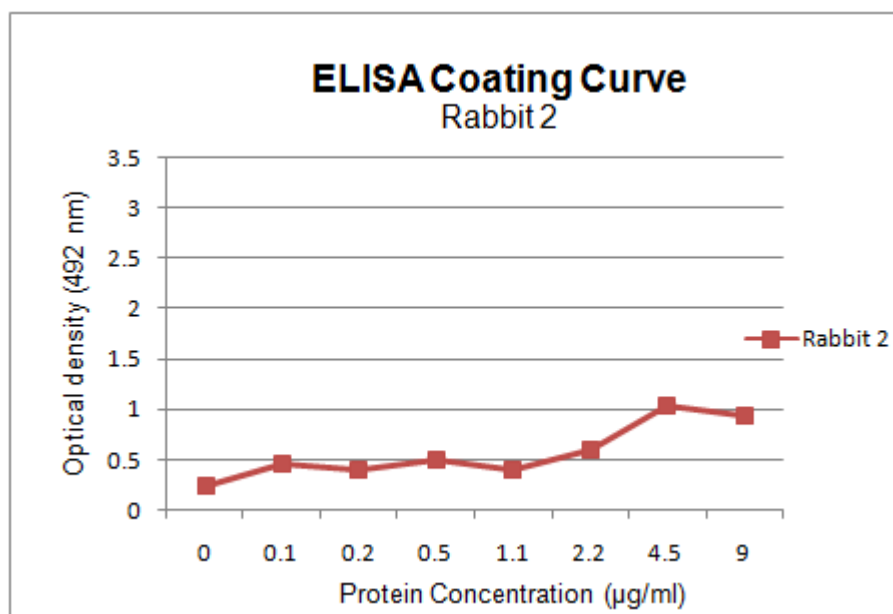


**Figure 4.7** Coating curve of Rabbit 1 for the determination of optimal antigen coating.



**Table 4.2:** Absorbance readings from ELISA for Rabbit 2

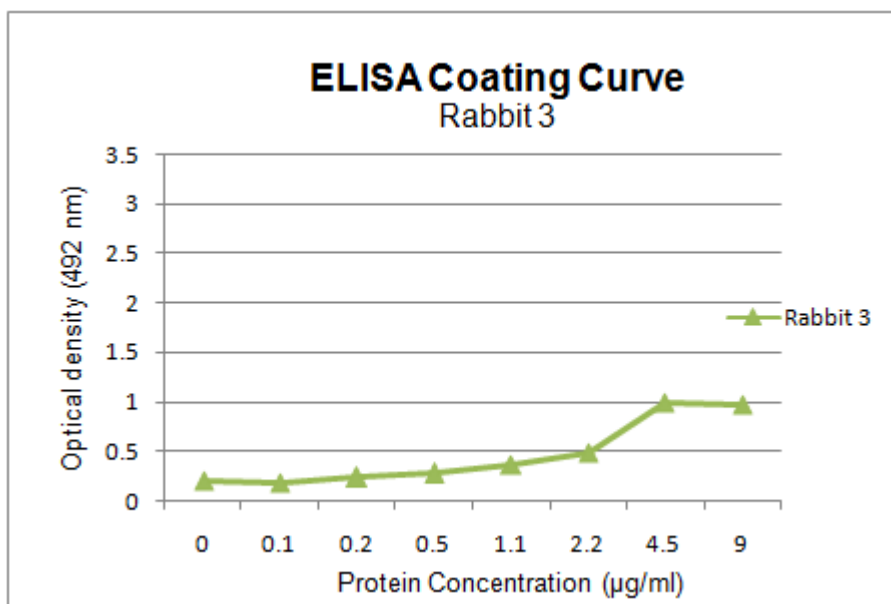
Coating Antigen Conc. ( $\mu\text{g/ml}$ )	Optical density (OD) readings (492 nm)			Average OD
<b>9</b>	0.931	0.826	1.067	<b>0.941</b>
<b>4.5</b>	0.552	1.184	1.347	<b>1.027</b>
<b>2.2</b>	0.121	0.620	1.072	<b>0.604</b>
<b>1.1</b>	0.652	0.416	0.116	<b>0.394</b>
<b>0.5</b>	0.638	0.436	0.438	<b>0.504</b>
<b>0.2</b>	0.458	0.366	0.349	<b>0.391</b>
<b>0.1</b>	0.380	0.611	0.381	<b>0.457</b>
<b>0.0</b>	0.170	0.347	0.209	<b>0.242</b>



