Migration of *Treponema pallidum* through a keratinocyte layer

by

Kavitha Naidoo (Mrs)

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Declaration

This study represents original work by the author and has not been submitted in any form to another University. The work of other investigators cited in this thesis has been duly acknowledged.

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Mrs. K. Naidoo

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Dedication

To my family and friends who have motivated and supported me through the years – Thank you. To my husband, I just want you to know that the journey here was so much easier knowing you were by my side. Thank you for your patience and understanding.

Most of all my biggest thank you is to God who is my strength – this one is for you.

Presentations originating from this dissertation

1. AstraZeneca faculty Research day – oral presentation – UKZN – Durban (SA) - 2007
2. 17th International Society for Sexually Transmitted Diseases Research – poster presentation – Seattle (USA) - 2007
3. FIDSSA – oral presentation – Cape Town (SA) – 2007
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Table of Contents

List of Figures and Tables

ABSTRACT

Treponema pallidum is the causative agent of the sexually transmitted disease, syphilis. The organism can not be cultured in vitro, which has inhibited the understanding of the pathogenesis of syphilis. There has been no evidence of a treponemal toxin but adherence of large numbers of treponemes is able to destroy cell monolayers of different cell types (Fitzgerald *et al*, 1982). Non-pathogenic treponemes failed to adhere to cultured cells and this suggests that adherence is associated with virulence of *T. pallidum* (Fitzgerald *et al*, 1977). In this study we explored the interaction of *T. pallidum* with HaCaT cells which are immortalized human keratinocytes with characteristics equivalent to their natural counterpart. The adhesion assay confirmed binding of the organism to HaCaT cell monolayers. Migration assays and electron microscopy revealed that *T. pallidum* migrates through a confluent keratinocyte layer and western blotting experiments that differentiate between soluble and insoluble occludin confirmed that *T. pallidum* does not loosen the tight junctions. It is concluded that *T. pallidum* passes through the keratinocyte layer by trans-cellular rather than inter-cellular migration.

CHAPTER ONE

INTRODUCTION

Treponema pallidum is an organism belonging to the order of the *Spirochaeteles*. While *T. pallidum* sub species *pertenue, endemicum and carateum* are responsible for the non-venereal diseases yaws, endemic syphilis and pinta respectively; *T. pallidum* sub species *pallidum* is responsible for venereal syphilis,
(Radolph *et al*, 2006).

Treponema pallidum is a helically coiled bacterium that is 5-15 μm long and has a diameter of 0.1 μm to 0.2 μm, (Radolph *et al*, 2006). The optimal pH range for the survival of the organism is between 7.2 and 7.4 and optimal temperature ranges between 30°C and 37°C. The organism is highly susceptible to environmental conditions such as mild heat, cold and desiccation (Radolph *et al*, 2006).

T. pallidum sub species *pallidum* enters its host through mucous membranes or skin. Syphilis presents in stages, initially as a genital tract lesion at the site of entry between 10 to 90 days post infection and heals spontaneously. If not treated progression to the second stage i.e. secondary syphilis occurs. Symptoms associated with secondary syphilis include disseminated lesions, swollen lymph glands, headaches, muscle aches and fatigue. Should the disease go untreated, it may progress to latent syphilis with a minority of patients progressing to tertiary syphilis where clinical signs include cardiovascular and neurological problems, (http://www.cdc.gov/std/Syphlis/STDFact-Syphilis.htm, Radolph *et al*, 2006).
Classically the diagnosis of syphilis is made by darkfield microscopy of a specimen obtained from a syphilitic chancre and by means of serological tests such as the

Treponema pallidum Particle Agglutination (TPPA), *Treponema pallidum* Haem-agglutination Assay (TPHA) and Rapid Plasma Reagin (RPR) are used. However, in research laboratories the polymerase chain reaction is rapidly replacing these techniques.

Previous attempts to culture *T. pallidum* have been relatively unsuccessful. The methodology described in some reports has either been non-reproducible or involved non-pathogenic strains (Fitzgerald *et al*, 1975).

The in vitro growth of *T. pallidum* has been attempted in the presence of cultured tissue cells with varying degrees of success relative to the type of cells used. Motility of *T. pallidum* was maintained for ten days on testicular epithelium whereas growth was unsuccessful on fibroblasts and on primary cell cultures of syphilitic and normal testis (Fitzgerald *et al*, 1975). This inability to culture *T. pallidum* has stunted the progress into the understanding of the pathogenesis of syphilis.

Cultivation of *T. pallidum* was based on the assumption that this organism was a strict anaerobe and that oxygen limited its survival. It was reported that optimal metabolic activity and rate of protein synthesis occurred in the presence of ten to twenty percent oxygen, but it was inhibited after a few hours under strict anaerobic conditions (Fieldsteel *et al*, 1977). *T. pallidum* was found to grow in rabbit testicular cells under aerobic conditions, attaching to the cell surface and entering the cell (Fitzgerald *et al*, 1975). It is possible that the requirement for a greater oxygen concentration is related to the presence of mammalian cell as these would require a higher oxygen concentration.

There has been no evidence of a treponemal toxin and although there are a large number of treponemes present in early infection, there is minimal damage to the host (Fitzgerald *et al*, 1982). Non-pathogenic treponemes failed to attach to cultured cells and this suggests that attachment may be a virulence factor (Fitzgerald *et al*, 1977).

This study aims at determining the factors involved in the pathogenesis of primary syphilis.

The hypothesis of the study states that *T. pallidum* enters the human host by passing through a keratinocyte layer by loosening of the tight junctions**.**

The objectives the study aimed to achieve:

1. To determine whether *T. pallidum* adheres to keratinocytes
2. To determine whether *T. pallidum* passes through a keratinocyte layer
3. To determine the mechanism by means of which *T. pallidum* passes through a keratinocyte layer

CHAPTER TWO

LITERATURE REVIEW

2.1 Historical background

Humans are the reservoirs for the causative agent of syphilis. The sexually transmitted form of syphilis, caused by the spirochete *T. pallidum*, is a worldwide problem. The incidence is particularly high in populations that have a high rate of partner change. After the implementation of penicillin as the standard treatment for syphilis, the incidence in the industrialized world decreased until 1958. At this point the situation changed as shown by a steady increase in incidence. In the late 1980s in the United States of America, increased substance abuse was strongly associated with an increase in the number of syphilis cases. In an effort to curb further spread, the Centers for Disease Control and Prevention (CDC) introduced a surveillance program and this has proved to be a useful tool, (http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=mmed.section.2010).

The prevalence of syphilis amongst first-time antenatal clinic attendees in the Kwa-Zulu Natal region decreased between 1998 and 2007 from 15.8 to 0.6 %, respectively, (http://www.hst.org.za/healthstats/33/data). However, this is still high when compared to rates in developed countries. (http://www.cdc.gov/std/Syphilis2006/Syphilis2006Short.pdf). However, the number of patients presenting with primary syphilis decreased as well from 40 % in 1998 to 3.5 % in 2007 in the KZN population attending the STD clinic at the Prince Cyril Zulu Centre for Communicable Diseases, Durban, South Africa (Fig. 1).

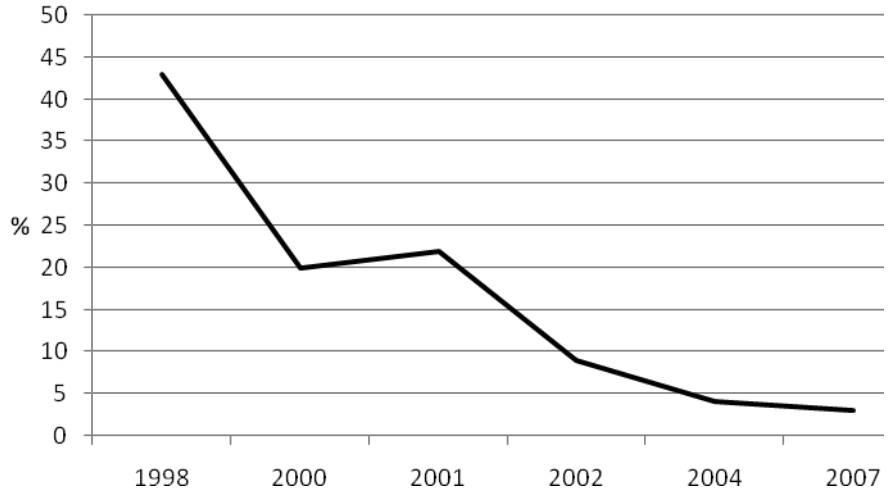


Figure 1: Prevalence of syphilis in genital ulcer patients in Durban from 1998 till 2007 (courtesy of A.Willem Sturm)

2.2 Morphological characteristics of *T. pallidum*

Until recently, the classification of spirochetes was based on a limited number of characteristics. This was largely due to the inability to isolate and culture these organisms. With the introduction of new isolation techniques as well as tissue culture as a means of growing the organisms, coupled with molecular and immunological techniques, classification is now advancing. The non-cultivable species can now be identified by polymerase chain reaction **(**PCR**)** using a comparison of 16S rRNA gene sequences (Edwards *et al*, 2003).

The genus *Treponema* belongs to the order *Spirochaeteles*. Spirochetes are morphologically different from other bacteria. They are thin cells with a basic helical

shape with coils that vary in tightness between species. The distinct feature of spirochetes is the presence of axial filaments within the periplasmic space. The number varies between species and ranges from 2 – 8 per organism. Flagella are anchored with their hook piece in the cell wall with the free end in the periplasm. This results in the characteristic corkscrew motility that allows the organism to move through viscous environments (Edwards *et al*, 2003).

The cells of the species *T. pallidum* are 6-15 μm in length with a diameter of 0.1 to 0.2 μm. They show the typical structure of a Gram negative bacterium consisting of a protoplasmic cylinder surrounded by the peptidoglycan-cytoplasmic membrane complex and an outer membrane. The space between the cytoplasmic and outer-membrane forms the periplasmic space which contains the axial filaments. As compared to other Gram negative organisms, the outer membrane of *T.pallidum* has a low density of surface exposed transmembrane proteins. Each end of the bacterium has three flagella. In contrast to the outer membrane, the cytoplasmic membrane is rich in lipoproteins, (Radolph *et al*, 2006).

2.3 Clinical manifestations of *T. pallidum* infection

T. pallidum is the causative agent of the sexually transmitted disease, syphilis. Syphilis is a disease that occurs in stages. Each of these stages presents with different symptoms and signs. Our knowledge regarding the development of syphilis stems from a study in African American prisoners who were left untreated for the purpose of the study. The

duration of the study was from 1932 – 1972. Although penicillin, the drug of choice for the treatment of syphilis, became available in the mid 1940's, the study subjects were denied treatment. During his presidency, Bill Clinton apologized on behalf of the American people for this study (http://www.cdcgov/tuskegee/timeline.htm).

2.3.1 Primary syphilis

The first stage is primary syphilis. This presents as a syphilitic ulcer or chancre at the site of infection. The chancre appears between 2 – 6 weeks after infection. The lesions are described as firm, round, indurated and painless. They are usually found on or in the genitalia, rectum, urethra or mouth. The chancre spontaneously heals after 3 to 6 weeks. If untreated the disease progresses to secondary syphilis, (http://www.cdc.gov/std/Syphilis/STDFact-Syphilis.htm), (Radolph *et al*, 2006).

2.3.2 Secondary syphilis

Secondary syphilis appears approximately 9-11 weeks after the appearance of the chancre and presents with general symptoms of infection and a skin rash that develops in one or more areas of the body. There is a possibility that syphilitic rashes appearing on other parts of the body may resemble rashes of other diseases. In addition to the rashes, symptoms such as fever, swollen lymph glands, sore throat, weight loss, muscle aches and fatigue may be experienced, (http://www.cdc.gov/std/Syphilis/STDFact-Syphilis.htm). Lesions referred to as condylomata lata contain high concentration of

treponemes. They are flat, moist and non-indurated. Other symptoms include patchy alopecia and mucous patches in the mouth, (Radolph *et al*, 2006). If untreated the disease progresses to latent syphilis.

2.3.3 Latent and tertiary syphilis

This stage occurs when the secondary symptoms are no longer detectable. The infection remains in the body and may consequently damage internal organs through the formation of necrotic areas known as gummata. When this damage becomes apparent as symptoms and signs, the disease has reached its final or tertiary stage. This happens only in a subset of patients with latent syphilis. The symptoms depend on the organ system that is most damaged and range from impairment of vision, paralysis, dementia, cardiovascular manifestations and constrained muscle movement, (http://www.cdc.gov/std/Syphilis/STDFact-Syphilis.htm)

2.3.4 Congenital syphilis

An infected mother can pass the disease on to her unborn child. The infection in the newborn is called congenital syphilis. There is a considerable chance of stillbirth and although a child may be born without symptoms, failure to treat asymptomatic patients or those presenting with congenital syphilis will lead to progression of the infection resulting in the symptomatic disease referred to as late congenital syphilis (http://www.cdc.gov/std/Syphilis/STDFact-Syphilis.htm). In congenital syphilis the

treponemes are transmitted transplacentally into the blood of the unborn baby. Therefore, the disease is systemic and the primary syphilitic stage does not occur (Radolph *et al*, 2006).

