The effect of HIV and *Neisseria gonorrhoeae* on the tight junctions of cervical epithelial cells

by

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ABSTRACT

Introduction: *Neisseria gonorrhoeae* and HIV are major public health concerns globally. The interaction between these diseases is unclear. To determine the effect that *N. gonorrhoeae* and HIV have on the tight junctions of cervical epithelial cells, a cervical epithelial cell line was infected with *N. gonorrhoeae* only, HIV only and with *N. gonorrhoeae* and HIV simultaneously.

Methods: The ME180 cervical cell line was grown to confluence and infected with*N. gonorrhoeae* only, HIV only and with *N. gonorrhoeae* and HIV simultaneously. Following infection, *N. gonorrhoeae* and HIV tansmigration assays and the blue dextran permeability assay were also performedto determine the effect that exposure to the microbes would have on the intact cervical epithelial layer. The tight junction gene expression assays, blue dextran permeability assay and immunofluorescence staining was performed to determine the effect that exposure to the different microbes had on the tight junctions.

Results: The results of this study showed that exposure of the cervical epithelial layer to *N. gonorrhoeae* alone, HIV alone and to *N. gonorrhoeae* and HIV simultaneously did not affect the paracellular permeability of the epithelial layer. The results showed that a small percentage of *N.gonorrhoeae*and HIV was able to migrate across the epithelial layer. With the simultaneous infection of *N.gonorrhoeae* and HIV, the presence of HIV did not seem to influence the migration of *N. gonorrhoeae,* as compared to infection with *N. gonorrhoeae* only, while the presence of *N.gonorrhoeae* seemed to cause the HIV to pass through the epithelial layer less efficiently than with exposure to HIV only.

Discussion: The overall results suggest that since exposure to these microbes does not seem to affect the tight junctions of the intact epithelial layer and does not affect the paracellular permeability, the migration of the microbes across the epithelial layer was possibly through transcytosis.

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ABBREVIATIONS

AHRI	-	Africa Health Research Institute
AIDS	-	Acquired Immunodeficiency Virus
BHI	-	Brain Heart Infusion
BSA	-	Bovine Serum Albumin
cDNA	-	Complementary Deoxyribonucleic Acid
CFU	-	Colony Forming Unit
DEPC	-	Diethyl Pyrocarbonate
DNase	-	Deoxyribonuclease
dsDNA	-	Double-stranded Deoxyribonucleic Acid
EDTA	-	Ethylenediaminetetratacetic Acid
FBS	-	Foetal Bovine Serum
FRT	-	Female Reproductive Tract
HIV	-	Human Immunodeficiency Virus
HSV-1	-	Herpes Simplex Virus 1
IC	-	Internal Control
IgA	-	Immunoglobulin A
JAMs	-	Junctional Adhesion Molecules
LOS	-	Lipooligosaccharide
LPS	-	Lipopolysaccharide
MOI	-	Multiplicity of Infection
mRNA	-	Messenger Ribonucleic Acid
NAATs	-	Nucleic Acid Amplification Tests
PBS	-	Phosphate Buffered Saline
RNA	-	Ribonuclucleic Acid
RNase	-	Ribonuclease
STDs	-	Sexually Transmitted Diseases
STIs	-	Sexually Transmitted Infections
TEER	-	Transepithelial Electrical Resistance
UNAIDS	-	United Nations Programme on HIV/AIDS
WHO	-	World Health Organization
ZO-1	-	Zonula Occludens-1

CHAPTER 1: INTRODUCTION

The aetiological agent of gonorrhoea is the obligate human pathogen *Neisseria gonorrhoeae.* Epidemiological data have shown that gonorrhoea is still a major public health concern globally, as approximately 78 million new cases of gonorrhoea were reported for 2017 (WHO 2019). Another major global public health concern is infection with Human Immunodeficiency Virus (HIV). The 2018 United Nations Programme on HIV/AIDS (UNAIDS) report estimated that in 2017 about 36.9 million of the global populationwere infected with HIV, with approximately 1.8 million cases occurring in that year (UNAIDS 2018). In South Africa, these two diseases pose a huge public health threat. Gonorrhoea and HIV are both sexually transmitted diseases (STDs). A strong associationbetween STDs and HIV have been observed, however, the interaction between these diseases is unclear. Research has attempted to elucidate the relationship between gonorrhoea and HIV. Studies have suggested that a bidirectional relationship exists between STDs and HIV (Chun et al. 2013). While some studies have suggested that STDs increase the risk of acquiring HIV, other studies have suggested the converse.

An intact epithelial layer should serve as an effective barrier against pathogenic microbes. However, studies have shown that HIV-1 could migrate across an intact epithelial layer and lead to infection. HIV usually enters host cells by binding to the CD4 receptors on the host cells. However, the genital epithelial cells do not express these receptors, which suggests that HIV-1 would not be able to enter these cells through the usual route of entry. Studies have suggested that HIV-1 can enter epithelial cells through endocytosis, which involves transporting macromolecules within vesicles across a cell. Transcytosis of HIV-1 was reported by Kinlock et al. (2014), when they showed that HIV-1 could be transported across the cell via the endocytic recycling pathway. Interestingly, they showed that endocytosed native virus successfully transcytosed through the cell, whereas the endocytosed heat-inactivated HIV-1 was processed through the degradation pathway (Kinlock et al. 2014).

An alternate mechanism of HIV-1 movement across the epithelial layer was suggested by other studies. One study suggested that exposure of epithelial cells to HIV resulted in the disruption of the tight junctions of the cells, which enabled microbial entry via the paracellular route (Nazli et al. 2010). This was also demonstrated inanother study which showed that exposure of oral epithelial cells to HIV-1 caused disruption to the tight junctions, thereby enabling human papillomavirus to move across the epithelial layer (S. M. Tugizov et al. 2013). Furthermore, a different study showed that HIV exposure

caused disruption to both the tight and adherens junction proteins and facilitated paracellular movement of HSV-1 across the epithelial layer, as well as enabling cell to cell movement of HSV-1(Sufiawati and Tugizov 2014).

This study aimed to determine the effect that infection with *N.gonorrhoeae* only, HIV only and *N. gonorrhoeae* and HIV simultaneously would have on the tight junction of cervical epithelial cells. The hypothesis was that HIV would cause disruption to the tight junctions, thereby enabling *N. gonorrhoeae* together with HIV to cross the epithelial layer by means of the paracellular pathway.