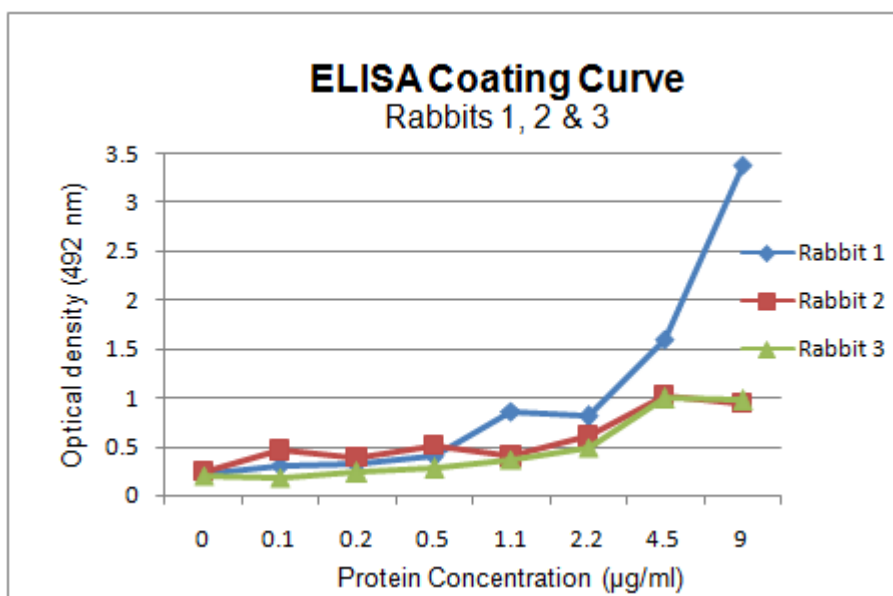
**Figure 4.8:** Coating curve of Rabbit 2 for the determination of optimal antigen coating.

**Table 4.3:** Absorbance readings from ELISA for Rabbit 3

Coating Antigen Conc. (µg/ml)	Optical density (OD) readings (492 nm)			Average OD
<b>9</b>	0.976	0.241	1.687	<b>0.968</b>
<b>4.5</b>	1.349	0.992	0.634	<b>0.991</b>
<b>2.2</b>	0.473	0.652	0.356	<b>0.493</b>
<b>1.1</b>	0.303	0.431	0.358	<b>0.364</b>
<b>0.5</b>	0.343	0.361	0.115	<b>0.273</b>
<b>0.2</b>	0.233	0.255	0.211	<b>0.233</b>
<b>0.1</b>	0.192	0.137	0.208	<b>0.179</b>
<b>0.0</b>	0.233	0.178	0.193	<b>0.201</b>



**Figure 4.9:** Coating curve of Rabbit 3 for the determination of optimal antigen coating.



**Figure 4.10: Coating curves for Rabbits 1, 2 and 3 plotted together.**

Figure 4.10 shows the combined results. The coating curve with the serum of rabbit 1 (figure 4.7) did not reach a plateau (maximum saturation of antigen by antibody) and therefore its optimal antigen coating has to be beyond 9 µg/ml.

The coating curves with the sera of rabbit 2 (figure 4.8) and rabbit 3 (figure 4.9), followed a similar pattern and reached a plateau at 4.5 µg/ml. Based on these results, the antigen concentration chosen for antibody capture was 5 µg/ml.

#### **4.5 Immune response of the rabbits**

In order to evaluate the immune response of each rabbit and finally to select the rabbit with the highest antibody (Ab) titre, an ELISA (3.11) was performed on sera before and after immunization. The values obtained were used to determine the positive/negative (P/N) ratios for each rabbit.

Tables 4.4, 4.5 and 4.6 show the results. In each table, lanes A, B and C contain the ELISA readings after immunization while lanes D, E and F show the readings before immunization. The P/N value based on the calculated averages is shown in the bottom lane.

**Table 4.4:** ELISA – Antibody titre for Rabbit 1

		<b>Coating Ab conc.</b>	<b>1/5</b>	<b>1/10</b>	<b>1/20</b>	<b>1/40</b>	<b>1/80</b>	<b>1/160</b>	<b>1/360</b>
Positive (P) value	<b>A</b>		3.068	2.291	1.464	0.776	0.416	0.231	0.141
	<b>B</b>		3.111	2.203	1.344	0.775	0.429	0.248	0.140
	<b>C</b>		3.035	2.221	1.342	0.721	0.408	0.234	0.133
Negative (N) value	<b>D</b>		1.966	1.246	0.675	0.353	0.206	0.125	0.084
	<b>E</b>		1.997	1.191	0.665	0.364	0.221	0.122	0.078
	<b>F</b>		2.054	1.239	0.656	0.366	0.206	0.118	0.080
	<b>P/N</b>		<b>1.531</b>	<b>1.826</b>	<b>2.079</b>	<b>2.097</b>	<b>1.979</b>	<b>1.953</b>	<b>1.71</b>