2.4 Diagnosis of Syphilis

Diagnosis of syphilis is based on clinical presentation of disease; observation of the organism from specimens of ulcer discharge or impressions using darkfield microscopy, serological tests, and with recent advances in molecular biology, techniques such as PCR has made it possible to identify a low bacterial load in a specimen.

Direct immunofluorescent staining (DFA) for the detection of *T. pallidum* on touch preparations of genital ulcers and PCR on ulcer discharge proved to be more sensitive and specific than darkfield microscopy for the diagnosis of primary syphilis. Limitation of the microscopic method was the presence of large numbers of leukocytes obscuring vision (Jethwa *et al*, 1995). A further limitation to microscopy as a diagnostic test is the difficulty to distinguish the presence of non-syphilitic treponemes.

2.4.1 Microscopy

T. pallidum, due to its narrow diameter, cannot be visualized using light microscopy. Darkfield microscopy is used to detect the presence of spirochetes whose motility is characteristic of *T. pallidum* in material taken from a syphilitic chancre. The accuracy of

this method is strongly dependant on training and experience of personnel (http://www.cdc.gov/std/Syphilis/STDFact-Syphilis.htm). This diagnostic test lacks specificity as most cases of syphilis are found in the developing world where experience is lacking and training is problematic. Sensitivity of the test is largely dependant on the stage of progression of the chancre.

2.4.2 Serological tests

Once infected the host's body develops an immune response against the organism. This leads to a production of antibodies. There are currently several commercially available kits to detect antibodies to *T. pallidum*. These are accurate and relatively inexpensive. The different tests can detect antibodies long after the patient had been infected and treated successfully as low levels of antibodies will remain in the bloodstream. Reliability of tests depends on the interpretation of results. The list of tests available include Treponema pallidum Hemagglutination Assay (TPHA), Treponema pallidum Particle Agglutination Assay (TPPA), the Fluorescent Treponemal Antibody Absorption Test (FTA-ABS), Immunoblot Assays, Enzyme Linked Immunosorbent Assays (ELISA), Venereal Disease Research Laboratory (VDRL) test and the Rapid Plasma Reagin (RPR). The last two are non-treponemal tests that detect antibodies against lipids from human endothelial cells that are released during vascular damage. These lipids are also found in the surface layer of the spirochetes (http://www.rapid-diagnostics.org/rti-syphilis-non-treponemal.htm) and this may play a role in the immune response as well. A positive test in either of the two indicates active disease if one of the treponemal tests is

also positive. In 2006 Muller *et al* reported the qualitative accuracy of the above tests to be higher for TPPA (98.1%) and Elisa's (95%).

2.4.3 Molecular Tests

In recent years molecular techniques such as polymerase chain reaction (PCR) have become the test of choice for the diagnosis of primary syphilis. Centurion-Lara *et al* in 1997 developed a reverse transcriptase PCR, which amplifies a 366bp region of the 16S rRNA gene of *T. pallidum*. The PCR was found to detect a single organism by Southern analysis. When RNA was extracted from an aliquot of 1000 organisms/mL prior to dilution the PCR was able to detect a dilution of 10^{-2} to 10^{-3}.

2.5 Treatment of Syphilis

T. pallidum is susceptible to penicillin. In primary and secondary syphilis a single intramuscular injection of 2.4 mega units of benzathine penicillin is effective. For latent and tertiary syphilis 3 such injections are given. Congenital syphilis and neurosyphilis are treated by continuous intravenous application of penicillin-G. Patients allergic to penicillin are treated with azithromycine or tetracycline.

2.6 *T. pallidum* in-vitro

Understanding the pathogenesis of *T. pallidum* has been stunted by the inability to successfully grow the organism in-vitro. In the laboratory, *T. pallidum* can only be maintained by intra-testicular passage in rabbits. It was therefore thought that rabbit testicular cells could be used to grow *T. pallidum* in vitro.

Walter Perry in 1948 cultured rabbit testicular tissue onto cover-slips and infected the cells obtained with *T. pallidum*. The cells that grew included fibroblasts, epithelial cells and macrophages. In these infected cell cultures, the development of 'giant'multi-nucleated cells was noted; these were absent in uninfected tissue culture. These 'giant' cells were thought to resemble cells associated with granulomatous lesions and were indicative of tissue response to treponemal infection. Viability of *T. pallidum* was maintained as observed by phase contrast microscopy up till 5 –7 days post inoculation. The organisms remained virulent as was shown by the formation of chancres in healthy rabbits inoculated with fluid from infected cultures 10 days post inoculation.

Fitzgerald et al, 1975 showed that separation of *T. pallidum* from the rabbit testicular material is important in successful maintenance of *T. pallidum* in vitro, since contamination with acellular tissue components interfered with viability of the treponemes. Equally important is the role of multiplying cells as maintenance of viability of the treponemes was much shorter in tissue culture media only. Monolayer cultures of rabbit testicular cells retained motility of *T. pallidum* for almost twice the time than that

of treponemal cultures incubated without cells. Similar observations were done in the presence of other cell types (leucocytes and erythrocytes) as well as pieces of testicular tissue. The researchers found an initial sharp decrease in the number of treponemes inoculated onto cultured cells which was absent in experiments without cells. They therefore concluded that the decrease was not an effect of lysis of the bacteria but attachment and invasion of *T. pallidum* to the cells. Primary cultures of normal rabbit testicular cells showed greater attachment of treponemes than the cervical cell line ME-180. The same researchers showed in a publication in 1977 that normal rabbit testicular cells suspensions were able to support treponemal viability but there was a decline in the virulence after 6 hours. However, a greater rate of decline in virulence was found in the absence of these cells.

In infections in the human host, *T. pallidum* has been found in many different tissues. Therefore, different cell lines have been used over the years to determine invasive properties of the organism. Jones et al in 1976 succeeded in the growth and subculture of pathogenic *T. pallidum* on Baby Hamster Kidney cells (BHK-21) under serum free conditions. Monolayers were required at a confluency of 50% for successful growth and subculture. A subculture frequency of once every 24 hours proved necessary to maintain continued cultivation of significant numbers of virulent treponemes. This work remained non-reproducible and therefore the validity of the results is questionable. Fitzgerald (1981b), in a review paper suggested that the multiplication observed may be a result of contamination with non-pathogenic treponemes.

T. pallidum shows an affinity for many different cultured cell types as it was able to adhere to 19 cell lines (Fitzgerald *et al*; 1977). Non-motile treponemes failed to adhere to cells, indicating that only live treponemes do so. Infection of non-viable cells and viable cells with *T. pallidum* revealed that within a few hours over a hundred treponemes adhered to viable cells but only 1-3 per non-viable cell. The finding that treponemal viability was essential for adhesion was confirmed when rabbit testicular cells were inoculated with heat inactivated and non-heat inactivated *T. pallidum*. It was found that the unheated treponemes rapidly attached to cells while heat inactivated treponemes failed to attach (Fitzgerald *et al*, 1982). Two possibilities exist. The attachment of treponemes to its host maybe an active process as the clustering of treponemes would require movement. In addition, the same authors concluded that morphological destruction of cells is likely to occur at the point of attachment and is a result of toxic activity by *T. pallidum*. Or during heating there are changes in configuration of the adhesion which is a protein.

As imperative as it is for the use of cell culture to the in-vitro viability of *T. pallidum*, equally important is the density of the cell culture at the time of inoculation. *T. pallidum* survives longer in the presence of multiplying host cells as compared to mature nearly or fully confluent cell monolayers (Wong *et al*, 1983).

Morphological destruction of cell lines occurs with treponemal infection. Increased vacuolization, rounding-off of cells, loss of nuclear detail as well as detachment of cells was observed (Fitzgerald *et al*, 1982).

In addition, *T. pallidum* has a greater affinity for endothelial cells as compared to epithelial cells. *T. pallidum* adhered to endothelial cells as well as HeLa (epithelial) cells in a linear fashion over time, with a two fold higher adhesion rate to endothelial cell as opposed to epithelial cells for the duration of the 6 hrs studies. *T. pallidum* (Nichols strain) was found to pass through a layer of endothelial cells grown on polycarbonate inserts (refer to Fig. 3, page 38 for a diagram showing a polycarbonate insert) more readily than the non-pathogenic *Treponema phagedenis* biotype Reiter (Thomas *et al*, 1988).

The invasive properties of *T. pallidum* were further studied by infecting an in vitro model of layered murine abdominal wall tissue. *T. pallidum* was found to traverse this tissue barrier, with peak counts at 20-22 hours post inoculation. It was found that the treponemes inoculated onto the epithelial surface traversed the tissue layer after 14 hours while no treponemes were present below the chamber when inoculated onto the connective tissue surface (Riviere *et al*, 1989).

2.7 The association of *T. pallidum* to human keratinocytes

Fitzgerald *et al* in 1982 found that primary cultures of human foreskin cells incubated with viable treponemes, resulted in adhesion of the organisms to the cultured keratinocytes. Destruction of the cells indicated as rounding of cells, loss of morphological structure and detachment from the solid surface was also observed. Once it was established that *T. pallidum* actively attached to cultured keratinocytes,

determining the mode of attachment would aid in clarifying the pathogenesis of primary syphilis.

2.8 Mechanism of adhesion

T. pallidum is capable of adhering to numerous cell lines in vitro. For the purpose of pathogenesis it is important to determine the means by which adhesion takes place. The basement membrane is an essential component of the dermis comprising of different acellular components. The ability of treponemal adherence to and passing through these components leading to subsequent entry into the circulatory system is important in understanding the pathogenesis of syphilis. Fitzgerald *et al* in 1984 found treponemal attachment to fibronectin and laminin with increasing number of bound organisms with the increasing concentration of these extra-cellular connective tissue matrix (ECM) components. Tests with collagen IV, collagen I and hyaluronic acid yielded similar results with attachment proportional to the concentration of ECM components and time of incubation.

Pre-incubation of *T. pallidum* with normal rabbit serum and serum with anti-treponemal antibodies showed a higher level of adhesion to ECM components in the absence of antibodies. This difference was not found for hyaluronic acid. The most likely explanation is that antibodies block specific adhesions indicating that the adhesion to hyaluronic acid is non-specific.

Fibronectin, a 220 kDa glycoprotein, is responsible for cell adhesion and cell repair. It also plays a role in blood clotting. It is present in an insoluble form in the ECM and in a

soluble form in body fluids. Alderete *et al* (1980) demonstrated re-arrangement of surface proteins of *T. pallidum*, when binding to fibronectin. Although fibronectin binding sites on *T. denticola* are located along the length of the treponemal cell, these appear to become clustered at one end when this organism attached to fibronectin (Ellen *et al*, 1994). A similar mechanism may explain why it was observed that *T. pallidum* adheres with the tip end to Hep 2 cells (Thomas *et al*, 1985).

Full genome sequencing has made it possible to determine the open reading frames of *T. pallidum.* This in turn has created the opportunity to investigate the different properties of its different proteins. It was discovered that the two treponemal proteins, Tp 0155 and Tp 0483, bind fibronectin and play a role in mediating *T. pallidum*-host cell interactions (Cameron *et al*, 2004).

A recognized laminin binding protein of *T. pallidum*, Tp 0751, was found to attach a multitude of laminin isoforms present on different host cells. This explains why *T. pallidum* interacts with a variety of tissues during infection. It was proposed that interaction with laminin facilitates treponemal traversal of basement membranes and progression of disease with subsequent tissue invasion (Cameron *et al*, 2005).

2.9 Interaction of *T. pallidum* with host cells and pathogenesis of syphilis

It has been demonstrated that *T. pallidum* adheres to cultured cells and is capable of traversing tissue barriers. Numerous studies have been conducted to determine the contributing factors to *T. pallidum* invasiveness.

Suspensions with viable treponemes were found to rapidly breakdown mucopolysaccharide indicating the presence of a mucopolysaccharidase. Serum taken between 10 and 35 days from rabbits that were inoculated intra-testicularly with *T. pallidum* tested positive for antibodies to bovine and streptomyces hyaluronidase using the Ouchterlony immuno-diffusion assay (Fitzgerald *et al*, 1979). *S*erum from the same rabbits was used in an adhesion inhibition experiment. Adhesion was indeed inhibited in the presence of serum. The authors concluded that *T. pallidum* interacts with cultured cells by means of the mucopolysaccharidase on the surface of *T. pallidum* and the acidic mucopolysaccharide on cells (Fitzgerald *et al*, 1979). The genome of *Treponema pallidum* sequenced in 1998 by Fraser *et al* does not contain hyaluronidase.

Olsen *et al* (1984) found that *Treponema denticola* attached to actively dividing epithelial cells but pre-exposure of epithelial cells to hyaluronidase resulted in decreased adhesion. This finding indicated that hyaluronic acid might be a receptor for attachment.