CHAPTER 2: LITERATURE REVIEW

2.1 *Neisseria gonorrhoeae*

2.1.1 Historical Background and Characteristics

Gonorrhoea was first described approximately 3500 years ago, making it one of the oldest diseases identified by man (J. L. Edwards and Apicella 2004; Hill et al. 2016). Galen (130 - 200 AD) named the disease proposing a name derived from the Greek words "gonos" meaning semen and "rhoia" meaning to flow, when he mistakenly identified the gonococcal discharge as "an unwanted secretion of semen without erection" (Oriel 1996). It was not until 1879 that the causative agent of this disease, *Neisseria gonorrhoeae* was discovered microscopically by Albert Ludwig Sigesmund Neisser (Ligon 2005). However, the organism was only cultivated in 1882 by Leistikow and Löffler (J. L. Edwards and Apicella 2004). *N. gonorrhoeae* is a gram-negative diplococcus of which the individual cells have flattened adjacent sides, giving it the appearance of a kidney or coffee bean. They are non-motile, non-sporulating and are aerobic organisms that grow well at 37°C in a moistened atmosphere that contains 3 to 5% CO_2 (Carr 2017). *N. gonorrhoeae* is an obligate human pathogen and the initial site of infection in men is the urethra and in women the endocervix and urethra (J. L. Edwards and Apicella 2004; Stevens and Criss 2018). The organism can also infect otherepithelialsurfaces, including the pharynx, conjunctiva and the rectum (Hill et al. 2016). *N.gonorrhoeae* has virulence factors that enhance its pathogenicity. These factors include the capsule (which helps to avoid phagocytosis), pili (which allows the organism to attach to cell surfaces) and lipooligosaccharide (LOS), which is a variant of lipopolysaccharide (LPS)and IgA-protease that cleaves immunoglobulin A (IgA) (Carr 2017). Both LOS and LPS contain lipid-A which is the main endotoxic component of these lipid-carbohydrate compounds.

2.1.2 Epidemiology

Neisseria gonorrhoeae infections are a major global health concern. According to the2019 World Health Organization (WHO) fact sheet, it is estimated that there were approximately 357 million new cases of the four curable sexually transmitted infections (STIs), i.e gonorrhoea, chlamydia infection, syphilis and trichomoniasis, occurring globally. Approximately 78 million of these cases were attributed to gonorrhoea (WHO 2019). In

South Africa, STIs continue to be a major public health problem. In 2017, the prevalence of gonorrhoea in this country was approximately 6.6% (3.8 – 10.8%) in females and 3.5% (1.7 – 6.1%) in males. The estimated incidence of gonorrhoea in South Africa in 2017 was approximately 2.3 (1.1 – 5.0) million cases in women and 2.2 (1.1 – 3.0) million cases in males (Kularatne et al. 2018).

2.1.3 Transmission and Clinical Manifestations

Gonorrhoea is a sexually transmitted disease that can be transmitted from an infected partner during vaginal, oral or anal sex. However, the disease can also be spread from an infected mother to her child during delivery. *Neisseria gonorrhoeae* is transmitted more efficiently to females from their infected male partners than from infected females to their male partners. This could be due to differences in the anatomy of the male and female genitalia, which allows the organism to have a longer period of contact with the female genital membranes following sex. The clinical manifestations of the disease depend on the site of infection (J. L. Edwards and Apicella 2004). The symptoms of gonococcal infection in females include vaginal discharge, dysuria,lower abdominal pain, rectal pain and abnormal uterine bleeding. In males, the symptoms include urethral discharge, dysuria and pain in the testicles or rectum. If the gonococcal infection is left untreated, women may experience pelvic inflammatory disease, chronic pelvic pain and tubal infertility, while men may present with epidydimitis, prostatitis, urethral stricture and rarely with infertility (Diez and Diaz 2011; Lenz and Dillard 2018; Ng and Martin 2005; Piszczek et al. 2015). Neonates that contract gonorrhoea during delivery present with neonatal conjunctivitis (ophthalmia neonatorum). If the conjunctivitis is left untreated, it can cause scarring and blindness (Alirol et al. 2017).

2.1.4 Diagnosis

Neisseria gonorrhoeae can be diagnosed in the laboratory through presumptive and confirmation tests. The presumptive tests include Gram staining and culturing the organism on selective media followed by the oxidase test, the catalase test and the colistin resistance test. The organism produces a positive reaction in the oxidase, catalase tests and is resistant to colistin. The confirmation tests include biochemical tests, serological tests and nucleic acid amplification tests (NAATs) (Ng and Martin 2005). The Gram stain and culture method were the initial diagnostic tests used for gonorrhoea until the late 1980s. The Gram

stain involves firstly collecting a swab sample from either the cervix, urethra, rectum or vagina. The swab is then rolled carefully over an area of a microscope slide, heat-fixed and stained using the Gram stain procedure, which includes staining the smear with crystal violet (i.e a primary stain), then applying iodine (a mordant), followed by decolourisation with ethanol and finally by staining with a counterstain eg. diluted carbolfuschin. The slide is then viewed under the microscope using an oil immersion objective. Gram-positive cells are seen as purple cells (as they have thicker peptidogylcan walls and retain the crystal violet stain) and gram-negative cells are seen as pink cells (as they have a thinner peptidyglycan layer, which results in the alcohol decolorising the cell thereby allowing the cells to be counterstained with carbolfuschin). The Gram stain is a rapid method of detection with a sensitivityof $\pm$95% in males with visible discharge. This is comparable to culture for symptomatic urethral gonorrhoea in this group. However, there is decreased sensitivity for specimens collected from females (i.e 40-60%), as well as in males for specimens other than urethral swabs and in non-symptomatic males or males with dysuria only. For those the sensitivity of microscopy is very low and thus not recommended. Bacterial culture involves inoculating the specimen onto a medium that supports the growth of *N. gonorrhoeae* like New York City agar, Thayer Martin agar or chocolate agar containing antimicrobials that do not inhibit the growth of *N. gonorrhoeae*, while preventing the growth of contaminating bacteria and fungi. The media are incubated at 35-37°C in a moistened environment containing CO_2 (4 – 6%). Bacterial culture is considered the gold standard for diagnosing gonorrhoea with a sensitivity of 85 – 95% and a specificity of up to 100% (Meyer and Buder 2020; Muralidhar 2015). The other advantages of using bacterial culture is the relatively low cost and that it produces viable organisms that can be used for testing for antimicrobial susceptibility. The disadvantage of this method is that it requires the collection of an invasive specimen that must be transported under stringent conditions to ensure that the organism remains viable. Nucleic acid amplification tests, for the detection of gonorrhoea, were introduced for routine use in the early 1990s. Nucleic acid amplification tests are used to detect *N. gonorrhoeae* nucleic acid using polymerase chain reaction. Several FDA-approved commercial kits are available, including Abbott RealTime CT/NG (Abbott, Des Plaines, USA), cobas CT/NG (Roche Diagnostics, Indianopolis, USA) and Aptima Combo2 Assay (Hologic, San Diego, USA).The advantages of NAATs over bacterial culture is that the sensitivity is higher (i.e>95% inswab specimens or urine from males), which makes it appropriate for screening purposes

as it allows accurate diagnosis of symptomatic and asymptomatic infections. The other advantages are that it does not require invasive specimens and the collected specimens do not need to be transported under as stringent conditions as for bacterial culture. The disadvantages of NAAT is that it is more expensive, inhibition of the reaction may occur resulting in false negatives, the assay is complex and requires high technical skills and quality control, there is a lack of antibiotic resistance information and it can produce both false-negative and false-positive results (Meyer and Buder 2020; Verma and Sood 2016; Whiley et al. 2006).