**Table 4.5:** ELISA – Antibody titre for Rabbit 2

		<b>Coating Ab conc.</b>	<b>1/5</b>	<b>1/10</b>	<b>1/20</b>	<b>1/40</b>	<b>1/80</b>	<b>1/160</b>	<b>1/360</b>
Positive (P) value	<b>A</b>		1.529	0.826	0.537	0.320	0.214	0.144	0.101
	<b>B</b>		1.406	0.952	0.522	0.342	0.381	0.144	0.102
	<b>C</b>		1.413	0.878	0.516	0.341	0.218	0.140	0.100
Negative (N) value	<b>D</b>		0.392	0.275	0.188	0.136	0.096	0.066	0.056
	<b>E</b>		0.375	0.263	0.186	0.132	0.096	0.066	0.052
	<b>F</b>		0.393	0.275	0.190	0.137	0.094	0.068	0.052
	<b>P/N</b>		<b>3.748</b>	<b>3.266</b>	<b>2.792</b>	<b>2.476</b>	<b>2.842</b>	<b>2.14</b>	<b>1.893</b>

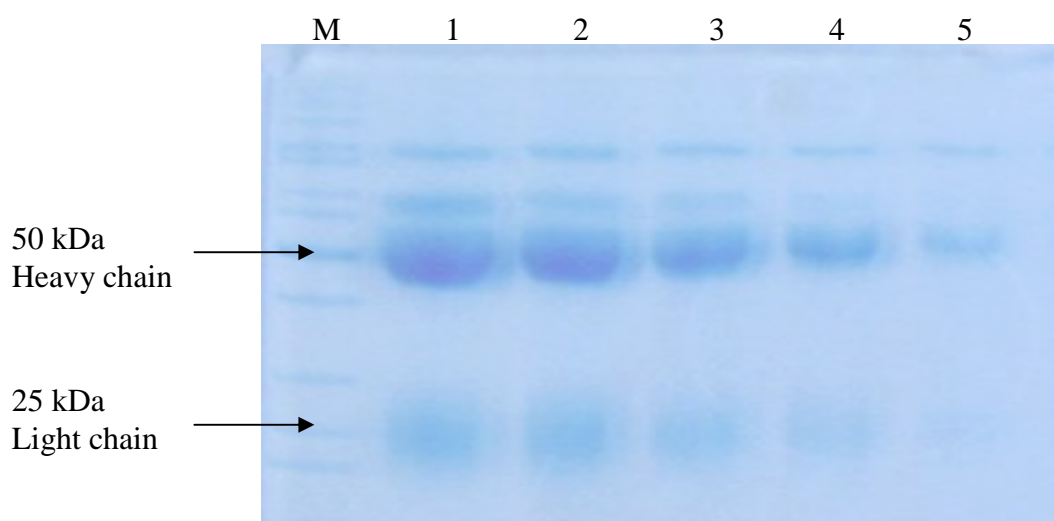
**Table 4.6:** ELISA – Antibody titre for Rabbit 3

		<b>Coating Ab conc.</b>	<b>1/5</b>	<b>1/10</b>	<b>1/20</b>	<b>1/40</b>	<b>1/80</b>	<b>1/160</b>	<b>1/360</b>
Positive (P) value	<b>A</b>		0.873	0.514	0.307	0.211	0.129	0.096	0.071
	<b>B</b>		0.930	0.538	0.306	0.218	0.137	0.097	0.071
	<b>C</b>		0.920	0.540	0.295	0.209	0.140	0.095	0.073
Negative (N) value	<b>D</b>		0.472	0.394	0.287	0.186	0.142	0.109	0.071
	<b>E</b>		0.451	0.382	0.265	0.191	0.144	0.104	0.070
	<b>F</b>		0.490	0.365	0.263	0.181	0.135	0.107	0.070
	<b>P/N</b>		<b>1.927</b>	<b>1.395</b>	<b>1.114</b>	<b>1.143</b>	<b>0.964</b>	<b>0.899</b>	<b>1.019</b>

The difference between pre- and post-immunisation absorbance values of the serum of rabbit 2 was significantly higher as compared to the other 2 rabbits and indicates that a strong immune response had occurred. This is reflected in the higher P/N value obtained with serum of rabbit 2 as compared to the others. Therefore, this rabbit's serum was used in the rest of the study.

#### 4.6 Purification of IgG antibodies

The IgG fraction of the serum from rabbit 2 was purified by means of Protein A affinity chromatography. To show the level of purification the harvest was mixed with sample reducing buffer and subjected to SDS-PAGE. The resulting heavy (H) and the light (L) chains are shown in figure 4.11.



**Figure 4.11: SDS Page gel showing the presence of affinity purified IgG fractions of rabbit serum under reducing electrophoresis conditions.**

Lane M: Molecular marker, 20 kDa – 200 kDa;

Lanes 1 – 6: sequential 1 ml fractions of purified IgG

#### **4.7 Determination of optimal capture and detection antibody concentration**

For the development of the antigen detection test, the capture antibody needs to be fixed to a solid phase while the detection antibody needs to be labelled. Since the chosen test system was ELISA (3.12), microtitre plates provided the solid phase while horseradish peroxidase was used as the label. The purified IgG fraction of rabbit 2 was used as capture as well as detection antibody. In order to determine the optimum concentration for capture and detection antibody, a checkerboard titration was performed with varying concentrations of both antibodies and a constant concentration of antigen (5 µg/ml).