Once *T. pallidum* invades the skin it rapidly spreads through the circulatory system. Hyaluronidase was found to be associated with treponemal dissemination. *T. pallidum* has a surface related hyaluronidase. Anti – hyaluronidase inhibited attachment of *T. pallidum* to capillaries. Capillaries have a fairly thick hyaluronic acid layer on its outer

surface as compared to cultured cells. Amniotic membranes mimic blood and lymph vessels in its structure. Treatment of treponemes with anti-hyaluronic acid anti-serum before inoculation onto the hyaluronic acid rich side of the amniotic membrane inhibited treponemal penetration significantly. In vivo experiments showed that anti-hyaluronidase treatment of *T. pallidum* inhibited dissemination of treponemes from one rabbit testis to the other (Fitzgerald *et al*, 1987).

Endothelial cells lie below keratinocytes. It has been hypothesized that *T. pallidum* attaches to keratinocytes, passes through and invades the endothelial cells below. Sections of endothelial cell monolayers at 2 – 6 hours post inoculation viewed by electron microscopy revealed that intercellular junctions retained integrity although treponemes were present in 7 of 15 intercellular junctions that were visualized. Treponemes were present on the apical surface but more treponemes were found between the monolayer and plastic support than in the intercellular junctions (Thomas *et al*, 1988).

A section of complete abdominal wall of mice placed between the halves of a dialysis cell was used to determine transmigration. The experiment was carried out with several species of oral spirochetes and with *T. pallidum subsp pallidum*. Using darkfield microscopy and scintillation counting, *T. pallidum subsp pallidum* was found to transverse the tissue barrier while none of the other treponemes were able to pass through. When this test was performed with a mix of different treponemes, it was found that *T. pallidum* passing through did not create the opportunity for other treponemes to migrate, (Riviere *et al*, 1991).

The ECM component present in the largest quantity in the human dermis is type I collagen. *Treponema palllidum* was found to stimulate production of an interstitial collagenase (MMP 1) in human dermal fibroblast culture. MMP 1 mRNA expression was found to be up-regulated significantly in fibroblast cultures incubated with *T. pallidum*. The ability of *T. pallidum* to stimulate MMP 1 production in fibroblasts may well be a virulence factor of the organism. The MMP 1 would degrade the collagenous dermis after it invades human skin, (Chung *et al*, 2002).

2.10 Factors influencing the success of *T. pallidum* culture in-vitro

Even though viability of *T. pallidum* has been maintained in vitro using tissue culture, whether proliferation of the organism occurs still needs to be confirmed.
Although success was reported when cotton tail rabbit epithelial cells (Sf1Ep) at a confluency of 25% were inoculated with $1x10^6$ *T. pallidum*. With this system Fieldsteel *et al*, (1981) reported a 49.3 fold increase after incubation for 9 days. These findings were confirmed by Norris in 1982. Apart from this single report, in vitro replication of treponemes has only been observed with non-pathogenic species. Research to determine optimal growth parameters for *T. pallidum* is essential to enable future studies on this organism.

2.10.1 Atmospheric conditions

The observation that the presence of rabbit testicular tissue components prolonged the survival of *T. pallidum* suggests anaerobic metabolism. However, it was found that incubation under anaerobic conditions (95% nitrogen- 5% carbon dioxide) did not increase the rate of motility (Fitzgerald *et al*, 1975).

Cox *et al* in 1974, set out to either prove or disprove that *T. pallidum* was an anaerobic organism by measuring oxygen consumption and studying terminal electron transport with the aid of metabolic inhibitors of oxygen. The rate of oxygen uptake was found to be dependant on treponemal concentration, similar to that of the control aerobic spirochete Leptospira. Oxygen uptake was inhibited by cyanide and azide indicating cytochrome oxidase activity and this has led to the assumption that toxicity of air is a result of the accumulation of oxidized intermediates and not oxygen itself. In addition anaerobic conditions merely prevent the accumulation of these toxic intermediates.

When *T. pallidum* was exposed to oxygen there was an increase in the degradation of glucose and pyruvate to carbon dioxide. The end products of metabolism of glucose in the presence of oxygen were lactate, pyruvate and acetate where as under anaerobic conditions there was production of pyruvate and lactate with little accumulation of acetate. Under aerobic conditions there was a continual increase in end products where as under anaerobic conditions there was no further increase after 4 hours incubation. This suggests that oxygen plays a role in treponema metabolism. There was a linear increase

in protein synthesis over 24 hours in the presence of 20% oxygen but under anaerobic conditions protein synthesis ceased within 3-6 hours. There was a high rate of motility under low oxygen concentrations (1 – 20% oxygen) but a higher concentration of oxygen or anaerobic conditions rendered the treponemes non-motile (Baseman *et al*, 1976).

T. pallidum inoculated onto normal rabbit testicular cells were incubated under aerobic conditions as well as in 95% nitrogen – 5% carbon dioxide. At 24 and 48 hours post inoculation, tissue cells were inoculated intradermally into rabbits. Fifty six percent of the sites with culture grown under aerobic conditions were positive while with cells grown under the adjusted atmospheric conditions, 97% were positive. It was concluded that the anaerobic conditions maintained a higher level of virulence.

In the presence of reducing agents, incubation under aerobic conditions resulted in 100% virulence of intradermally infected rabbits. In addition, the cultured cells remained unaffected by the reducing agents. In contrast, exclusion of air in a reduced tissue culture environment supported survival only up to 48 hours incubation after which the treponemes died and tissue culture cells detached from the glass surface. In an effort to determine the limit of tolerance for oxygen, the optimal concentration was found to be 3% oxygen in a combination mixture of 95% nitrogen and 5% carbon dioxide which had no effect on the cell culture and maintained viability for up to 3 weeks, (Fitzgerald *et al*, 1977).

Gradient cultures revealed that the most number of motile treponemes were found in the area of the graph where the dissolved oxygen concentration was 1.5%. An increase

oxygen concentration of 3.5% showed a decrease in viability with a decrease in multiplication of treponemes over time as compared to a 1.5% oxygen concentration, (Fielsteel *et al*, 1981). Sf1Ep cultures inoculated with *T. pallidum* and incubated at a temperature of 33°C showed an optimum atmospheric range of 1.5 – 5% oxygen in which replication of *T. pallidum* was its highest ranging from 22.4 to 27.7 fold, (Fielsteel *et al*, 1982).

Under anaerobic conditions maximal attachment to cell lines was observed after 2-4 hours while attachment peaked at 24 hours under microaerophilic and anaerobic conditions. Motility retention was highest in anaerobic cultures. Oxygen limits the survival of *T. pallidum*. While DTT, a reducing agent, increased the viability of *T. pallidum* in vitro, it reduced it's attachment to BRGO cells, (Wong *et al*, 1983).

Since atmospheric condition was vital in the success of treponemal culture, the influence of atmospheric conditions during *T. pallidum* extraction was tested. There was no difference in aerobically and anaerobically extracted treponemes on the basis of motility after 24 and 48 hours incubation. However when treponemes were incubated anaerobically for 36 to 48 hours and then subjected to microaerophilic conditions (3% oxygen), treponemes survived longer, (Wong *et al*, 1982).

2.10.2 Temperature requirements

Determining optimal temperature requirements of *T. pallidum* for in vitro culture is relevant for the success of proliferation of the organism in in-vitro. The possibility exists that the establishment of infection in the human host is dependant on attachment to the cells of the dermis. Human skin has a temperature of approximately 33°C. Once the treponemes pass through the keratinocyte layer, they enter the circulatory system invading many tissues in the process. The host temperature is 37°C. Animal models are used to maintain a viable passage of *T. pallidum* for experimental purposes. The animal of choice is the New Zealand white rabbit. *T. pallidum* inoculated into the testes proliferates in an environment at 33°C, the body temperature of rabbits.

In vitro inoculation of *T. pallidum* onto cells obtained through culture of whole rabbit testes and Hep-2 cells displayed linear increase of attachment with increasing temperatures ranging from 25 to 37°C, (Hayes *et al*, 1977).
Fieldsteel *et al* in 1982 who claimed successful culture of *T. pallidum* in vitro found the optimum temperature range for the growth of *T. pallidum* to be 33 to 35° C in the presence of Sf1Ep cells and 10% foetal bovine serum in Basal Reduced Minimal medium (BRMM).

2.10.3 Nutritional requirements: Serum

It was found that foetal bovine serum at a concentration of 20% in a tissue culture system using Sf1Ep cells was optimal for treponemal replication. Calf serum was lot dependant producing varying results. Only one lot was comparable to foetal bovine serum where the average increase in the number of treponemes was 13.7 fold in calf serum and 16.5 fold in foetal bovine serum, (Fieldsteel *et al*, 1982). Variations in batches of calf serum may be related to variability in the protein concentration, the protein profile as well as the presence of antimicrobial agents used in the animals before serum collection.

Under microaerophilic and aerobic conditions it was found that the percentage motility of *T. pallidum* was directly proportional to the serum level in the media with 20% serum being the optimal concentration. When cells were starved of serum for 24 hours prior to infection, it was observed that a greater number of treponemes attached per cell, (Wong *et al*, 1983).

2.10.4 Nutritional requirements: Others

Treponemal survival was found to be superior using cultured cells as compared to media alone. In addition, the toxicity of oxygen for *T. pallidum* required the use of reducing agents in the media. Media requirements were assessed over the years to determine the optimal medium for growth of *T. pallidum*. However, this has still not been achieved.

It was found that the addition of superoxide dismutase to infected cell cultures prolonged the motility of *T. pallidum* and retained virulence of 24 hour cultures as indicated by skin lesions after 21 days of intradermal infection of rabbits. In contrast no lesions were formed after skin inoculation with culture without superoxide dismutase. This was evidence that superoxide dismutase eliminates the toxic effects of oxygen on the organism (Fitzgerald *et al*, 1975).

Addition of a combination of glutathione (1.2mg/ml) and cysteine (0.12mg/ml) effectively prolonged motility of *T. pallidum* inoculated onto normal rabbit testicular cells. The effects of this combination of reducing agents in the presence of air greatly increased survival and virulence of *T. pallidum*. All 12 intradermally infected sites became positive but only 33% of sites infected with cells without reducing agents produced positive lesions. Dithiothreitol (DTT) is a known reducing agent. DTT, added at a concentration of 2mM, prolonged the motility of *T. pallidum* (66 %). Addition of 4mM DTT resulted in precipitate formation in the media after 48 hours incubation resulting in DTT depletion. It was found that 2 mM of DTT retained the motility of *T. pallidum* best with 42 % motility after 140 hours of incubation. No precipitation was noted as seen with 4mM DTT (Fitzgerald *et al*, 1977).

In addition to the reducing agents the addition of normal rabbit serum or foetal bovine serum increased lesion formation (96% and 82% respectively). This effect was not seen with bovine serum albumin. Further testing using various concentration of foetal bovine serum concluded that the optimal range for maintenance of motility was between 5 – 30% foetal bovine serum.

When the various types of media were tested, maximal attachment of *T. pallidum* was observed using EMEM under both aerobic and anaerobic conditions. In addition, motility of *T. pallidum* was significantly greater in the presence of EMEM (Wong *et al*, 1983). Addition of pyruvate to the culture medium significantly increased the attachment of *T. pallidum* to cultured cells (Wong *et al*, 1984).

2.11 Structure of *T. pallidum* – EM observations

Ultra-thin cross-sections of *T. pallidum* viewed by electron microscopy indicated a diameter of 163 nm for the body and the diameter of the axial filaments lying outside the limiting membrane of the organism was 21 nm. In contrast, the non pathogenic *T. denticola* was a larger organism with an overall diameter of 224.9 nm. *T. phagedenis*, biotype *Reiter* was much larger than the previous two with an overall diameter of 331 nm. In addition, the axial filaments were seen in the space between the outer and inner membrane for both the non-pathogenic treponemes, (Sykes *et al*, 1973).

A report by Johnson *et al* in 1973 disagrees with the findings by Sykes *et al*, 1973. The electron micrographs reveal that *T. pallidum* does have an outer envelope and the axial filament are present between the outer envelope and the cell membrane, therefore *T. pallidum* is not morphologically different from the non-virulent *T. denticola* and *T. reiteri*.

Monolayer cultures of rabbit testicular cells inoculated with *T. pallidum* showed invasion of the cell line by *T. pallidum* at 30 minutes post inoculation. Intact organisms were detected intra and extracellularly up to 7 days post inoculation. Invasion is a virulence factor as no intracellular organisms of the non-pathogenic *Treponema* species were observed (Fitzgerald *et al*, 1975).

T. pallidum was found to adhere to normal rabbit testicular cells by one or both terminal ends as well as by body spirals (Hayes *et al*, 1977).

Scanning electron micrographs of Sf1Ep cells infected with *T. pallidum* showed that after attachment there were colonies of treponemes present on the surface of cells, the number of colonies increased over time. After 12 days incubation the cell monolayer deteriorated resulting in the break up of these treponemal colonies, (Fieldsteel *et al*, 1981). Concentrations of attached treponemes on Sf1Ep cells were also observed at the Dept of Medical Microbiology, UKZN, South Africa using fluorescent staining. Whether these are really bacterial colonies defined as groups of bacteria resulting from the multiplication of one colony forming unit is for debate.