2.1.5 Treatment and Emerging Resistance

Gonorrhoea can usually be treated with antimicrobials, however, this pathogen has been classified as a "superbug", which has been able to develop high-level resistance to many of the available antimicrobials (including sulphonamides, penicillin, tetracyclines and ciprofloxacin) over the years (Costa-Lourenco et al. 2017). This has limited the treatment options for this disease. In 2016 the WHO guidelines recommended dual-therapy with ceftriaxone and azithromycin, which could possibly slow down the development of resistance to these drugs (Costa-Lourenco et al. 2017; WHO 2016). Since there is no vaccine for gonorrhoea and the threat of developing high-level resistance to all antimicrobials is possible in the coming future, new treatment options need to be explored. Currently, efforts are being made to combine the available antimicrobials in novel ways, produce new antibiotics and discover alternative therapies to decelerate resistance development (Suay-Garcia and Perez-Gracia 2018).

2.2 Human Immunodeficiency Virus

2.2.1 Overview and Classification

A new disease, Acquired Immunodeficiency Syndrome (AIDS), was first discovered in 1981. However, the etiological agent of this disease, Human Immunodeficiency Virus (HIV) was only identified in 1983(Barre-Sinoussi et al. 1983; Sharp and Hahn 2011). HIV is a retrovirus, which belongs to the family of Retroviridae and genus Lentivirus (Barre-Sinoussi 1996; German Advisory Committee Blood 2016). There are 2 types of HIV, viz. HIV-1 and HIV-2. Globally, the most frequently occurring type is HIV-1. HIV-2 is less

virulent than HIV-1 and is the most widespread HIV type found in West Africa, where it was first isolated in 1986 (Clavel et al. 1986; Santoro and Perno 2013). HIV-1 includes four groups, viz. M (main), O (outlier), N (nonmajor and nonoutlier) and P.Group M has been further subdivided into nine subtypes or clades (viz. A, B, C, D, F, G, H, J and K) (Junqueira and Almeida 2016). Group M also consists of at least 58 circulating recombinant forms (Santoro and Perno 2013).

2.2.2 Epidemiology

HIV infection is a global health problem. The UNAIDS global estimate for the number of people living with HIV in 2017 was 36.9 million (31.1 million–43.9 million), approximately 54% of which were in eastern and southern Africa. Globally, there were approximately 1.8 million (1.4 million–2.4 million) new cases of HIV infection in 2017, with 45% of these cases in eastern and southern Africa. In 2017, the estimated number of HIV infected individuals in the South African population was approximately 7.2 million (6.6 million – 7.2 million) and there were approximately 270 000 (240 000 – 300 000) new HIV infections (UNAIDS 2018). In 2017, 27% of the South African HIV infected population aged 15 to 49 years were living in KwaZulu Natal (National Institiute of Communicable Diseases 2018).

2.2.3 HIV Structure

The spherical HIV virion (Figure 2.1) is enclosed by a host-derived lipid bilayer. The lipid bilayer contains envelope protein complexes that are made up of a surface glycoprotein (gp120) and a transmembrane protein (gp41). The matrix protein (p17) is attached to the inside of the membrane. Within thevirion is the major capsid protein, p24, which encloses two single-stranded viral ribonucleic acid (RNA) copies as well as three enzymes,viz. protease, reverse transcriptase and integrase (Barre-Sinoussi 1996; Fanales-Belasio et al. 2010).

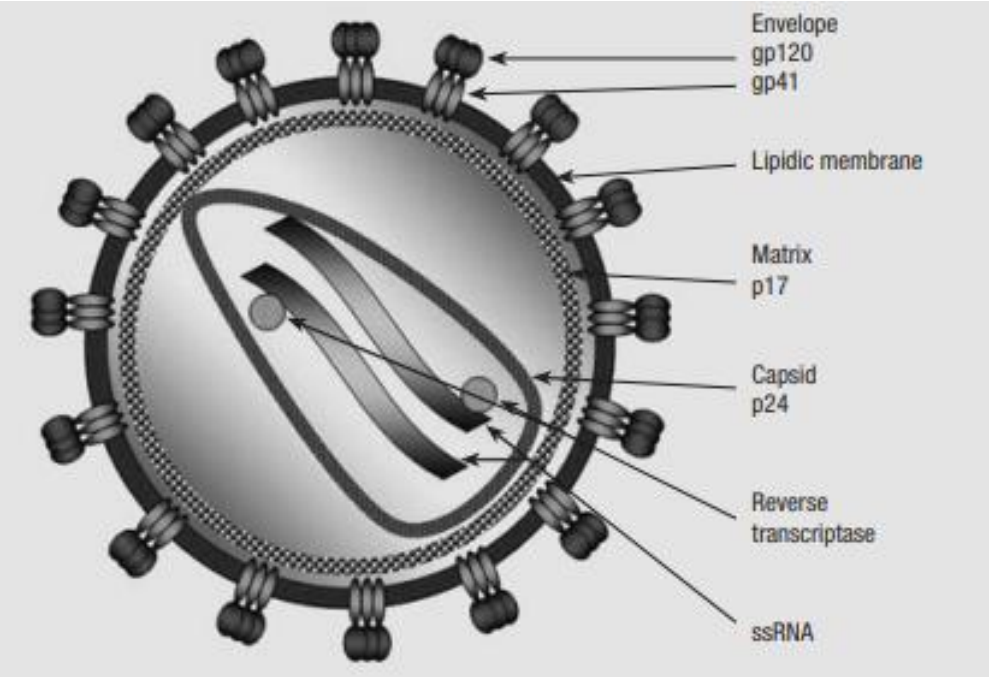


Fig 2.1 Structure of the HIV. Taken from Fanales-Belasio et al. (2010).

2.2.4 HIV Life Cycle

The replication cycle of HIV (Figure 2.2) commences with the attachment of the virus to the host cell surface. Binding occurs when the HIV gp120 protein attaches to the CD4 receptor on the host cell surface. This causes gp120 to undergo a conformational change, exposing a domain that is able to bind to co-receptors (most commonly CCR5 and CXCR4) on the host cell. This binding is followed by penetration of the host cell membrane by gp41, which thereafter undergoes a structural change allowing the HIV membrane to fuse with the host cell membrane, releasing the viral contents inside the host cell. Within the cells, the viral reverse-transcriptase then reverse transcribes the viral RNA into double-stranded deoxyribonucleic acid (dsDNA). The dsDNA is thereafter transported to the nucleus, where the viral integrase incorporates it into the host cell DNA. The host cell then produces viral messenger ribonucleic acid (mRNA) and this is transported into the cytoplasm. In the cytoplasm, the viral mRNA is translated into viral proteins and also actsas viral genomic RNA that migrates towards the cell membrane, where assembly and

budding of the immature virion occurs. The viral protease then cleaves the viral proteins, producing mature virions(Barre-Sinoussi 1996; Fanales-Belasio et al. 2010).