Table 4.7 shows the results of the checkerboard titration. The absorbance readings obtained with antigen did not differ from those with PBS indicating that no antigen capture had taken place.

**Table 4.7:** ELISA checkerboard titration to determine optimal capture and detection affinity purified antibody concentrations.

Detection Antibody conjugate (µg/ml)	Capture Antibody Concentration (µg/ml)															
	100µg	100µg	80µg	80µg	60µg	60µg	40µg	40µg	20µg	20µg	10µg	10µg	5µg	5µg	1µg	1µg
		PBS		PBS		PBS		PBS		PBS		PBS		PBS		PBS
<b>100µg</b>	0.766	0.862	0.791	0.689	0.647	0.728	0.668	0.650	0.634	0.643	0.634	0.700	0.695	0.576	0.478	0.425
<b>50µg</b>	0.467	0.547	0.492	0.556	0.451	0.541	0.482	0.500	0.415	0.475	0.430	0.473	0.492	0.476	0.336	0.393
<b>25µg</b>	0.422	0.416	0.458	0.384	0.398	0.460	0.409	0.369	0.330	0.317	0.333	0.319	0.346	0.331	0.282	0.311
<b>12.5µg</b>	0.293	0.256	0.268	0.277	0.280	0.325	0.366	0.264	0.299	0.241	0.237	0.231	0.220	0.247	0.191	0.187
<b>6.25µg</b>	0.224	0.204	0.206	0.233	0.206	0.186	0.241	0.191	0.230	0.198	0.183	0.189	0.193	0.184	0.158	0.154



#### **4.8 Evaluation of biological activity of purified antibody**

Since no antigen capture had occurred in the checkerboard titration (table 4.7) the biological activity of the purified IgG fraction which was used as capture antibody was evaluated. The results are shown in Table 4.8.

The top panel shows that after coating, the capture antibody is present in the bottom of the wells. The ELISA readings after coating with BSA are significantly lower than after coating with the capture antibody. The bottom panel shows that the detection antibody is able to detect both vervet and goat anti-rabbit antibodies that were used for coating the wells.

These experiments confirm that the raised rabbit antibodies do effectively coat the microtitre wells and that the conjugate is working.

**Table 4.8:** Evaluating biological activity of affinity purified antibody and labelled antibody

<b>Detection Antibody</b>	<b>Capture Antibody (50 µg/ml)</b>	<b>ELISA absorbance readings done in triplicate</b>		
Goat-anti-rabbit-HRP- conjugate (Sigma-Aldrich), 1:10000	Rabbit-anti-58 kDa HSP- IgG	>3.299	3.299	>3.299
	<b>Control – BSA</b>	0.095	0.094	0.138
HRP-rabbit2-IgG-anti-HSP (developed in laboratory), 1:50	Vervet-anti-rabbit-IgG	>3.299	>3.299	>3.299
	Goat-anti-rabbit-IgG	2.987	3.048	2.840
	<b>Control – BSA</b>	0.099	0.100	0.097

## **Chapter Five: Discussion**

This study is one of two that aimed at the development of an immuno-chromatography based test for the exclusion of chancroid and syphilis in patients with genital ulcer disease. This test was deemed essential to avoid treatment with anti-bacterial agents that are not needed now as chancroid and syphilis have almost disappeared in most settings.

There are various approaches to the development of such a test. The study reported here is the first of these. This approach was not successful and therefore one of the other options needs now to be explored.

The approach attempted here is based on the use of a secreted protein and a polyclonal purified IgG antibody to be used as capture as well as detection antibody. The 58.5 kDa HSP was chosen as the antigen. This was based on the fact that this protein is secreted by *H. ducreyi* in large amounts. Since the concentration of bacterial cells in ulcer secretions is low, the use of cell bound antigens was considered to be prone to fail.

Microscopic characteristics and colony morphology of the *H. ducreyi* isolates used for antigen isolation was consistent with previous publications (Sturm and Zanen, 1984; Albritton, 1989; Morse, 1989). Since these were well characterized isolates, no further identification test were applied.

Sodium dodecyl sulphate polyacrylamide gels were used to locate and separate the proteins from the bacterial sonicate sample. The protein of interest, the 58.5 kDa heat shock protein

was clearly visible as a large band which fell in the 60 kDa region, as described previously (Parsons *et al*, 1992; Brown *et al*, 1993). The abundance of the heat shock protein was confirmed by the large band with great intensity in comparison to other proteins found in the bacterial lysates. Others also found this HSP present intracellularly (Brown *et al*, 1993; Odhav, 1996; Frisk *et al*, 1998) as well as present in large amounts (Parsons *et al*, 1992; Brown *et al*, 1993). In this study, a large yield of the 58.5 kDa heat shock protein was obtained when the organism was harvested at its optimal growth temperature of 33°C. This was also observed by Brown *et al* (1993), when *H. ducreyi* cell lysate was analysed (Brown *et al*, 1993).