Purified basement membrane isolated from rat kidney cortex and rat retinal vessels infected with *T. pallidum* revealed that treponemes attached to the basement membrane at their ends in much the same way as it did to the cultured capillary tissues and remained motile. These observations were made using scanning and phase contrast microscopy. In

addition, experiments illustrated that *T. pallidum* attaches to the extracellular matrix of different cell lines (Fitzgerald *et al*, 1984).

Freeze fracture techniques revealed that the outer membrane of *T. pallidum* contained scarce intramembraneous particles (IMPs). Due to its uniform size and random distribution it suggests that this may represent a single integral membrane protein. IMPs on the convex faces encircle the organism in spiral arrays suggesting that it may be associated with the periplasmic endoflagella. The fundamental structure of the outer membrane permits contact of antibodies to a restricted number of surface exposed proteins and represents a mode of evasion of host humoral defenses, (Radolf *et al*, 1989).

2.12 Immunogenicity of *T. pallidum*

In most micro-organisms, antigens with which immune effector molecules react are located on the surface of the cell. The application of immuno-electron microscopy (IEM) on treponemes of which the outermembrane was disrupted by repeated centrifugation and resuspension as well as incubation with Triton X-100 (0.1%), revealed that treponemes without an outer membrane were more immunoreactive to syphilitic immune rabbit serum than *T. pallidum* with intact outermembranes. In addition, antibodies directed against the 47kDa immunogen of *T. pallidum* found that this immunogen was intra-cellularly located. A radio-immunoassay, (*T. pallidum* surface specific radioimmunoassay (TpSSRIA)), was developed to measure the binding of antibodies to treponemes with and without outer membranes. With this method it was confirmed that

the major immunogens of *T. pallidum* were not surface exposed. It was hypothesized that the outer membrane of *T. pallidum* contained small amounts of transmembrane proteins and the major immunogens are anchored by lipids to the cytoplasmic membrane, (Cox *et al*, 1992). This structure would allow *T. pallidum* to evade the immune system

CD 8 cells were detected in primary and secondary syphilitic lesions. These CD 8 cytotoxic lymphocytes were activated as indicated by the detection of mRNA for granzyme B and perforin by reverse transcriptase PCR (Voorhis *et al*, 1996). It is therefore likely that, in the absence of a treponemal toxin, the tissue damage seen in syphilis is an immune mediated bystander effect.

CHAPTER THREE

METHODOLOGY

3.1 Proliferation and storage of Human Epithelial Cells

The HaCaT cell line (keratinocyte) was used. Professor N. E. Fusenig of the Cancer Research Center in Hieldelburg, Germany kindly donated this cell line to the Department of Medical Microbiology, Nelson R. Mandela School of Medicine, Durban in 1995. No antimicrobial agents were used in any of the cell cultures.

3.1.1 Resuscitation of cell line

The growth media for HaCaT cells is Roswell Park Memorial Institute (RPMI) 1640. A vial with frozen cells was removed from cryo-storage and thawed in a water-bath set at 37°C. Once thawed, the content was decanted into 10 mL of RPMI 1640 containing 10 % foetal bovine serum. This was then centrifuged at 1500 rpm for 10 min to remove the cryo-preservation medium. The supernatant was decanted and the pellet resuspended in 20 mL RPMI 1640 containing 10% foetal bovine serum in a 75cc flask. Cells were incubated at 37°C and observed every day for changes in morphology. The media was changed every three days or earlier if a change in pH occurred (as indicated by the pH indicator in the medium), until the monolayer reached confluency.

Confluent monolayers were rinsed thrice with phosphate buffered saline (PBS), pH 7.0 to remove all traces of foetal bovine serum. Two milliliters of 0.1 M EDTA was added to the monolayer and the flask was rotated to evenly distribute the EDTA. The flask was then incubated at 37°C for 5 minutes. Thereafter the EDTA was decanted and 2 mL of

trypsin-EDTA was added. Once again the flask was rotated to evenly distribute the trypsin. The trypsin-EDTA covered cell monolayer was then further incubated at 37°C for approximately 2 minutes or until cells detached when gently tapped. Thereafter, 60 mL of 10% foetal bovine serum in RPMI 1640 was added and the cells were gently mixed with the medium to obtain a homogenous suspension. Twenty milliliters of this suspension was then dispensed into each of three 75 cc flasks.

3.1.2 Cryo-preservation of cells

To maintain a stock of frozen cells the confluent monolayer was washed with PBS (pH 7.0) to remove all traces of foetal bovine serum. The monolayer was detached from the bottom of the flask as detailed above. Once detached, the trypsin action on the cells was stopped by addition of 1 mL of foetal bovine serum. This suspension was then aliquoted into a sterile tube and an equal volume of cryo-preservation medium (RPMI 1640 containing 20% glycerol) was added drop by drop. This suspension was then gently mixed and aliquoted into cryo-vials. These cryo-vials were placed in a - 20°C freezer for one hour and then up to 1 month at - 70°C or placed in liquid nitrogen for long-term storage.

3.2. Proliferation and Extraction of *T. pallidum*

The Nichol's strain of *T. pallidum* with a known concentration 2 x10^8 bacteria/mL was used to infect rabbit testis in order to proliferate the organism and achieve an inoculum

for the experiments. This strain was kindly donated by Dr. Allan Pillay, CDC, Atlanta, GA, USA.

3.2.1 Infection of Rabbit Testes

The propagation and extraction of *T. pallidum* in rabbit testes was as described by Fitzgerald *et al*, 1977, with slight modification.

The treponemal suspension obtained from CDC was a crude extract of infected rabbit testes containing *T. pallidum*, testicular cells and spermatozoa. Infection of rabbits was done at the Biomedical Research Unit at the Westville Campus, UKZN, under the supervision of trained personnel. The treponemal suspension was transported on ice from the Department of Medical Microbiology to the Biomedical Research Unit where it was thawed. An aliquot was viewed under darkfield microscopy for motility to determine viability of the organism prior to infection of rabbit testis. Rabbits of approximately 3 months old were anaesthetized by a single intramuscular injection of a mix of 0.6 mL xylazine (20 mg/mL) and 0.6 mL ketamine (100 mg/mL). Each of the testes was injected with 500 μL (~1 $x10^8$) of the treponemal suspension. The rabbit was observed daily for changes in behavior (eating and drinking) as well as enlargement of the testis. From day 4 to 11 post-infection the rabbit was administered cortisone acetate intramuscularly at a concentration of 6 mg/kg. When the orchitis reached its peak (approximately 12 days post inoculation) the rabbit was anaesthetized again and then euthanized by either intravenous or cardiac injection with 4 mL pentobabitone sodium (200 mg/mL). The rabbit testes were then removed and placed in Eagle's Minimal Essential Medium (EMEM)

containing non-essential amino acids and sodium pyruvate. This was then transported to the Department of Medical Microbiology for further processing.

3.2.2 Storage of *T. pallidum*

T. pallidum viability is best preserved in tissue. Therefore on arrival at the Department of Medical Microbiology, one of the testicles was placed in freezing fluid (50% fetal calf serum in PBS (pH 7.0) with 15% glycerol and 1 mM dithiotreitol) and stored at -70°C. The second testicle was used to perform the tests.

3.2.3 Extraction of *T. pallidum* from rabbit testes

The testicle was washed with PBS (pH 7.0) and freed from any non-testicular tissue using a sterile scalpel blade. The testicle was then minced using scalpel and forceps. This was placed in a beaker containing 5 mL of EMEM enriched with 20 % fetal bovine serum. The beaker was placed in an anaerobic jar with a CampyGenTM Compact sachet. This sachet produces a microaerophilic atmosphere within 15 minutes (Fig. 2).

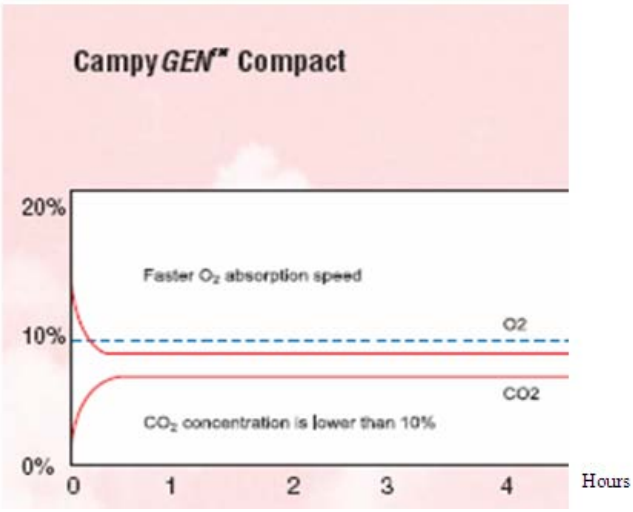


Figure 2: The above graph illustrates the time taken to achieve optimal microaerophilic conditions using the CampyGen TM Compact. At 15 minutes the system reaches microaerophilic conditions, (with permission www.oxoid.com).

This anaerobic jar was then placed on a rotary shaker for 30 min at 37°C. Following this the suspension was centrifuged at 500 x g for 10 min to separate the treponeme containing fluid from the testicular tissue. An aliquot of the supernatant was viewed under darkfield microscopy to assess viability of the treponemes.

3.3 Adhesion assay

The adhesion assay was performed to determine if *T. pallidum* was able to adhere to keratinocytes and the rate at which adherence occurs. Each experiment was done three times in triplicate.

HaCaT cells were seeded into the wells of a 24-well tissue culture plate and allowed to grow till approximately 80% confluency was reached. At this point the monolayer was washed three times in PBS (pH 7.0) and inoculated with 1 mL of a dilution of the

treponemal suspension in EMEM containing 20% foetal bovine serum. The plates were incubated at 37^{o}C under microaerophilic conditions using the CampyGenTM Compact system. At different times post inoculation (15 min, 30 min, 45 min, 60 min and 120 min) the non-adherent treponemes were removed by washing three times in PBS, (pH 7.0). The cells containing the adherent treponemes were detached from the bottom of the well by addition of 150 µL of trypsin-versene to the monolayer followed by incubation at 37°C for 15 min. The plates were then tapped gently to release the cells and trypsin action was stopped by the addition of 50 µL foetal bovine serum. The treponeme containing cell suspension was aliquoted into Eppendorf tubes. To collect remaining cells, the well was flushed with 100 µL PBS (pH 7.0) and this was added to the respective Eppendorf tubes. Quantitation of the number of adherent treponemes was done using Real Time PCR.

3.4 Migration Assay

HaCaT cells were grown in fetal bovine serum (10%) enriched RPMI 1640 on polycarbonate tissue culture inserts with a pore size of 0.3 µm (Fig. 3). Confluency was determined by monitoring the trans-epithelial electrical resistance (TEER) across the monolayer using the Milli-Cell ERS System. A constant TEER reading indicated a confluent monolayer (Hashimoto et al, 2008). Once 100 % confluency was reached, approximately four days post seeding, the monolayer was rinsed with PBS (pH 7.0) and placed in a well of another tissue culture plate containing 1 mL of EMEM with 20% fetal bovine serum. The cells were then inoculated with 150 µl of treponemal suspension

(approximately $3.69x10^6$ treponemes) in EMEM containing 20% fetal bovine serum. The inoculated cells were incubated at 37°C under microaerophilic conditions. At different time points post inoculation (0 hrs, 3 hrs, 6 hrs, 12 hrs, 24 hrs, 48 hrs) the inserts were placed into another well and the media below the insert, which contained the treponemes that passed through, was transferred into an Eppendorf tube. Quantitation was done using Real Time PCR.

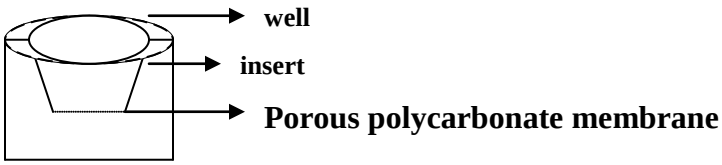


Figure 3: Polycarbonate insert within a well of a 24 well tissue culture plate.

One set of 3 wells was used for measurements at each time point. The medium from beneath the membrane harvested at each time point was stored at + 4^oC overnight. All of these were processed further at the same time.

3.5 DNA Extraction

DNA was extracted using Probetec® lysis buffer. This extracts DNA from both prokaryotic and eukaryotic cells. The cell suspensions containing treponemes were centrifuged at 12000 x g for 10 minutes and the supernatant discarded. The pellet was resuspended in 200 μL of Probetec® lysis buffer and placed in a heating block (113°C) for 30 min, (Caliendo *et al*, 2005).