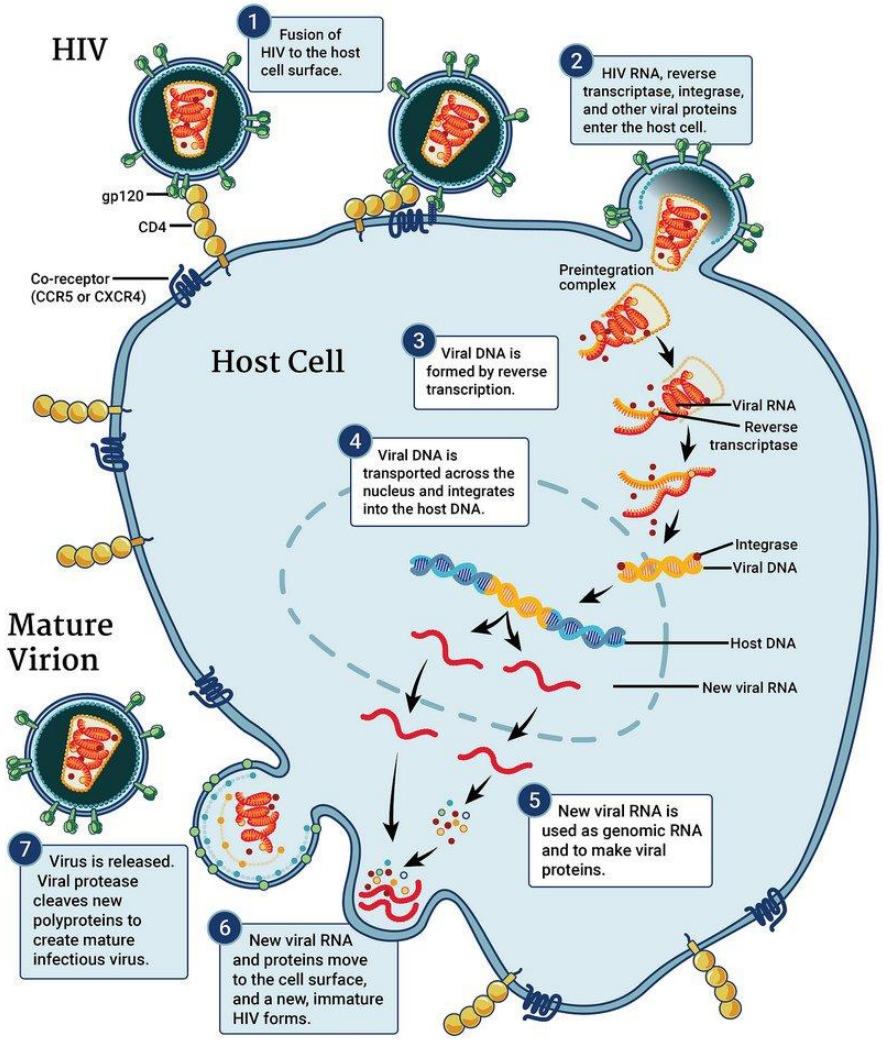


Figure 2.2 HIV Life Cycle. The HIV life cycle startswith the viral envelope fusing with the host cell membrane. This enables the viral contents tobe released into the host cytoplasm, where the viral RNA is reverse-transcribed into dsDNA. The viral dsDNA is then incorporated into the host cell DNA. Following replication of the host cell, viral mRNA is produced and this is then translated into viral proteins. The viral proteins and viral genomic RNA migrates towards the cell membrane, where budding of immature virions takes place. Cleavage of the viral proteins within the virion by protease results in the formation of mature virions. Taken from https://www.niaid.nih.gov/diseases-conditions/hiv-replication-cycle (NIH).

2.2.5 Transmission and Clinical Manifestations

HIV can be transmitted sexually, through parenteral exposure or through mother-to-child transmission (Berkley 1991; Patel et al. 2014). Sexual transmission of HIV occurs during unprotected oral, vaginal or anal sex when mucus membranes are exposed to infected body fluids or when the virus enters the bloodstream. Parenteral exposure of HIV includes exposure through needle-stick injuries, needle-sharing during intravenous drug use and receiving infected blood, blood products or organs. The spread of HIV from a mother to a child can occur during pregnancy, during delivery or through breastfeeding (German Advisory Committee Blood 2016; Malani 2016; Patel et al. 2014).Following infection, the HIV clinical course follows three phases, viz. acute HIV infection, chronic HIV infection and AIDS (Figure 2.3) (An and Winkler 2010). The acute phase of infection usually develops two to four weeks following exposure to the virus and the infected individual usually presents with flu-like symptoms including fever, rash, lymphadenopathy, pharyngitis and weight loss (Fanales-Belasio et al. 2010). During the acute phase of infection, the viral load increases rapidly and the CD4+ lymphocyte count declines drastically since the virus attacks and replicates within these cells. When an antiviral immune response is elicited, the viral load begins to decline. At the same time, the CD4+ lymphocyte count begins to increase, however the count is lower than it was before the infection. The high viral load present in the acute phase results in the newly infected individual being highly infectious. The chronic phase of infection developsa few weeks following initial infection and it is associated with a decrease in the viral load. The individual is asymptomatic in this phase of infection. During the chronic phase, the CD4+ lymphocyte count gradually decreases until the count drops to < 200 cells/µL, which is the onset of the third phase of infection (AIDS). The decrease in the immune cells increases the susceptibility to opportunistic pathogens. The symptoms of AIDS include lymph node swelling, severe weight loss, fever, as well as respiratory and gastro-intestinal symptoms. The chances of developing cancers, including Kaposi's sarcoma and lymphomas, are also increased in these individuals. If left untreated, individuals with AIDS usually surviveabout three years (Bhatti et al. 2016; Fanales-Belasio et al. 2010).