The two different *Haemophilus ducreyi* strains (A54 and A68) of sonicate samples tested had comparable protein profile patterns when screened on SDS PAGE gels and this remained consistent for all sonicate samples tested. In support of this, Lindquist and Craig (1988) had reported that heat shock proteins are a highly conserved group in prokaryotic organisms (Lindquist and Craig, 1988).

Two approaches can be applied to obtain purified proteins: elution from gels after gel electrophoresis of lysates of *H. ducreyi* and cloning of the gene in a rapid and easy growing organism like an *E. coli* that is able to secrete the gene product in large amounts.

We chose for the first, mainly since expertise with the cloning technique was not available. The method employed to passively elute polyacrylamide gel pieces (Nurhadi *et al*, 2003), was found to be a cost effective and rapid process. However in order to gain a substantial amount of protein for the entire project, a large number of gels were required and therefore SDS

PAGE had to be done on a daily basis. This process was found to be laborious and time consuming.

The ProteoSpin detergent kit was used to purify the protein eluted from the polyacrylamide gel pieces to effectively remove SDS present in the protein elution buffer, as well as the SDS particles that may have bound to the protein. This was necessary as purified protein free of SDS was required for downstream processes, such as the coating of ELISA plates. Even though the use of the kit was a rapid process and protein was concentrated in a smaller volume, a substantial amount of protein was lost, as the amount of protein measured prior to using the kit was found to be much higher than the final purified protein after the use of the kit. This loss of protein was attributed to the large dilution processes which were required for some of the purification steps as stipulated by the kit's instruction insert.

The immunoglobulin G (IgG) which was purified by means of Protein A affinity chromatography had to be passed through the affinity column twice as one passage was not sufficient to remove all of the contaminating proteins. This was evident when the purified IgG fraction was screened on SDS page gels. Even after two passages through the affinity purification column, a small amount of contaminating proteins was still observed. No further passages through the purification column were entertained since this can reduce biological activity and result in IgG denaturation as the elution buffer used had a very low pH.

A good immune response was achieved with serum of one of the 3 rabbits immunized. This was before the purification process. However when the IgG fraction was purified as described

above no reaction was observed with the purified HSP. The control wells (PBS) exhibited similar values to those of the test wells. Even the immediate neutralization of the low pH of the IgG elution buffer by dialysis had resulted in no antibody activity being detected.

The purified non-conjugated antibodies did still have capacity to coat the microtitre wells. Also, the purified conjugated antibody could be captured by the anti-rabbit IgG antibodies raised in 2 other animals. This indicates that the Fc portion was undamaged in the purification process. However, the antibodies were ineffective in capturing the HSP antigen. This can only be explained by the fact that the Fab portion had been made biologically inactive by the purification process.

The problem with the low pH of the elution buffer is not uncommon, as Murphy *et al* (1986) had a similar experience. They were unable to detect IgG antibodies to P6 and indicated that their elution conditions (0.1 M glycine, pH 2.5) could have played a role in IgG antibody denaturation (Murphy *et al*, 1986). Akkoyunlu *et al* (1996) had reported on a loss of bactericidal activity against *Haemophilus influenzae* when affinity-isolated human anti-PDm antibodies which were eluted in an elution buffer of low pH were no longer found to be bactericidal (Akkoyunlu *et al*, 1996).

In the current project, an attempt was made to overcome this problem by purifying the serum with a commercial antibody purification kit, PURE-1A (Sigma-Aldrich, USA). However when the final purified antibody fraction was screened on a SDS-PAGE gel under reducing conditions, it was found that a large amount of non-specific proteins were present and the 25

kDa light chain band and 50 kDa heavy chain band of IgG were not detectable. It did not make much difference when the same purified fraction underwent purification for a second time with the PURE-1A purification kit. These findings were reported to the manufacturer of the commercial antibody purification kit. In this project, the limiting factor was with an inefficient purification system and as Huse *et al* (2002) appropriately pointed out, “occasionally, a method recommended for the purification of a member of a antibody class, which should work, does not.” He goes on to state that these antibodies may not even bind or bind insufficiently during chromatography (Huse *et al*, 2002).

A number of elution buffers are employed to recover proteins from affinity columns. The role of such an elution buffer is to break the chemical bonds involved in protein-protein interaction so that the target protein reverts back into its active form (Firer, 2001). When using affinity chromatography for purification, pH elution is the commonly used method to remove protein bound to ligands (Wikibooks).

Antibody purification methods employ a change in the pH of the elution buffer (usually a low pH) which in some instances can result in significantly reduced antibody activity. Even though these harsh elution conditions may reduce antibody activity, the yield and purity of the antibody is usually good. This is different for milder elution conditions, where antibody activity is retained with reasonable purity but antibody yield may be reduced (Harkins, 2001).