3.6 Real-Time PCR

The primers described by Chen *et al*, 2006, targeting the polymerase A gene of *T. pallidum* were used. These were synthesized by Applied Biosystems for use in the ABI SDS 7000^{TM} equipment. The master mix comprised of 2.5 µL each of 18 µM forward and reverse primer, 2.5 µL of 5 µM probe, 25 µL of a 2 x Universal PCR Mix, 12.5 µL water and 5 µL DNA. Real-time PCR was carried out in a 96 well optical reaction plate, using the ABI SDS 7000^{TM} system. Ten fold serial dilutions of a known concentration of *T. pallidum* were used to construct a standard curve. The ABI SDS 7000 is a fully automated system that generates a standard curve for each test series and automatically calculates the CT values. Figure 4 is a standard curve produced by the instrument during a test run.

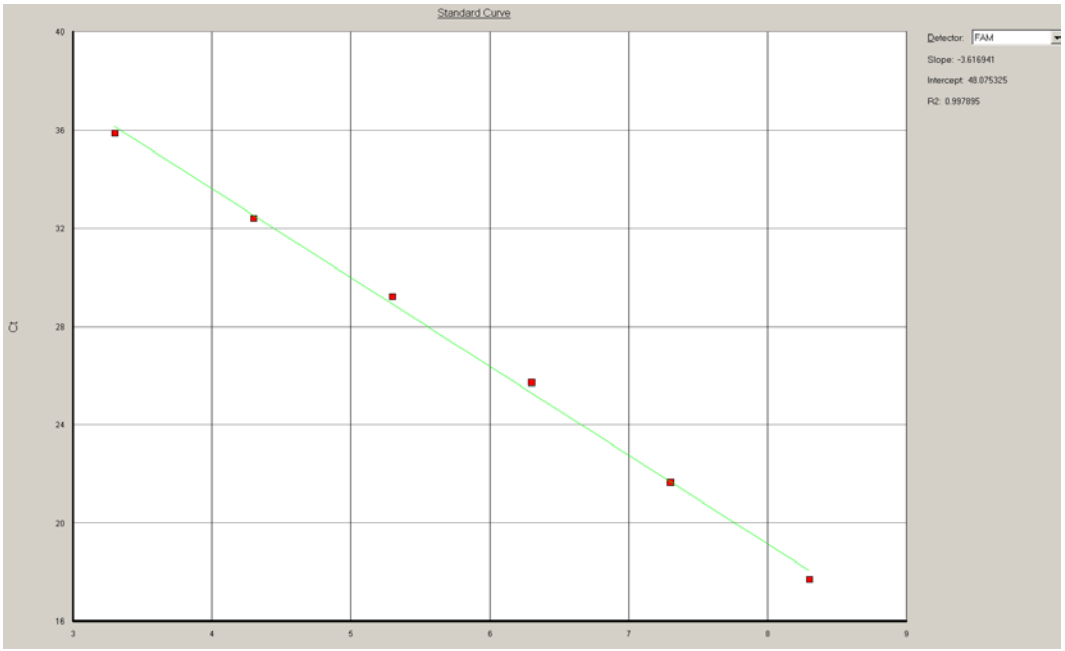


Figure 4: Representation of a standard curve produced by the ABI SDS 7000 for quantitation assays

3.7 Invasion Assay

To determine whether *T. pallidum* invades keratinocytes, transmission electron microscopy was employed. For this assay 25 cm^3 tissue culture flasks were seeded with HaCaT cells. Once confluent the monolayer was washed with PBS (pH 7.0) and inoculated with 1 mL of a treponemal suspension in EMEM containing 20% foetal bovine serum. The cells were incubated at 37°C under microaerophilic conditions. At different time points post inoculation, (3 hrs, 6 hrs, 9 hrs, 12 hrs, 16 hrs, 20 hrs), the monolayer was washed once with PBS (pH 7.0) and fixed by incubation for 2 hrs at room temperature in 2 mL of 2% gluteraldehyde in EMEM. The cells were further processed for transmission electron microscopy as described in table 1.

Table: 1 Processing steps for transmission electron microscopy

STEP	REAGENT	TEMPERATURE	TIME
Wash (twice)	EMEM (5 mL)	Room temp (22-24°C)	5 min each
Wash	Sodium Cacodylate buffer 0.2 M, pH 7.2 (5 mL)	Room temp (22-24°C)	5 min
Post fixation	1% Osmium tetroxide	Room temp (22-24°C)	45 min
Wash (twice)	Sodium Cacodylate buffer 0.2 M, pH 7.2 (5 mL)	Room temp (22-24°C)	5 min each
Dehydration	50 % Ethanol	Room temp (22-24°C)	10 min
Dehydration	70 % Ethanol	Room temp (22-24°C)	10 min
Dehydration	90 % Ethanol	Room temp (22-24°C)	10 min
Dehydration (twice)	100 % Ethanol	Room temp (22-24°C)	10 min
Infiltration	Ethanol : spur resin (1:1)	Room temp (22-24°C)	30 min
Infiltration (twice)	spur resin	60°C	60 min
Embedding	spur resin	60°C	24 – 48 hrs

Blocks were trimmed and cut into sections and viewed using the JEOL 1011 transmission electron microscope.

3.8 Occludin assay

To determine the means by which *T. pallidum* passes through a keratinocyte layer, the occludin assay was performed. Occludin is a 65 kDa trans-membrane protein. This assay

looked at an increase in soluble occludin, where soluble occludin is an indicator of the disruption of tight junctions.

3.8.1 Triton extraction

The methodology used for the extraction of occludin was based on the publication by Chen *et al*, 2003. For the occludin assay, 24-well tissue culture plates were seeded with HaCaT cells. Once confluent the monolayer was washed with phosphate buffered saline and infected with 1 mL of a diluted treponemal suspension in EMEM containing 20% foetal bovine serum and incubated at 37°C under microaerophilic conditions. At different times post inoculation (0 hrs, 1 hr, 2 hrs, 4 hrs, 8 hrs, 12 hrs, 16 hrs, 20 hrs), the supernatant was discarded and the monolayer was washed three times with cold PBS, pH 7.0. The cells were scraped off the culture plate and centrifuged at 2500 rpm for 10 min. The supernatant was discarded and 200 µL of extraction buffer (50 mM MES, 3 mM EGTA, 5 mM $MgCl_2$, and 0.5% triton X-100 at pH 6.4) was added to the pellet. After gentle pipetting on ice, it was further incubated for 30 min on ice. Thereafter the tubes were centrifuged at 12,500 rpm for 30 min to recover the soluble occludin in the supernatant. Extraction buffer (200 µL) was added to the pellet, mixed gently and incubated for 30 min on ice. Thereafter the tubes were centrifuged at 12,500 rpm for 30 min to recover the insoluble occludin in the supernatant.

3.8.2 Protein blots

Protein blots were performed on the extracts of keratinocyte monolayers produced as described in 2.8.1, as per protocol of Chen *et al* (2003). Proteins were separated by SDS PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with blocking buffer (0.5% skimmed milk in PBS, pH 7.0) for one hour at room temperature. After three washes in PBS (pH 7.0) containing Tween-20 (rinsing and diluting solution), the membrane was incubated overnight in the cold room with (2µg/mL) rabbit anti-human occludin, followed by incubation with 1:10,000 peroxidase-labelled goat ant-rabbit IgG for 45 min. ECL detection, using luminol was done on the BioRad ChemiDoc system.

3.8.3 Immunostaining

To confirm the results of the protein blots an immunostaining experiment was performed according to Spoerri *et al*, 2006. In this experiment the redistribution pattern of occludin when subjected to detergent was examined. Confluent HaCaT cell monolayers grown in 24-well tissue culture plates were inoculated with *T. pallidum*. At different times post inoculation (0 hrs, 1 hr, 2 hrs, 4 hrs, 8 hrs, 12 hrs, 16 hrs, 20 hrs) the wells were washed with PBS (pH 7.0) three times, and fixed for 10 min at – 20^{o}C with 1 mL of a freshly prepared mix of 96 % methanol and 4 % acetone. This was followed by addition of 500 µL of 0.2 % Triton X-100 in PBS (pH 7.0) for 5 min at room temperature to permeabilise the cells. After three washes with PBS (pH 7.0) the monolayer was then blocked with 0.5

% BSA in PBS (pH 7.0). A further three washes with PBS (pH 7.0) was done followed by incubation with fluorescein isothiocyanate (FITC) conjugated mouse anti- human occludin (2µg/mL) for 1 hour at room temperature on a rotary platform in a dark room. After three washes with PBS (pH 7.0) the cells were viewed at 200 x magnification and photographed using a fluorescent microscope.

3.9 Ethical considerations

Ethical approval for the use of animal models was obtained from the UKZN research committee, animal ethics sub-committee (Ethics no. 013/07/Animal). The numbers of rabbits required was kept at a minimum. Rabbits were anaesthetized before invasive procedures.

3.10 Data Analysis

Statistical analysis was done using SPSS Version 15.0. The Post Hoc Bonferroni test was applied to determine differences of bacterial adhesion over time. Significance was determined as a *P* value less than 0.05.

Inter-test variation was assessed by calculating the coefficient of variation (CV). A CV of ≥1 verifies a significant difference.

CHAPTER FOUR

RESULTS

4.1 Adhesion of *T. pallidum* to HaCaT cell monolayers

The adhesion assay was performed 3 times in triplicate. Figure 5 shows the results as the average of all 9 experiments (mean standard deviation shown at each time point) while the statistical evaluation is shown in Table 2.

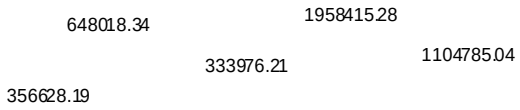


Figure 5: Adhesion of *T. pallidum* to HaCaT cell monolayers. Mean ±SD shown at each time interval.

The data show that *T.pallidum* does adhere rapidly to keratinocytes. Adhesion approaches saturation at 15 minutes. However, there was still a significant increase in the number of adhered treponemes after 30 minutes of exposure. No further increase was found between 30 and 120 minutes.

Table 2: Post Hoc Bonferroni adjusted multiple comparison test between the different time points using log adhesion as the dependant variable

Time 1 (min)	Time 2 (min)	Mean Difference	Standard Error	*P* =
15	30	-0.5924	0.12488	< 0.001
30	45	0.1599	0.12488	1
45	60	-0.2139	0.12488	0.944
60	120	-0.0288	0.12488	1

The experiments were shown to be reproducible with no significant variance between tests as shown in table 3.

Table 3: Inter-test variation of 9 observations of adhesion of *T. pallidum* to a HaCaT monolayer

Time (min)	Mean (SD) no. of organisms	CV
15	5.68 (0.24)	0.04
30	6.28 (0.14)	0.02
45	6.12 (0.12)	0.02
60	6.33 (0.47)	0.07
120	6.36 (0.21)	0.03

4.2 Transmigration of *T. pallidum*

Confluent monolayers of HaCaT cells grown on the membrane of Transwell^{TM} inserts were inoculated with approximately 3.69×10^6 *T .pallidum* cells. Figure 6 is a picture of an uninoculated cell monolayer at a constant TEER stained with FITC labeled anti human E-cadherin. This clearly shows that there is confluency and that the cells do adhere to each other.

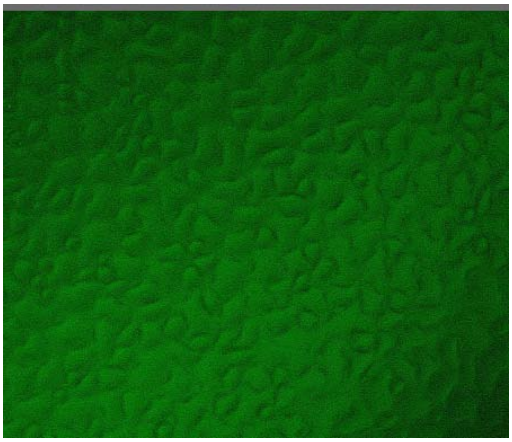


Figure 6: Uninfected HaCaT cells stained with FITC labeled anti human E- cadherin. This figure shows that HaCaT cells do form confluent monolayers

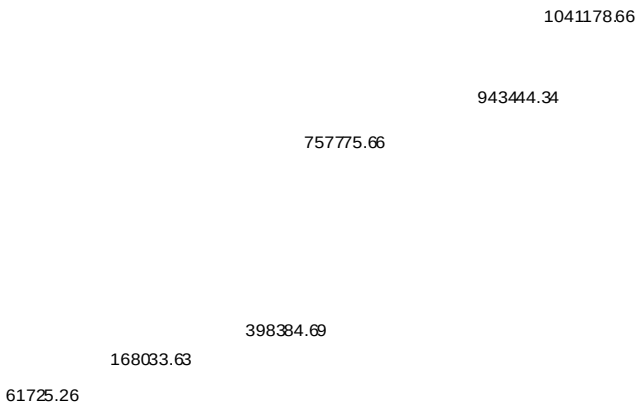


Figure: 7 Migration of *T. pallidum* through a keratinocyte layer

Figure 7 shows the absolute number of *T. pallidum* organisms that have passed through a keratinocyte layer over time. Table 4 shows the cumulative percentage of *T. pallidum* that

passed through the keratinocyte layer. After 24 hours of incubation, more than 70 % had traversed the cell monolayer.