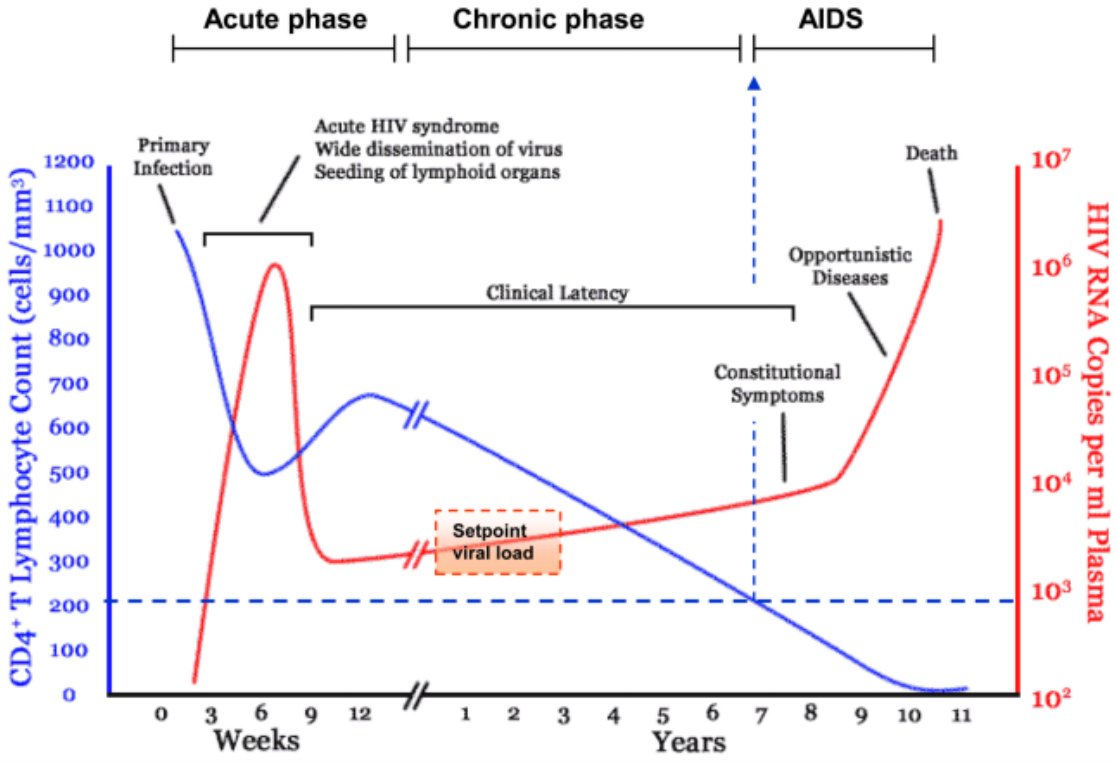


Fig 2.3 HIV Clinical Course. The illustration shows the 3 phases of HIV infection, viz. the acute phase (primary infection), the chronic phase (asymptomatic phase) and AIDS. Taken from An and Winkler (2010).

2.3 Endocervical Cells

The female reproductive tract (FRT) is composed of the upper and lower FRT. The lower FRT is composed of the vagina and ectocervixand both of these arecovered by non-polarised squamous epithelial cells. The upper FRT is composed of endocervix, uterus, salpinx and ovarium. The lining of the endocervix and uterus is composed of a monolayer of polarised columnar epithelial cells (Wang et al. 2017).The epithelial cells form the first line of defense against invading pathogens and guards against themby the production of mucus, secreting cationic antimicrobial peptides and by maintaining the integrity of the barrier through the formation of tight junctions (Stevens and Criss 2018). The endocervix cells are held together by the epithelial junctional complexes, which prevent the paracellular movement of invading pathogens (Blaskewicz et al. 2011). However, the epithelial junction complex does not remain static and is able to assemble and disassemble to enable paracellular movement of solutes and

ions. This paracellular permeability is regulated by extracellular and intracellular stimuli through signal transduction and reorganization of actin cytoskeleton (Rodgers and Fanning 2011). In a similar manner, pathogens have been shown to induce regulation of the apical junction, thereby altering the paracellular permeability (Nazli et al. 2010; Tapia et al. 2017).

2.4 Tight Junctions

Epithelial junctional complexes, which are responsible for attaching adjacent cells to each other, consists of the adherens junctions, tight junctions, gap junctions and desmosomes (Figure 2.4) (Bartle et al. 2018; Vedula et al. 2009). The functions of the adherens junction are to initiate and stabilise adhesion between adjacent cells, regulate actin cytoskeleton and provide intracellular signaling. The adherens junction is composed of the cadherins and catenins (Hartsock and Nelson 2008; Miyoshi and Takai 2008). Cadherins (eg. E-cadherin) are the transmembrane proteins of the adherens junctions and the catenins (eg. β-catenin) are the cytoplasmic proteins that connect the cadherins to the actin cytoskeleton. When the adherens junctions form, it causes the formation of the tight junctions. However, maintenance of the tight junction organisation does not require E-cadherin (which is the core transmembrane protein of the adherens junction) (Hartsock and Nelson 2008). The actin cytoskeleton of the cell is composed of the actin filaments, their accessory proteins and their regulatory proteins. The actin cytoskeleton is responsible for defining the shape of the cell, changing the shape of the cell during movement, regulation of intracellular motility and enabling cells to adhere to one another(Fletcher and Mullins 2010; Svitkina 2018). Tight junctions are the areas between the plasma membranes of adjacent epithelial and endothelial cells, where the two cells join together to form a barrier. The tight junctions have two functions. The first function is to act as a selective barrier that controls paracellular movement (i.e the gate function) and the second function is to prevent apical or basolateral intramembrane lipid diffusion thereby physically separating the apical and basolateral sections of the plasma membrane(i.e the fence function) (Anderson and Van Itallie 2009; Balda and Matter 2009; Chiba et al. 2008; Miyoshi and Takai 2008). The tight junctions consist of the transmembrane proteins (which regulate the paracellular permeability) and the associated cytoplasmic proteins (which connect the transmembrane proteins to the actin cytoskeleton). Transmembrane proteins include occludins, claudins, tricellulin and junctional adhesion molecules (JAMs), while the cytoplasmic proteins include zonula occludens-1 (ZO-1), ZO-2 andZO-3 (Balda and Matter 2008; Chiba et al. 2008).

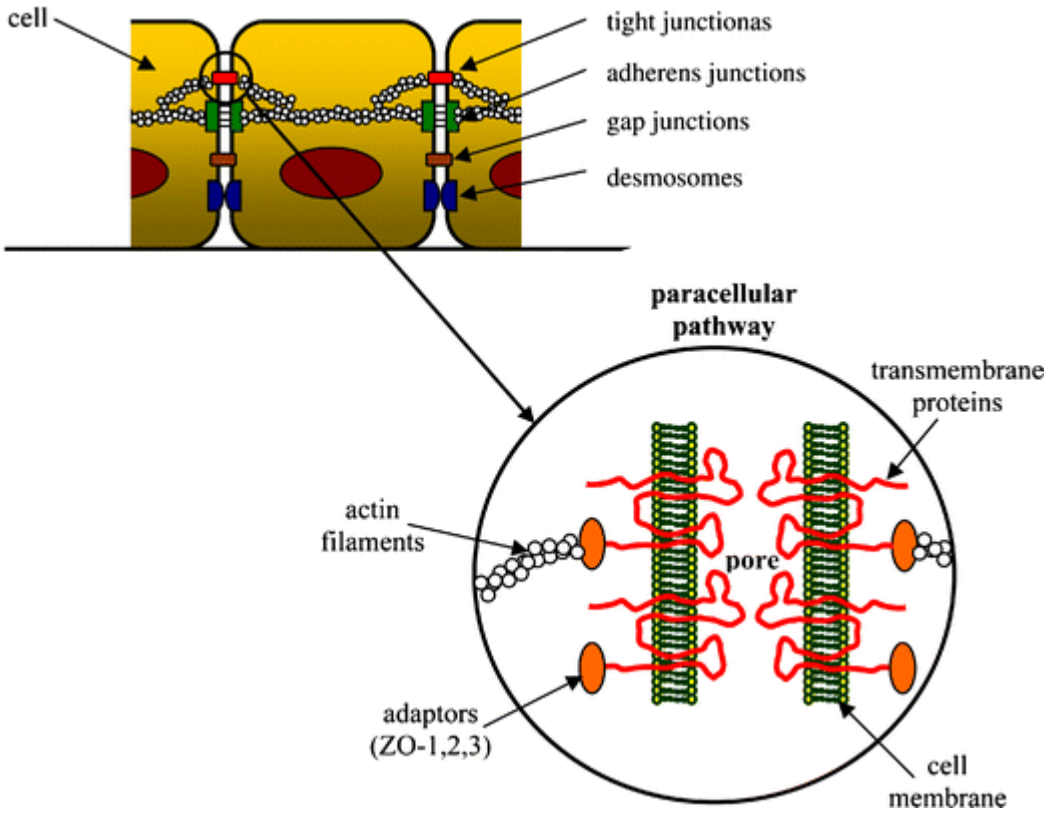


Fig 2.4 Tight Junction. This diagram shows the tight junction of epithelial cells, which is a component of the epithelial cell junctional complex. The tight junction is made up of transmembrane proteins, which are linked to the cytoplasmic proteins (adaptors), which are attached to the cell actin filaments. Taken from Vedula et al. (2009).