Since the pH elution method is associated with a decrease in pH to elute bound protein (Wikibooks), it is advised to test various elution buffers on an affinity column before use (Huse, 2002); however this may have cost implications and be time consuming (Firer, 2001).

In this study, a high affinity ligand, Protein-A was used which binds specifically to the Fc-portion of the IgG molecule. Due to its high affinity, protein-A columns require denaturing conditions with extremely low pH for the elution of the IgG antibodies. This low pH results in conformational changes of the antibody, and this may cause antibody degradation and aggregation. The use of milder elution conditions could avoid this problem (Arakawa *et al*, 2004). However when using a high affinity ligand like Protein-A, milder conditions may result in partial elution. In other words, it would be difficult to elute antibody with a milder pH elution buffer if it has a high affinity to the chromatography media being used.

The requirement of stringent elution buffers for the use of high affinity interactions could result in partial denaturation and a decrease or loss of activity of the released target protein (Firer, 2001).

Arakawa *et al* (2004) has demonstrated the use of arginine as an efficient eluent for the elution of antibodies when low pH elution buffers are utilized. Arginine seems to prevent protein aggregation during refolding; as observations showed that arginine does not affect the stability of native proteins and that the solubility of denatured proteins is increased (Arakawa *et al*, 2004).

Uptima mild elution medium is a non-denaturing elution medium which is also available for improved affinity purifications under neutral stabilizing conditions. This elution medium



firstly offers the advantage of increased antibody yields by 97% from immunoadsorbents without the loss of biological activity, and secondly preserves the antibody's biological activity by 97%. Uptima mild elution medium is commercially available (Interchim, France).

The use of Differential Scanning Calorimetry (DSC) is shown to be a promising method in making downstream purification processing of proteins more cost effective and reproducible in a robust way. A stability study conducted by Diosynth Biotechnology demonstrated that DSC maintained stability and protection of the molecule by means of stabilizing buffering elution conditions (Acharya, Diosynth Biotechnology-USA).

Any of the methods described above could be used to purify antibody, as opposed to the use of a low pH elution buffer for the purification of antibody. However due to time constraints and the limited amount of antigen available for testing, the above mentioned methods for antibody purification analyses was not possible in this study. Even if the search for an effective elution process had been continued, the Protein-A affinity media used had showed a certain degree of affinity to non-specific antibodies, probably due to the hydrophobic properties of these non-specific antibodies to the column, and hence further optimization would be required. Since the rabbit serum had been used up in the different experiments and the purified material was no longer active against the antigen of interest, we could no longer proceed to the development of the chromatography test strips without starting from scratch.

## **Chapter Six: Conclusion**

This study attempted to develop a point-of-care test for the exclusion of chancroid in patients presenting with genital ulcer disease. The success of such an attempt depends on a number of things: the antigen, the use of purified naturally produced antigen versus antigen obtained after gene cloning and the use of polyclonal versus monoclonal antibodies. In this study, the use of naturally produced antigen and polyclonal antibodies for both antigen capture and detection was tried. This turned out not to be successful mainly because of problems with antibody purification which led to the loss of the Fab activity of the antibodies. This first attempt will be followed by the more demanding approach by the next student with antigen produced after gene cloning and monoclonal antibodies.

In this project, the choice of using the same polyclonal antibody as both the capture and detection antibody against the 58.5 kDa heat shock protein could have limited the test to a certain extent. The potential of this test may be greatly improved by the use of a specific monoclonal antibody (MAb) against the 58.5 kDa heat shock protein as either capture or detection antibody, or the use of two polyclonal antibodies, where the capture and detection antibodies are raised in different animal species.

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## Appendices

### Appendix 1: Media used to culture *H. ducreyi*

#### **A. Preparation of Modified Bieling Broth**

To make up 300 millilitres of Modified Bieling broth, you would require:

- 50 ml horse blood
- 50 ml dH<sub>2</sub>O
- 200 ml Columbia broth (autoclaved)
- 2 % (6 ml) IsoVitalex

Combine the 50 ml of horse blood with 50 ml of dH<sub>2</sub>O in a sterile glass bottle. Set aside for 30 minutes to allow for lyses of blood. Once blood is lysed, add this to the 200 ml of Columbia broth followed by 2% (6 ml) of IsoVitalex. Mix well.

Aliquot the broth into smaller quantities as required and store at room temperature.

#### **B. Preparation of Modified Bieling Agar**

To make up 1 litre of Modified Bieling agar, you would require:

- 165 ml horse blood
- 165 ml dH<sub>2</sub>O
- 24.6 g Columbia agar base
- 650 ml dH<sub>2</sub>O
- 20 ml IsoVitalex

Combine the 165 ml of horse blood with the 165 ml of dH<sub>2</sub>O in a sterile glass bottle. Set aside for 30 minutes to allow for lyses of horse blood.