Table 4: Migration (%) of *T.pallidum* through a HaCaT monolayer

time (hrs)	cumulative %
0	1.03
3	7.94
6	20.35
12	47.19
18	63.14
24	70.96

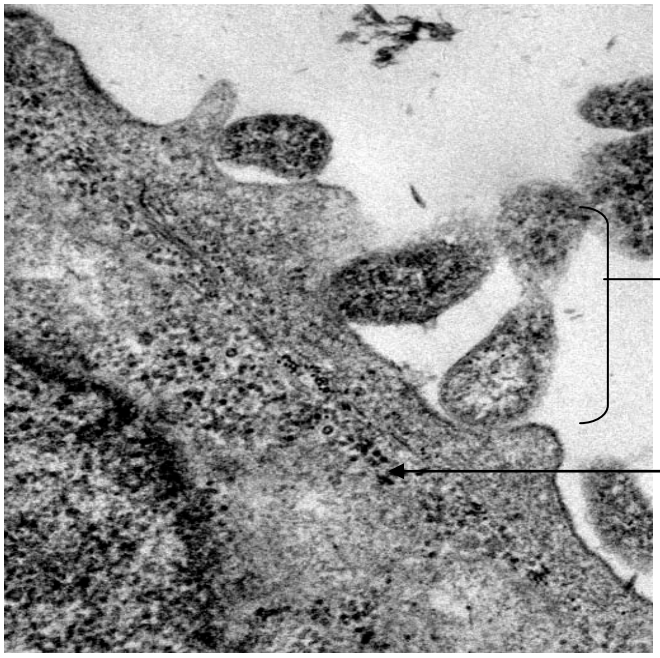
There were no significant differences between the 9 migration experiments at each time point ($p > 0.05$) as shown in table 5. This indicates minimal inter-test variation and high reproducibility. The variation at time 0 is due to the absence of data during one experiment as there were PCR inhibitors present in the specimen.

Table 5: Inter-test variation of 9 observations of migration of *T.pallidum* through a HaCaT monolayer

Time (hrs)	Mean (SD) no. of organisms	CV
0	38040 (61725)	1.6
3	293241 (168033)	0.6
6	750856 (398384)	0.5
12	1741486 (757776)	0.4
18	2325696 (943444)	0.4
24	2618337 (1041179)	0.4
Total	1318318 (1187536)	

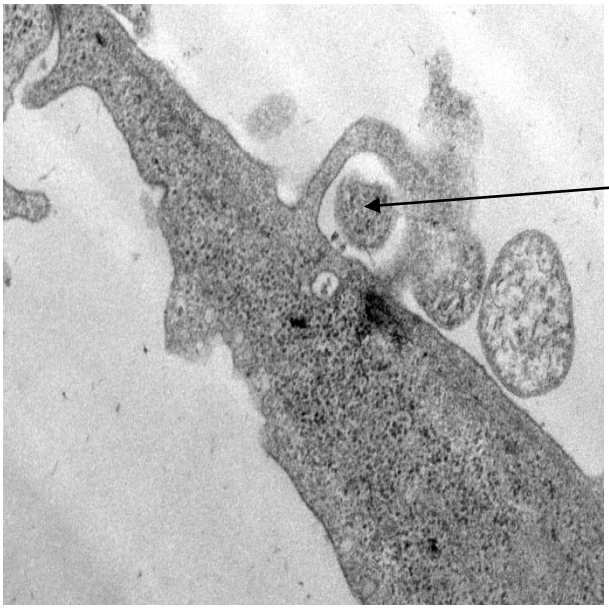
4.3 Transmission Electron Microscopy of HaCaT cells exposed to *T. pallidum*

Transmission electron microscopy was performed to observe the position of the *T.pallidum* cells in relation to the keratinocytes while they passed through the keratinocyte layer. The photographs show the results after 3 hours of incubation. The results at other time points are not shown as bacteria were observed in all positions at each time point from 3 to 24 hours. *T.pallidum* cells were found adherent to the cell surface of the keratinocyte (Fig. 8), in membrane bound compartments within the keratinocytes (Fig 9 and 10) and underneath the cells (Fig 11).



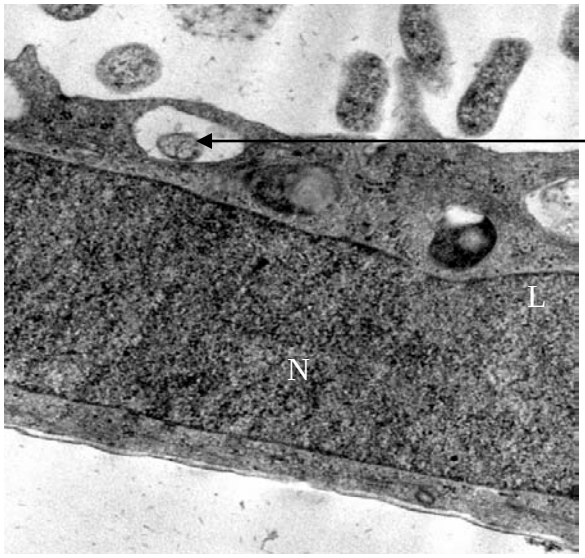
X60 000

Figure 8: Adhesion of *T. pallidum* to HaCaT cells surface 3 hrs post inoculation



X 40 000

Figure 9: Electron micrograph depicting typical axial filaments (The two dark dots between the section through a treponeme and the membrane of intra cellular vacuole) of *T. pallidum* in close proximity to plasmalemma of a HaCaT cell



X 30 000

Figure 10: Interaction of *T. pallidum* with a HaCaT cell at 3 hrs post inoculation. This electron micrograph illustrates T. pallidum within an intra cellular vacuole in close proximity to plasmalemma of the HaCaT cell. Nucleus (N) and lysosome (L).

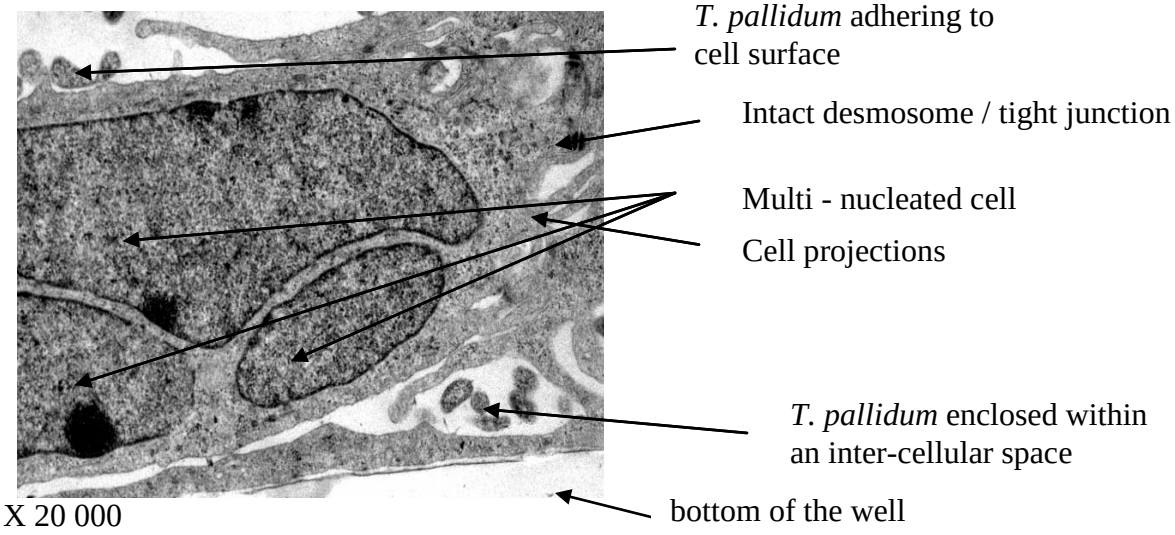


Figure: 11 Multinucleated HaCaT cell with *T. pallidum* adhering to its surface as well as underneath the cell

4.4 Effect of *T. pallidum* infection on tight junctions of HaCaT cell monolayers

4.4.1 Immunoblotting of occludin

Confluent HaCaT cell monolayers were exposed to *T.pallidum* to observe whether occludin was solubilised by the bacteria. Figure 12 and fig. 4 shows that this is not the case. Fig. 12 shows the presence of non-solubilised occludin after exposure to *T.pallidum* for up till 20 hours. Fig. 13 shows the absence of the solubilised protein over the same period of exposure. Fig. 14 shows the Western blot using a human occludin specific antibody. This confirms that the 65 kDa band in fig 12 is indeed occludin.

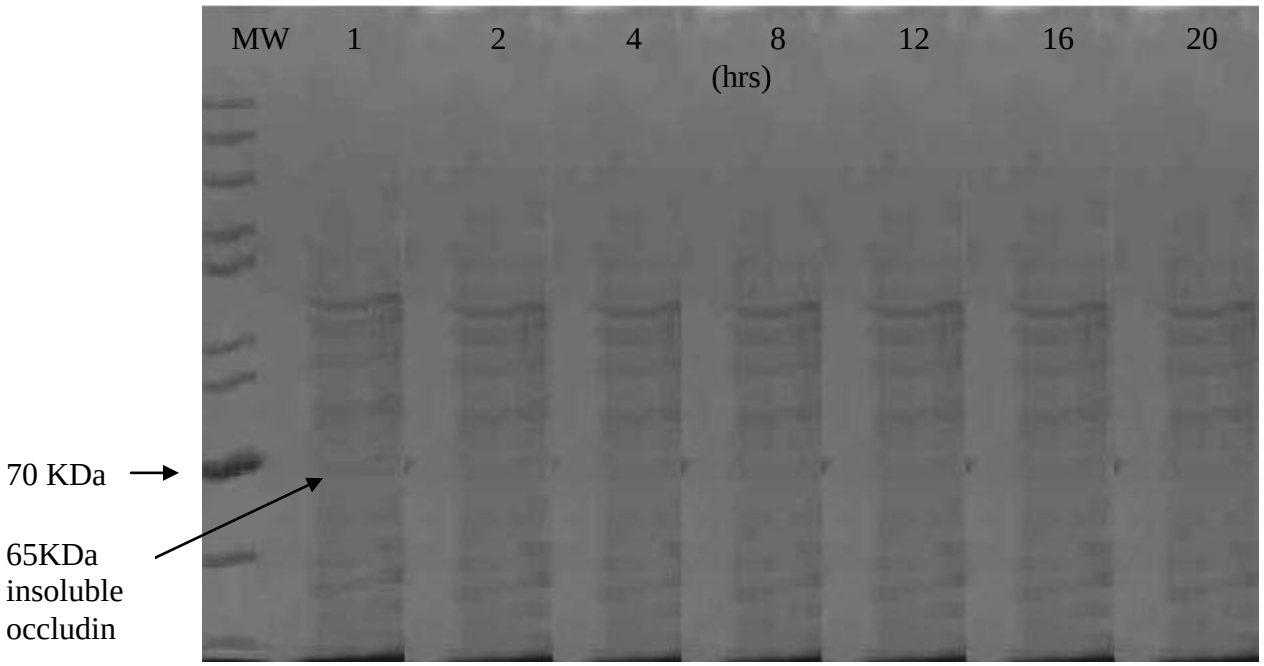


Figure 12: Detergent insoluble proteins run on SDS page and stained with coomassie blue.