2.4.1 Occludin

Occludin was the first identified intramembrane protein (Chiba et al. 2008). This protein is approximately 65kDa and is composed of four transmembrane domains, one intracellular loop,two extracellular loops and the N- and C-terminal cytoplasmic sections(Furuse 2010). The C-terminal domain is directly attached to ZO-1. The two extracellular loops controls the accumulation of this protein at the junctions and it also regulates paracellular permeability, as occludin controls the diffusion of small hydrophilic molecules through the pararcellular route(Balda and Matter 2008; Furuse 2010).

2.4.2 Claudins

Claudins are composed of 24 proteins, viz. claudins 1 – 24, which are 21 – 28kDa proteins. Claudins have four transmembrane domains, a short intracellular turn, two extracellular loops and the N- and C-terminal cytoplasmic sections. They are believed to be the backbone of the tight junction (Furuse 2010). These proteins are capable of completely tightening the paracellular gap for solutes, as well as forming pores for selective ion paracellular permeability (Krause et al. 2008). The claudin constitution within the tight junction regulates this ion selectivity through the paracellular route, since the changes in the expression of the claudin proteins are associated with changes to the ion selectivity (Balda and Matter 2008).

2.4.3 ZO-1

ZO-1 was the first tight junction protein that was identified (Stevenson et al. 1986). ZO-1 is an adaptor protein (Balda and Matter 2009). The transmembrane proteins are associated with ZO-1, which is found in the intracellular part of the plasma membrane. ZO-1 is associated with the actin filaments of the cytoskeleton, thereby enabling the tight junctions to connect the cytoskeletons of adjoining cells (Hartsock and Nelson 2008). ZO-1 has been shown to perform a crucial part in coordination of epithelial actomysin and formation of the unified apical surface (Odenwald et al. 2018).

2.5 STDs and HIV

A strong association between HIV-1 and STDs became evident when an increased spread of HIV-1 infection in regions where STDs were endemic was observed (Cohen 1998). Research has since suggested that a bidirectional relationship exists between HIV and “classic” STDs, including herpes, syphilis, gonorrhoea, trichomoniasis and chlamydia infection.A recent study showed that there was a high prevalence of asymptomatic STDs in a cohort of HIV-positive Zimbabwean women(Lowe et al. 2019). STDs may increase the risk of HIV acquisition and transmission by causing breaks in the protective mucosal barrier, recruitment of susceptible immune cells to the point of infection or by increasing HIV-1 shedding in genital secretions (Chun et al. 2013; Cohen 1998; Kalichman et al. 2011; Piot and Laga 1989; Rottingen et al. 2001). Johnson and Lewis (2008) reported that genital tract infections resulted in an increase in the shedding of HIV, especially when the infection resulted in leukocyte recruitment in the

genital tract (Johnson and Lewis 2008). Cohen et al. (1997) showed that urethritis in HIV-positive men increases their infectiousness. They found that HIV-positive men with urethritis had an eight-fold higher concentration of HIV RNA in semen when compared to HIV-positive men without urethritis. The highest concentration of HIV in semen was found in men infected with gonorrhoea. The CD4 counts and the HIV RNA concentration in the blood of the two groups of men were similar. The men with urethritis were given antibiotic therapy directed against STDs. Two weeks following treatment, they found a significant decrease in the HIV RNA concentration in the semen of the treated men, with no significant change in the semen of the control group within that two-week period. There was no change in the concentration of HIV RNA in the blood of the two groups of men(Cohen et al. 1997).Conversely, studies have shown that HIV may increase the risk of acquiring STDs, possibly resulting from disruption to the tight junctions of the protective epithelial barrier (Nazli et al. 2010; S. Tugizov 2016). Tugizov et al. (2013) demonstrated this by showing that exposure to HIV-1 resulted in disruption to the tight junctions of mucosal epithelia, which allowed paracellular migration of human papillomavirus. HIV-1 has also been shown to disrupt the tight and adherens junctions in oral epithelial cells, which may facilitate the paracellular and cell-to-cell movement of HSV-1 (Sufiawati and Tugizov 2014).

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials and Ethics Approval

The ME180 cervical cell line was used for the infection experiments. This cell line is a human cervical cell line that was obtained from American Type Culture Collection (ATCC® number HTB-33™). Serum samples containing the HIV used in the infection experiments was obtained from the Africa Health Research Institute (AHRI). The serum had been obtained from HIV subtype C infected, antiretroviral naïve patients participating in a research project of AHRI. *Neisseria gonorrhoeae* used was one of the clinical isolates from a former study carried out by Ramburan et al. (2019). The isolate used was piliated and opa-positive from a male patient presenting with urethral discharge (Rambaran et al. 2019).

Approval for the study was obtained from the University of KwaZulu-Natal Biomedical Research Ethics Committee (BE220/13).

3.2 Tissue Culture

ME180 cells were retrieved from frozen stock stored at -80°C. To resuscitate the cells, a vial of frozen cells was thawed in a waterbath at 37°C and transferred to a 25 cm^2 flask containing 10 mL of warm McCoy's 5Amedia (Gibco, Paisley, UK) with 10% Foetal Bovine Serum (FBS) (BioWhittaker, Walkerville, USA). The cells were incubated at 37°C in a humidified atmosphere with 5% $CO_2$and viewed daily with an inverted microscope at 20x magnification to monitor morphological changes, cell density and to check for possible contamination. The media was changed every two to three days until the cells reached approximately 90% confluency. Media changes were performed by discarding the spent media, rinsing the cells with warm phosphate buffered saline (PBS) (Oxoid, Hampshire, England), pH 7.3,and finally by adding freshly prepared prewarmed McCoy's 5A media (Gibco, Paisley, UK) containing 10% FBS. When 90% confluency was reached, the cells were passaged. This procedure involved firstly discarding spent media, followed by washing the cells three times with warm PBS to remove unattached cells and FBS (which would inhibit the action of trypsin). One milliliter of trypsin EDTA was then added to the flask and the flask was rotated to ensure proper coverage of trypsin over the cells. This was followed by incubation at 37°C for 1 minute. The flask was then gently tapped until the cells detached. One milliliter of FBS was added and the cells were either cryo-preserved, seeded into new flasks for propagation or