Combine the 24.6 g Columbia agar base with the 650 ml dH<sub>2</sub>O, mix well and autoclave. When the Columbia agar base mixture is warm, add the lysed horse blood followed by the 20 ml IsoVitalax. Mix well and aliquot the mixtures at 20 ml per Petri dish to make up 50 Modified Bieling agar plates.

### **C. Storage media for *H. ducreyi***

To make up 100 ml of storage media, you would require:

- 1 g Protease Peptone No3
- 90 ml dH<sub>2</sub>O
- 10 ml Glycerol

Dissolve 1 g protease peptone No3 in 90 ml of dH<sub>2</sub>O. To this add 10 ml of glycerol and mix well. Autoclave solution for 15 minutes at 121°C.

## **Appendix 2: Gram stain procedure**

A glass slide was prepared with a culture smear and subjected to the following:

- Slide was heat fixed and flooded with crystal violet for one minute. Excess dye was washed off gently with tap water.
- Slide was then flooded with iodine for one minute, after which excess iodine was removed over a sink and washed with acetone for a few seconds. Excess acetone was washed off with water.
- Slide was then flooded with carbol fuchsin for 30 seconds and then rinsed with water.
- Slide was then blotted dry with blotting paper and viewed under a light microscope.



### **Appendix 3: Preparation of SDS PAGE solutions**

#### **A. Acrylamide/Bis solution**

- 29 g Acrylamide (Biorad)
- 0.8 g Bis-methylene-acrylamide (Biorad)

Add the above to 100 ml dH<sub>2</sub>O. Filter and store at 4°C in the dark (30 days maximum use).

#### **B. Separating or resolving buffer (1.5 M Tris-HCL, pH 8.8)**

- 18.15 g Tris base

Add the above to 50 ml dH<sub>2</sub>O and adjust pH to 8.8 with 1N HCL. Bring volume to 100 ml with dH<sub>2</sub>O and store at 4°C.

#### **C. Stacking buffer (0.5 M Tris-HCL, pH 6.8)**

- 6 g Tris base

Add the above to 60 ml dH<sub>2</sub>O and adjust pH to 6.8 with 1N HCL. Bring volume to 100 ml with dH<sub>2</sub>O and store at 4°C.

#### **D. 10% SDS**

- 10g SDS

Dissolve 10 g SDS in dH<sub>2</sub>O with gentle mixing and bring volume to 100 ml with remainder dH<sub>2</sub>O.

### **E. Ammonium persulphate (10%) (APS)**

- 0.1 g Ammonium persulphate

Dissolve 0.1 g APS in 1ml of dH<sub>2</sub>O.

### **F. Sample reducing buffer**

- 2 ml 20% SDS
- 2.5 ml 0.5 M Tris-HCl, pH 6.8
- 1 ml b-mercaptoethanol
- 2.5 ml Glycerol

Mix the above reagents together and add a small amount of bromophenol blue dye powder to obtain a dark blue colour.

### **G. Fixing solution**

- 250 Isopropanol
- 100 ml Acetic acid

Mix the above reagents and bring volume to 1 litre with dH<sub>2</sub>O.

### **H. Coomassie staining solution**

- 1 g Coomassie blue
- 500 ml (50%) Methanol
- 100 ml (10%) Acetic acid

Mix the above reagents and bring volume to 1 litre with dH<sub>2</sub>O.

**I. Destaining solution 1**

- 500 ml (50%) Methanol
- 100 ml (10%) Acetic acid

Mix the above reagents and bring volume to 1 litre with dH<sub>2</sub>O.

**J. Destaining solution 2**

- 70 ml (7%) Methanol
- 100 ml (10%) Acetic acid

Mix the above reagents and bring volume to 1 litre with dH<sub>2</sub>O.

**K. 10 X Electrode buffer**

- 3.028 g Tris base
- 1 4.413 g Glycine
- 1 g SDS

Dissolve the above in 1 litre of dH<sub>2</sub>O and adjust pH to 8.3. Autoclave and store at 4°C.

#### **Appendix 4: Gel preparation for SDS-PAGE slab**

**Table 8.1:** Separating gel preparation - 12% for SDS-PAGE

<b>REAGENTS</b>	<b>Large gel</b>	<b>Small gel</b>
Distilled water	12.1 ml	3.35 ml
1.5 M Tris-HCL, pH 8.8	9.3 ml	2.5 ml
10% (w/v) SDS (Store RT)	400 $\mu$ l	100 $\mu$ l
Acrylamide/Bis solution	14.3 ml	4 ml
10% ammonium persulphate (fresh daily)	266 $\mu$ l	50 $\mu$ l
TEMED	26 $\mu$ l	5 $\mu$ l
<b>Total Monomer</b>	<b>36 ml</b>	<b>10 ml</b>