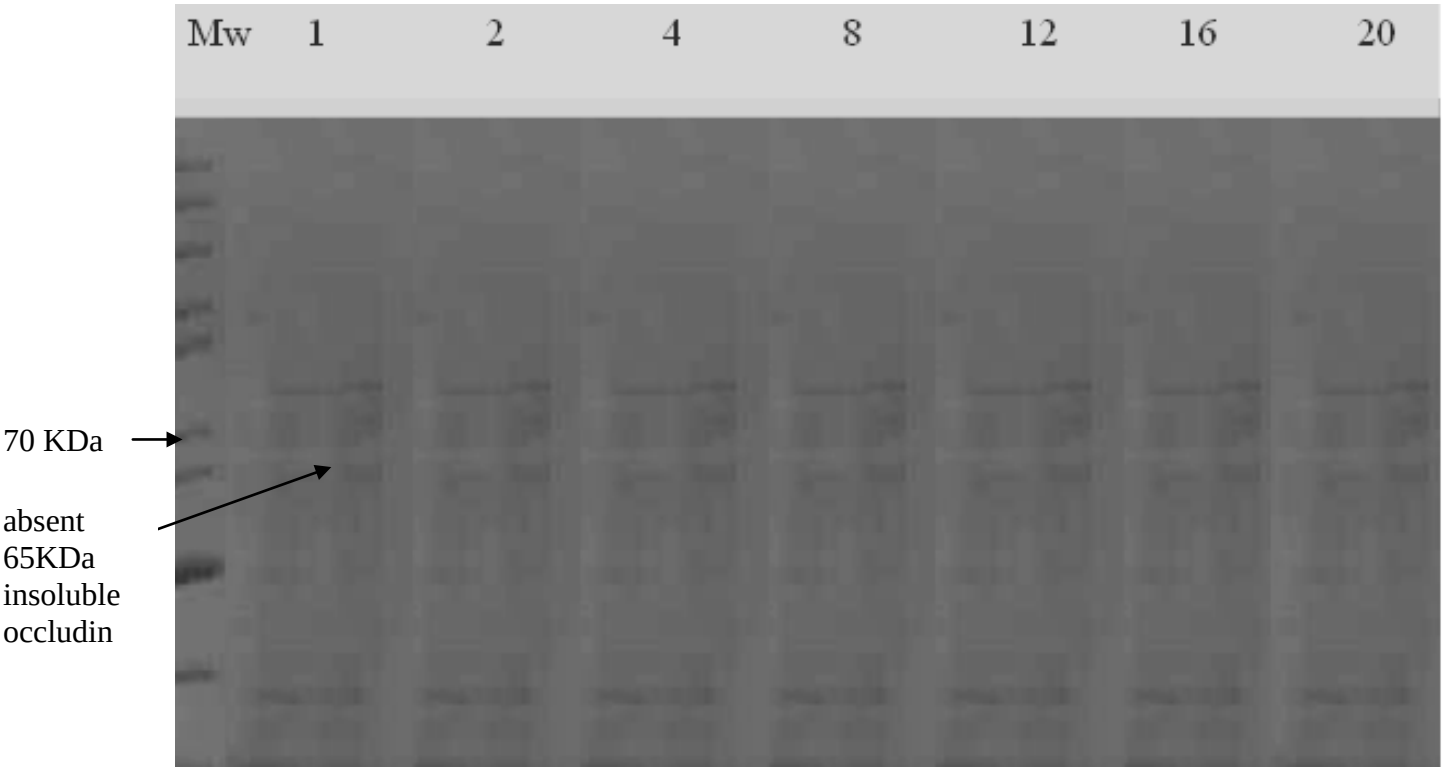


Figure 13: Detergent soluble proteins run on SDS page and stained with coomassie blue.

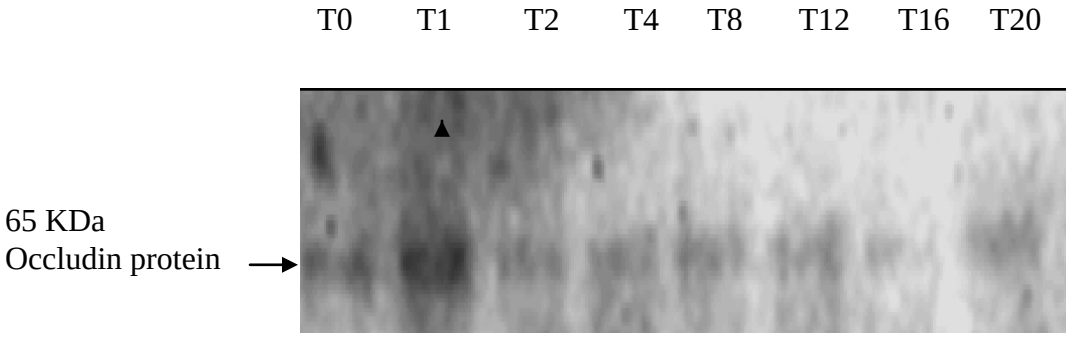


Figure: 14 Western blot of after protein extraction from the monolayer confirming that the 65 kDa protein shown in Fig. 12 is occludin.

Infection of HaCaT cell monolayers with *T. pallidum* had no effect on tight junction integrity. Detergent extraction of tight junction proteins separates disrupted occludin into the soluble fraction while intact occludin remains in the insoluble fraction. Figure 12 represents the insoluble fractions of total protein isolated from HaCaT cell monolayers infected with *T. pallidum.* A 65 kDa protein is present at all time points. Occludin is a 65KDa protein. To determine whether this 65KDa protein is indeed occludin, Western Blotting was performed (fig. 14). This positively identified the 65KDa protein as occludin.

4.4.2 Immunostaining of occludin

Direct immuno-fluorescence was performed on HaCaT monolayers to determine the positioning of the occludin protein. The pictures in Fig. 15 show the results at different time points. Occludin can be seen in between the cells in all pictures. This again confirms that the protein is not solubilised by treponemal activity.

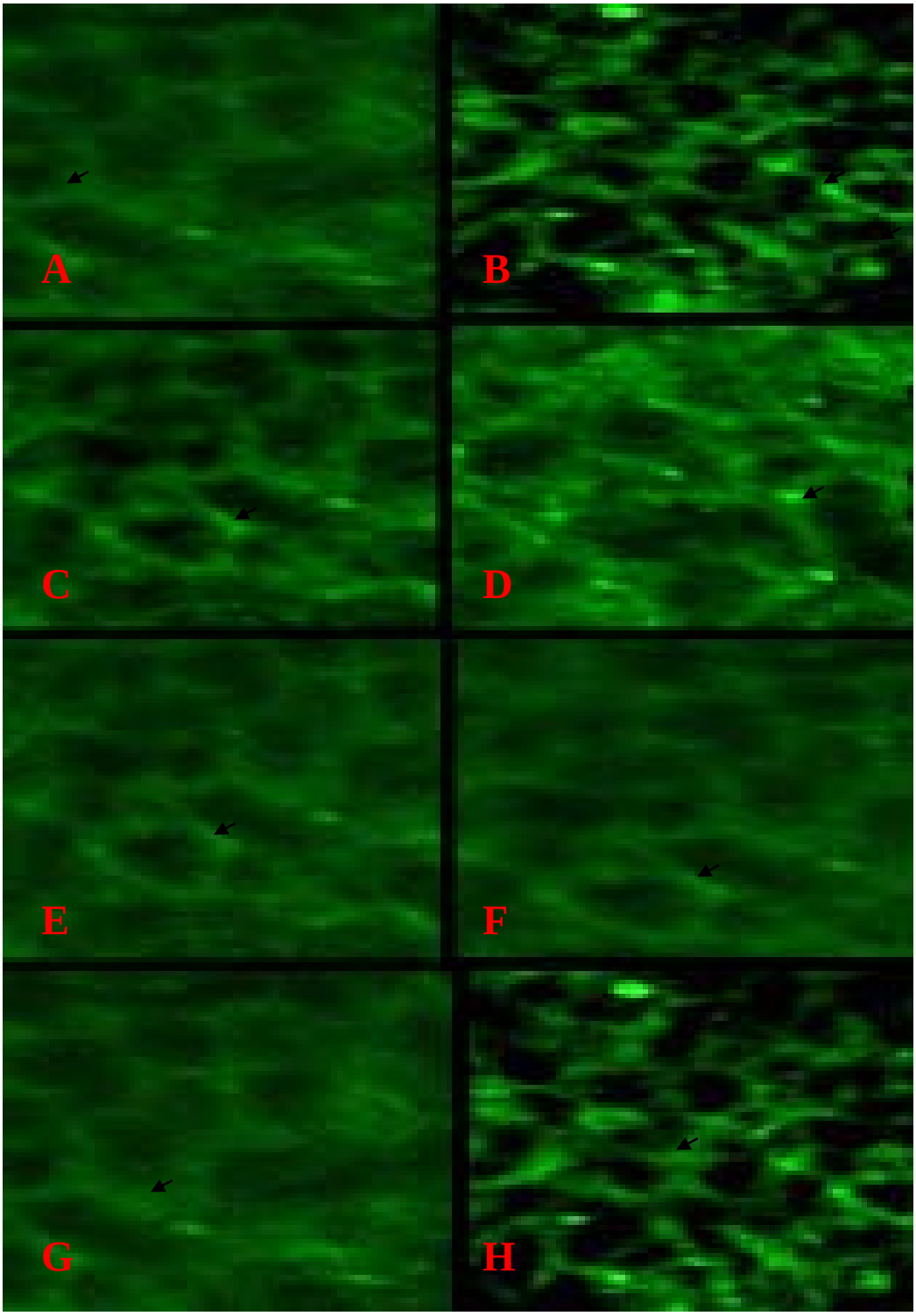


Figure 15: Immunofluorescent staining of occludin. Arrows represent intact occludin present between HaCaT cells infected with *T. pallidum.* The time post-inoculation: A – 1 hr, B – 2hr, C – 4 hr, D – 8 hr, E – 12 hr, F – 16 hr, G – 20 hr. H is the uninfected control

CHAPTER FIVE

DISCUSSION

The pathogenesis of syphilis is not clearly understood. Extensive studies in its single host, the human being, have resulted in a thorough understanding of disease progression from localized primary to generalized secondary syphilis followed by the tertiary stage. It has also been observed that although the disease is localized in the primary stage, the bacteria are already in the blood stream. Information on the mechanisms through which *T. pallidum* achieves this progressive spread in the human body is scarce. This is to a large extent because in-vitro studies are hampered by the inability to grow the organisms. It is likely that micro-abrasions of the epithelium which expose deeper located cell layers play a role in the penetration of *T. pallidum* through the epithelium (Fig 16).

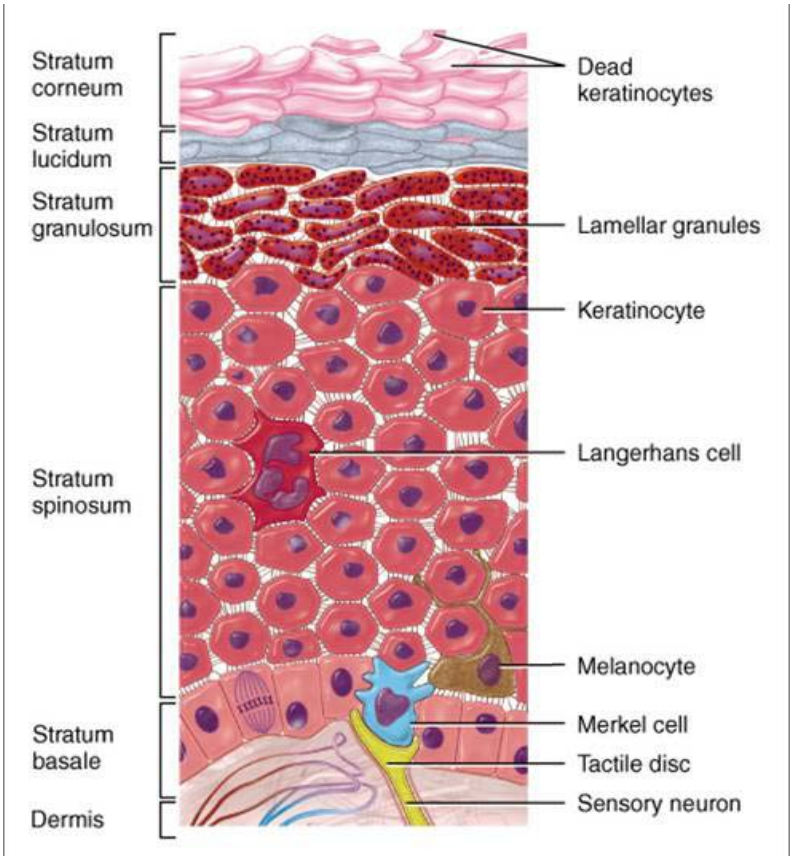


Figure 16: Different layers of the epidermis (http://www.imperial.edu/~thomas.morrell/Picture2.jpg)

The work presented here focuses on the mechanism through which *T. pallidum* enters the human body by studying the interaction between a human keratinocyte cell line and the Nichols strain of *T. pallidum* harvested from rabbit testicular tissue. This cell type is an obvious choice since a keratinocyte layer is in most patients the first barrier that the microbes have to pass to get into the host. HaCaT cells behave in much the same way as normal human keratinocytes as shown by Boukamp *et al*, 1988. HaCaT cells were able to form an epidermal tissue that maintained a differentiated, ordered structure when transplanted onto nude mice. In addition, differentiation specific keratins were expressed.

Fitzgerald *et al* reported in 1977 that *T. pallium* (Nichol strain) was capable of adhering to a variety of cell lines, including the HSE human epithelial cell line. It was noted that adhesion occurred almost immediately after inoculation. The failure of heat killed treponemes to adhere to cultured cells suggests that the adhesin is proteinaceous. The failure of adhesion of non- pathogenic treponemes to cultured cells further indicates that adhesion is a virulence attribute. While Thomas *et al,* 1988 found a linear relationship of adhesion of *T. pallidum* to HeLa cells over six hours, our study showed that maximum adhesion to keratinocytes was achieved at 30 min post inoculation with no significant increases thereafter. Our observation is not a technical limitation as in other experiments (data not shown) 10^8 organisms were detected. This difference may be explained on the basis of the different cells used. We used keratinocytes while Thomas *et al* used HUVEC, RAEC and HeLa cells. There are also differences in methods to quantify the treponemes.