counted using a haemocytometer and thereafter seeded into the experimental wells. To cryo-preserve the cells, the trypsinised cells were transferred to a sterile tube. An equal volume of ME180 freezing fluid was added drop by drop to the cells and gently swirled to mix. The mixture was then transferred into cryovials and placed in the -80°C freezer in a closed polystyrene box to allow it to slowly reach -80°C.To seed the correct number of cells for the experiments, the cells were counted using a haemocytometer and the trypan blue exclusion assay. A haemocytometer is a glass slide onto which a counting grid is etched using a laser (Figure 3.1). A glass coverslip was placed over the counting grid of the haemocytometer. Twenty microlitres of cell suspension was added to a tube containing 20 µL of 0.4% trypan blue solution (1:2 dilution) and a drop of this mixture was transferred under the glass coverslip. The cell suspension-trypan blue dye mixture moved under the coverslip due to capillary action. The cells were then viewed under the inverted microscope at 10x magnification. Viable cells are able to actively remove the trypan blue dye out of the cell and appear colourless, while the dead cells appear blue as they are not able to remove the dye from the cell. The cells were counted in each of four corner squares on the haemocytometer (Figure 3.1). For the cell count in each square, the cells within the square and on the top and left borders were included, while the cells on the bottom and right borders were excluded. The following formula was used to calculate the number of cells in each millilitre of the cell suspension:

$$\textbf{Number of Cells/mL} = \frac{\text{number of cells}}{4} \times 10^4 \times \text{Dilution Factor}$$

In this formula, the average number of cells in each of the four squares is first calculated by dividing the total number of cells in all four squares by four. Since the volume of each square is 1×10^{-4} mL, the average number of cells in each square is then multiplied by 10^4 to provide the number of cells per millilitre of cell suspension. Finally, to correct for the dilution of the cells with trypan blue, the number of cells per millilitre is multiplied by the dilution factor (i.e two) to obtain the number of cells per millilitre in the original cell suspension.

The required number of cells was then seeded into the experimental wells.

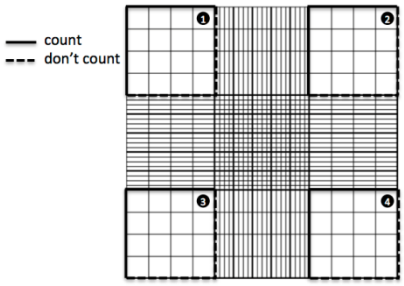


Fig. 3.1 Haemocytometer. The cell count is performed in the four corner squares of the counting grid. In each square, the cells within the square and on the top and left borders are included, while the cells on the bottom and right borders are excluded from the count. Taken from https://www.hemocytometer.org/hemocytometer-protocol/

3.3 Propagation and Storage of *N. gonorrhoeae*

Neisseria gonorrhoeae was regrown from frozen stocks by culturing the organism onto GC agar plates enriched with yeast autolysate. The plates were incubated for 24 hours at 37°C in an atmosphere with 5% CO_2 and saturated water vapour. With this culture a suspension was made in 10 mL Brain Heart Infusion (BHI) broth, with an optical density at 450 nm (OD_{450}) of 1. To allow settlement of clumps of bacteria, the tube containing the suspension was then placed upright for 1.5 hours at 37°C. Following this, the upper 4 mL of the broth, containing the single-cell suspension, was carefully transferred to a sterile tube. To confirm that a single-cell suspension was obtained, a Gram stain was performed. To determine the number of organisms in this suspension, 10-foldserial dilutions of the suspension were prepared in BHI broth and 100 µL of each dilution was spread on GC agar plates enriched with yeast autolysate (90mm) using a L-shaped spreader (Whitehead Scientific, Cape Town, South Africa). The plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 48 hours. Following incubation, the plate with a number of colonies estimated to contain between 20 and 200 colonies was chosen to determine the number of colony-forming units (CFU) of bacteria that were present in the basolateral media. The number of colonies was counted and the result multiplied by the dilution factor of the dilution used and multiplied by 10 to correct for the volume plated out. The test was done in triplicate. The number of colonies was calculated as the average of the 3 readings. To store the *N. gonorrhoeae* from the GC agar plates, 1 mL of *N. gonorrhoeae* storage media was firstly added to a cryovial and a loopful of bacterial culture was

collected from the plate using a sterile loop and added to the storage media. Sterile glass beads were then added to the cryovial, sealed and then vortexed to suspend the culture in the storage media. The suspension was then stored in the -80°C freezer. To prepare heat killed *N. gonorrhoeae*, *N. gonorrhoeae* suspended in the BHI broth was heated to 80°C for 30 minutes.

3.4 Measuring the Concentration of HIV

To determine the number of viral particles per millilitre in the serum samples obtained from AHRI, RNA was isolated from the serum and the HIV Real-TM Quant kit (Sacace Biotechnologies, Como, Italy) was used to quantify the RNA.

3.4.1 Viral RNA Extraction

Viral RNA was extracted from 140 µL of the serum sample using the QiaAmp Viral RNA Mini kit (Qiagen, Hilden, Germany). Negative and positive control samples supplied with the HIV Real-TM Quant kit (Sacace Biotechnologies, Como, Italy) were also prepared. The negative control sample contained 100 µL of the negative control reagent, while the positive control contained 90 µL of the negative control reagent and 10 µL of the positive control reagent. Five microlitres of the internal control (IC) from the HIV Real-TM Quant kit (Sacace Biotechnologies, Como, Italy) was then added to all the samples and mixed thoroughly. To each tube, 560 µL of Buffer AVL (containing carrier RNA) was added, mixed and then incubated at room temperature (which is between 22 - 23°C) for 10 minutes to enable lysis of the viral particles to occur. The tubes were then centrifuged briefly and 560 µL of absolute ethanol was added to each tube. After mixing, the sample was then transferred to the QiaAmp Mini column and centrifuged at 6000 xg for 1 minute using a Heraeus Fresco 21 microcentrifuge (ThermoFisher Scientific, Germany). Thereafter, 500 µL Buffer AW1 was added to the QiaAmp Mini column and centrifuged at 6000x g for 1 minute. This was followed by adding 500 µl of Buffer AW2 to the QiaAmp Mini column and centrifuging at 20 000 x g for 3 minutes. The QiaAmp Mini column was then transferred to a sterile microcentrifuge tube. To elute the RNA, 40 µL of elution buffer was added to the column and the sample was incubated at room temperature for 1 minute. The sample was then centrifuged at 6000x g for 1 minute and the eluted viral RNA was then stored at -80°C until required.