**Table 8.2:** Stacking gel preparation - 4% for SDS PAGE

<b>REAGENTS</b>	<b>Large gel</b>	<b>Small gel</b>
Distilled water	6.1 ml	3 ml
0.5 M Tris-HCL, pH 6.8	2.5 ml	1.25 ml
10% (w/v) SDS (Store RT)	100 $\mu$ l	50 $\mu$ l
Acrylamide/Bis solution	1.3 ml	650 $\mu$ l
10% ammonium persulphate (fresh daily)	50 $\mu$ l	25 $\mu$ l
TEMED	10 $\mu$ l	5 $\mu$ l
<b>Total Monomer</b>	<b>10 ml</b>	<b>5 ml</b>

## **Appendix 5: Preparation of protein elution buffer and IgG elution buffer**

### **A. Protein elution buffer**

- 0.25 M Tris-HCL, pH 6.8
- 0.1% SDS

Make up a solution of 0.25 M Tris-HCL, pH 6.8 and add 0.1% SDS.

### **B. IgG elution buffer**

- 0.1 M Glycine (7.5 g)

Dissolve the above in 1 litre of sterile dH<sub>2</sub>O and adjust pH to 3.

## **Appendix 6: ELISA solutions**

### **A. ELISA Coating buffer (Bicarbonate buffer)**

- 5.3 g Sodium Carbonate ( $\text{Na}_2\text{CO}_3$ )
- 4.2 g Sodium Hydrogen Carbonate ( $\text{NaHCO}_3$ )

Dissolve 5.3 g of  $\text{Na}_2\text{CO}_3$  in 900 ml  $\text{dH}_2\text{O}$ . To this solution dissolve 4.2 g of  $\text{NaHCO}_3$  and pH to 9.6. Adjust volume to 1 litre with additional  $\text{dH}_2\text{O}$  and store at  $4^\circ\text{C}$

### **B. ELISA Blocking buffer (3%)**

- 5.3 g Sodium Carbonate ( $\text{Na}_2\text{CO}_3$ )
- 4.2 g Sodium Hydrogen Carbonate ( $\text{NaHCO}_3$ )

Dissolve 5.3 g of  $\text{Na}_2\text{CO}_3$  in 900 ml  $\text{dH}_2\text{O}$ . To this solution add 4.2 g of  $\text{NaHCO}_3$  and pH to 9.6. Dissolve the 30 g of non-fat dry milk to the above solution and adjust the volume to 1 litre with additional  $\text{dH}_2\text{O}$ . Make up blocking buffer daily when required.

### **C. Phosphate buffered saline – PBS**

To make up 1 litre of PBS, the following is required:

- 8g NaCl
- 0.2 g KCl
- 1.44 g  $\text{Na}_2\text{HPO}_4$
- 0.24 g  $\text{KH}_2\text{PO}_4$

Dissolve the above reagents in 800 ml dH<sub>2</sub>O and adjust pH to 7.4. Bring volume to 1 litre and autoclave. Store at 4°C. Alternatively 1 PBS tablet can be dissolved in 100 ml dH<sub>2</sub>O, autoclaved and stored at 4°C.

**D. ELISA Wash buffer (1X Phosphate buffered saline tween-20-PBST)**

For every 100 ml of 1x PBS add 10 µl (0.01%) tween-20 to make up 100 ml ELISA wash buffer.

**E. ELISA Detection antibody diluent for IgG-HRP conjugate**

- 3 g
- Skimmed milk powder
- 100 ml PBST

Dissolve 3 g of skimmed milk powder in 100 ml PBST on the day of use.

## **Appendix 7: Horseradish peroxidase conjugation and reagent preparation**

### **A. Conjugation preparation**

1. Equilibrate a column (1.4 x 5cm) of Sephadex G-25 with 30 ml of 1 mM Sodium acetate buffer, pH 4.
2. Dissolve 8 mg Horseradish Peroxidase (HRP) in 1 ml of distilled water and leave aside.
3. Add 50  $\mu$ l of 0.2 M NaIO<sub>4</sub> freshly dissolved in distilled water and vortex. (The mixture should immediately turn green). Let the above activation mixture stand for 20 minutes at room temperature.
4. Remove the excess NaIO<sub>4</sub> from the activation mixture by desalting on the Sephadex G-25 column.
5. Add the prepared HRP to the column and allow for activated HRP to drip directly onto a continuously vortexed test tube containing 16 mg of antibody (previously dialysed against NaHCO<sub>3</sub> buffer, pH 9.5, overnight).
6. Let conjugated antibody stand for 4 hours at room temperature, after which add an equal volume of conjugate stabiliser buffer to the prepared HRP-antibody conjugate and store in refrigerator.

### **B. Conjugate stabiliser buffer**

- Glycerol
- 20% Human serum albumin
- Tris-saline buffer, pH 8.0



Mix 50 ml glycerol with 49 ml tris-saline buffer, pH8.0. Add 1 ml 20% human serum albumin and stir on a magnetic stirrer until well mixed. Add mixture to a suitable container and store in refrigerator.