The migration assay was designed to determine whether *T. pallidum* was capable of transmigration through a keratinocyte layer. The reliability of this migration assay was dependant on the ability of the cells to form confluent monolayers. This was observed as shown in fig. 6. Data from the migration assay reveals that *T. pallidum* is capable of traversing a monolayer of human keratinocytes. This suggests that the microbes might be able to pass other epithelial barriers as well. . This is consistent with experiments performed by Riviere *et al*, 1991 in which *T. pallidum* was found to traverse an in vitro model of a mouse abdominal wall and that of Thomas *et al*, 1988, who demonstrated the ability of *T. pallidum* to traverse endothelial cell monolayers. Different from ours, work by Fitzgerald *et al* (1982) suggests that passing through epithelial barriers might result from cell destruction. They observed this on primary foreskin cells that include keratinocytes. However, the inoculum was approximately 100 times higher than what we used. While this does explain the destruction of the epithelial lining at the site of the lesion as well as the observation that the number of treponemes in a chancre is very high, it cannot account for entry of treponemes at the time of infection as the number of transmitted organisms is unlikely to be high enough. Our work shows that the organisms traverse the keratinocyte layer in a time dependent fashion, leaving the keratinocytes intact as indicated by the presence of occludin in the insoluble fraction (fig 12).

Numerous organisms pass through cellular barriers by disruption of tight junctions, (Chen *et al*, 2003). Experimental results of Thomas *et al*, 1988 suggested that *T. pallidum* passes through endothelial cells by invasion of intercellular junctions as indicated by electron micrographs of *T. pallidum* present in intercellular junctions. However, intracellular

treponemes were also detected. This study shows *T. pallidum* in close proximity to the plasmalemma of the HaCaT cell

(fig 9), present intracellularly in a membrane bound compartment (fig 10) and beneath the monolayer (fig 11). Although this observation may not be conclusive on its own, it supports the results obtained with the transwell transmigration experiments and the occludin test. Detergent solubilisation of occludin was used to determine whether *T. pallidum* passed through intercellular junctions. Data revealed that occludin remained intact (present in the insoluble fraction, fig. 12) for the duration of the experiment although migration and electron microscopy results revealed that approximately 10 % of the inoculum of *T. pallidum* had already passed through the monolayer in 3 hr. In addition electron micrographs (fig 11) show the presence of intact tight junctions while treponemes are present inter-cellularly.

The classic teaching is that minor abrasions of the skin or mucosa are an important part of the pathogenesis of primary syphilis. Our findings do not oppose this since adhesion can only take place to viable cells. Therefore, abrasion of the epithelium to the level of viable cell layers remains essential.

The observations in this study conclude that *T. pallidum* is able to migrate through an intact layer of kerationocytes. This suggests that during natural infection *T. pallidum* transmigrates the intact epidermis. The ability of *T. pallidum* to adhere to different cell lines, (Fitzgerald *et al*, 1977), explains the ability of *T. pallidum* to concentrate in numerous tissues and organs during infection.

The specific interaction of *T. pallidum* with different cell lines, (Fitzgerald *et al*, 1977) and the ability to traverse the monolayer without disruption of tight junctions as seen in this study provides evidence that *T. pallidum* most likely passes through an epithelial barrier by specific interaction with the apical membrane and exits via the baso-lateral membrane. Therefore any changes to the surface of the epithelial cell will result in the failure of adhesion of *T. pallidum* and the subsequent failure to establish infection. The next step in this project would be the use of polarized cells which have differentiated apical and basal membranes to determine if in fact *T. pallidum* does adhere to the apical surface only and exits via the basal membrane.

Syphilis is known as the great imitator as patients have been reported to present with many different clinical manifestations (Fitzgerald *et al*, 1981). In this era of HIV, the diagnosis of syphilis has become more difficult as HIV changes the presentation of many diseases including syphilis (Hall *et al*, 2004). Karumudi *et al* (2005) stated that there was a higher rate of asymptomatic primary syphilis in HIV-infected individuals and that more HIV-positive patients presents with symptoms secondary syphilis.

In a review article by Lynn *et al* (2004) it has been reported that there was a shift from primary to secondary disease presentation in HIV-positive patients, in addition, there are reports of patients who have typical features of primary or secondary syphilis but have negative serological results. However, observations in Kwa-Zulu-Natal do not confirm this (Sturm *et al*, personal communication).

Rompalo *et al* (2001) reported on a group of 214 patients presenting with general ulcers over a period of 3 years to a STD clinic in Baltimore. These authors found a higher prevalence of primary syphilis in HIV-infected versus HIV-uninfected patients.
We found the opposite in 1245 patients reporting with genital ulcers to an STD clinic in Durban. HIV-infected patients had a lower risk of presenting with primary syphilis as compared to the HIV-uninfected. This is shown in Table 6 which summarises observations in patients with genital ulcer disease attending the Prince Cyril Zulu Centre for Communicable Diseases in Durban between 2000 and 2007 (Sturm et al, unpublished data).

Table 6: Etiology of ulcers in a cohort of HIV infected and HIV uninfected patients

	Prevalence (%)			
	HIV infected (n=931)	HIV uninfected (n=314)	RR (95% CI)	p=
T.pallidum	5	10	0.78 (0.65 - 0.78)	0.001
H.ducreyi	6	6	1.01 (0.88 - 1.15)	0.9
HSV	50	44	1.06 (0.99 - 1.13)	0.08
C.trachomatis (LGV)	12	8	1.09 (1.00 - 1.20)	0.07
no aetiology	30	34	0.95 (0.89 - 1.02)	0.2

RR = relative risk

There are several possible explanations for this observation. Firstly, HIV infected keratinocytes could have altered membranes, preventing the transmigration of treponemes. Another possibility is that keratinocytes in HIV infected patients have an altered chemokine expression profile, resulting in a different local immune response. Since tissue damage leading to ulceration is likely the result of the immune response (Fitzgerald *et al*, 1982, McBroom *et al* 1999), such a difference might prevent ulceration

but not dissemination of infection. This can be elucidated by in vitro studies using the methodology presented here.

CHAPTER SIX

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CHAPTER SEVEN

APPENDICES

7.1. Media and solutions

7.1.1 Cryo-preservation medium

7.1.1.1 Cryo-preservation medium for testes

5 mL Foetal bovine serum

1.5 mL Glycerol

10 µL DTT

Add the above reagents together and make up to 10 mL using PBS, pH 7.0. Store at 2 - 8º C

7.1.1.2. Cryo-preservation medium for HaCaT cells

8 mL RPMI 1640

2 mL glycerol

Add together the above reagents and store at 2 - 8º C.

7.1.2. Buffers

7.1.2.1. Phosphate buffered saline (PBS)

PBS tablets

Triple distilled water

Add 1 PBS tablet to every 100 mL triple distilled water. Dissolve tablet and autoclave solution. Store at 2 - 8º C.

7.1.2.2. Sodium Cacodylate buffer 0.2 M, pH 7.2 (5 mL)

$Na(CH_3)_2AsO_2*3H_20$ (MW – 195.92)	42.8 g
Double distilled water	1 L

Weight out $Na(CH_3)_2AsO_2*3H_20$ and dissolve in double distilled water. Make up to 1 L. Store at 2 - 8º C.

7.1.2.3 EDTA (0.5 M)

Triple distilled water	1 L
$Na_2EDTA-2H_20$	186.1 g

Dissolve in 700 mL water. Adjust pH to 8.0. Make up to 1 L. Autoclave and store at room temperature.

7.1.3. SDS PAGE solutions

7.1.3.1 Monomer solution

Acrylamide	60 g
Bis-Acrylamide	1.6 g
Triple distilled water	200 mL

Dissolve reagents in water. Store at 2 - 8º C in the dark.

7.1.3.2. Running Buffer

Tris	36.3 g
Triple distilled water	150 mL

Dissolve Tris in water and adjust pH to 8.8 using HCl. Autoclave and store at 2 - 8º C in the dark.

7.1.3.3. Stacking Buffer

Tris	3 g
Triple distilled water	50 mL

Dissolve Tris in water and adjust pH to 6.8 using HCl. Autoclave and store at 2 - 8º C in the dark.

7.1.3.4. 10% SDS

SDS	10 g
Triple distilled water	100 mL

Dissolve SDS in water. Store at room temperature.

7.1.3.5. 10%Ammonium persulphate

Ammonium persulphate	0.1 g
Triple distilled water	1 mL

Dissolve reagent in water. Should be freshly prepared but stable for up to 1 week when kept in the fridge in the dark.

7.1.3.6 Resolving Gel

Monomer solution	3.33 mL
Running buffer	2.5 mL
10% SDS	100 µL
Triple distilled water	4 mL
10% Ammonium persulphate	50 µL
TEMED	5 µL

Add the above reagents together into a beaker in order of appearance. As soon as the last reagent is added swirl to mix and load casting tray.

7.1.3.7 Stacking Gel

Monomer solution	670 µL
Stacking buffer	1.25 mL
10% SDS	50 µL
Triple distilled water	3 mL
10% Ammonium persulphate	25 µL
TEMED	2 µL

Add the above reagents together into a beaker in order of appearance. As soon as the last reagent is added swirl to mix and load casting tray.

7.1.3.8 10X Electrode Buffer

Tris	3.028 g
Glycine	14.413 g
SDS	1 g
Triple distilled water	1 L

Dissolve reagents in water and adjust pH to 8.3. Autoclave and store at room temperature.

7.1.3.9. Coomassie Stain

CCB	0.1 g
Methanol	50 mL
Acetic acid	10 mL
Triple distilled water	40 mL

Mix together CCB, methanol and acetic acid. Make up to 100 mL using triple distilled water.

7.1.3.10. Destaining solution

Methanol	50 mL
Acetic acid	10 mL
Triple distilled water	40 mL

Mix together methanol and acetic acid. Make up to 100 mL using triple distilled water

7.1.4. Western blot solutions

7.1.4.1 Resolving Gel Buffer

SDS	0.8 g
Trizma base	36.3 g
Triple distilled water	100 mL

Dissolve reagents in triple distilled water. Adjust pH to 8.8 using HCl. Store at 2 - 8º C.

7.1.4.2 Stacking Gel Buffer

SDS	0.4 g
Trizma base	6.05 g

Triple distilled water 100 mL

Dissolve reagents in triple distilled water. Adjust pH to 6.8 using HCl. Store at 2 - 8º C.

7.1.4.3 10X Blotting Buffer

Trizma base	30.3 g
Glycine	144 g
Triple distilled water	1 L

Dissolve reagents in triple distilled water. Adjust pH to 8.3. Store at 2 - 8º C.

7.1.4.3.1 1X Blotting Buffer

Methanol	400 mL
10X Blotting Buffer	200 mL
Triple distilled water	1.4 L

Mix together. Store at 2 - 8º C.

7.1.4.4 Rinsing and diluting Buffer

PBS, pH 7.0 100 mL

Tween 20 50 µL

Add Tween 20 to PBS. Mix well. Store at 2 - 8º C

7.2. List of reagents and consumables

Table 7: List of reagents and consumables used for the various experiments

NAME	COMPANY	CATALOGUE NUMBER
RPMI 1640	Lonza/ Biowhittaker	12-115F
Foetal bovine serum	GIBCO	10108-157
Phosphate buffered saline	Oxoid	BR0014G
EDTA	Sigma Aldrich	E5134
trypsin-versene	Lonza/ Biowhittaker	17-161E
Glycerol	Saarchem	SAAR2676520LC
Xylavet (xyalazine)	Intervet	_____
Anaket - V (ketamine)	Centaur Labs	_____
Cortisone acetate	King Edward VIII hospital dispensary	
Euthapent (pentobarbitone sodium)	Kyron Laboratories	_____
EMEM	Lonza/ Biowhittaker	12-662F
Dithiotreitol (DTT)	Fluka	43816

CampyGenTM Compact	Oxoid	CN0020C
Transwells	Corning	3415
Probetec® lysis buffer	BD	440453
2 x Universal PCR Mix	Applied Biosystems	4304437
Gluteralderhyde	Agar Scientific	R1012
Osmium tetroxide	Agar Scientific	R1017
Spurr kit	SPI-Chem™	_____
MES	Sigma	M3671-50g
EGTA	Sigma	E3889
$MgCl_2$	Saarchem	412 30 00 EM
triton X-100	Sigma	T-8787
PVDF membrane	Roche	3010031
Tween-20	Sigma	P9416-50mL
Anti-human occludin	Zymed Laboratories	71-1500Z
Peroxidase-labelled goat ant-rabbit IgG	Zymed Laboratories	81-6120
(FITC) conjugated mouse anti-human occludin	USBiological	05203
Sodium Cacodylic acid	Sigma	C0250
Acrylamide	BioRad	161-0107
Bis-Acrylamide	BioRad	161-0201
Tris (Trizma base)	Sigma	T8529

Sodium Dodecyl Sulphate (SDS)	Sigma	L3771
Ammonium persulphate	Sigma	A3678-100g
TEMED	Sigma	T9281
Glycine	Sigma	G8898
Coomassie Brilliant Blue G-250	BioRad	161-0406
Methanol	Saarchem	1030880
Acetic acid	Saarchem	1030763
Trizma base	Sigma	T1503-500g
Skimmed Milk Powder	Fluka	70166