3.4.2 Quantification of Viral RNA

Quantification of the extracted viral RNA was performed using the HIV Real-TM Quant kit (Sacace Biotechnologies, Como, Italy). Firstly, 300 µL RT-PCR-mix-1 HIV and 200 µL RT-PCR-mix-2was added to the tube containing DTT. For each PCR reaction, 12.5 µL of the mix prepared in the DTT vial was added to 0.5 µL of TaqF Polymerase and 0.25 µL of M-MLV to obtain a volume of 13.25 µL. Twelve and a half microlitres of viral RNA was added to each PCR reaction in a 96 well PCR microtitre plate and mixed by gently pipetting up and down. In each PCR run, 3 HIV quantitation standards (QS1 HIV, QS2 HIV and QS3 HIV), 3 IC quantitation standards (QS 1 IC, QS2 IC and QS3 IC) and 1 negative control (TE-Buffer) were included. These were prepared by adding 12.5 µL of each of the reagents to separate wells containing 13.25 µL of the PCR reaction mix. The PCR plate was sealed, centrifuged briefly and loaded onto the ABI 7500 Real Time PCR Instrument (Applied Biosystems) and the PCR was performed at the conditions shown in Table 3.1.

Table 3.1: PCR Cycling Conditions for Quantification of Viral RNA

Stage	Temperature	Time	Number of cycles
1	50°C	30 minutes	1
2	95°C	15 minutes	1
3	95°C	20 seconds	5
	52°C	30 seconds	
	72°C	30 seconds	
4	95°C	20 seconds	40
	55°C	40 seconds	
	72°C	30 seconds	

The concentration of HIV in each of the control and experimental samples was calculated using the formula:

$$\textbf{Copies HIV/mL} = \frac{\text{HIV DNA copies per specimen}}{\text{IC DNA copies per specimen}} \times \text{coefficient}^*$$

*The coefficient value is provided in the HIV Real-TM Quant Data Card provided with the kit and it is specific for each lot number.

3.5 Growing the Epithelial Cell Layer

ME180 cells were prepared as described in section 3.2. The cells were counted and 3.3 x 10^4 cells were seeded into the apical chamber of 6.5 mm Transwell® inserts (Corning, Tewksbury, Massachusetts), which were collagen-coated polytetrafluoroethylene membrane tissue culture inserts with a pore size of 3 µm and a surface area of 0.33 cm^2 (Figure 3.2) (D. Ye et al. 2015). The cells were allowed to proliferate until a fully confluent epithelial layer was obtained. To confirm this, the transepithelial electrical resistance (TEER) of the layer was tested daily until a steady state of TEER was obtained. To test the TEER, the Millicell® ERS-2 system (Millipore, Burlington, Massachusetts) was used. The Millicell® ERS-2 system electrode was firstly decontaminated in 70% ethanol for 15 minutes, allowed to air-dry for 15 seconds and then placed in McCoy's 5A media (Gibco, Paisley, United Kingdom). The electrode was then ready to use. To measure the electrical resistance across the Transwell® membrane, the shorter arm of the electrode was immersed in the apical chamber of the insert and the longer arm in the basolateral chamber of the well. The resistance readings (in Ohms) were then recorded for the wells containing cells, as well as for blank wells (which contained no cells). To calculate the resistance of the cell layers, the resistance reading of the blank was firstly subtracted from the resistance reading of the cells and this value was then multiplied by the area of the membrane (cm^2). When the readings reached a steady state of resistance, the epithelial layers were ready for infection. To further confirm that a confluent epithelial layer was obtained, ZO-1 staining of the cells was carried out. This procedure involved removing the media from the cells, followed by fixing the cells in 4% paraformaldehyde for 20 minutes at room temperature. The cells were then rinsed three times with PBS (pH 7.3) and treated with 0.1% Triton X-100 for 7 minutes at room temperature. This was followed by rinsing the cells three times with PBS and blocking the cells in 3% bovine serum albumin (BSA) blocking solution for 30 minutes at room temperature. A further three washes with PBS were done and the cells were then incubated for 1 hour at room temperature in Rabbit anti-ZO-1 IgG antibody (1mg/ml) (Abcam, Cambridge, United Kingdom), which was diluted to 0.01 mg/ml 3% BSA. After the incubation period, the cells were once again washed three times with PBS (pH 7.3) and incubated for 1 hour at room temperature in Alexa Fluor® 488 Goat Anti-Rabbit IgG H&L antibody (Abcam, Cambridge, United Kingdom) (2mg/ml), which was diluted 1 in 100 in 3% BSA. Following this incubation, the cells were washed three times with PBS (pH 7.3) and the membrane was excised from the Transwell® insert using a number 24 surgical blade (and transferred to a microscopy slide with the cells facing upwards. A drop of Invitrogen Prolong Gold Antifade mountant with

DAPI (ThermoFisher Scientific, Waltham, Massachusetts) was added to the cells and this was then covered with a glass coverslip. The cells were viewed under a fluorescent microscope (Leica DM3000, Wetzlar, Germany) at 100 x magnification. Whether the confluent epithelial layer was intact was determined using the Blue Dextran Permeability Assay. The epithelial cell layer was prepared as mentioned in section 3.2. When a steady state of electrical resistance was obtained, the media was removed from the apical chamber of the Transwell® insert and the insert was transferred to a well containing fresh media. One hundred microlitres of blue dextran dye (Sigma, St Louis, USA) was added to the upper chamber of the Transwell® insert. Blue dextran dye was freshly prepared in McCoy's 5A medium (Gibco, Paisley, United Kingdom) to obtain a final concentration of 2.3 mg/mL and the dye was filter sterilized using a 0.22 µm filter (Millipore, Burlington, Massachusetts). Following an incubation of 1 hour, 100 µL of media from the basolateral chamber was removed and the optical density of the dye was measured at 595 nm. The optical density was expressed as a percentage of the dye added to the apical side.

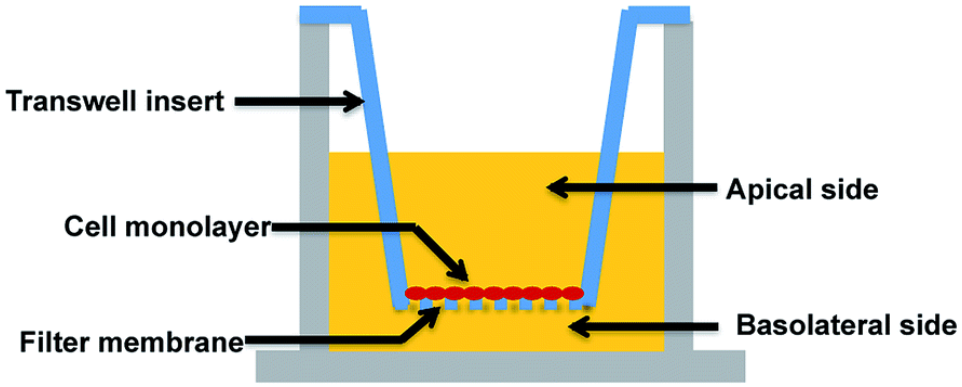


Fig 3.2 Transwell® insert. This is a diagram of the Transwell insert. The insert is placed into the well thereby creating an apical side and a basolateral side. The cells are seeded into the apical side. The cells attach to the filter membrane and form an epithelial layer. Taken from Ye et al. (2015).

3.6 Effect of Different *N. gonorrhoeae* MOIs on the Epithelial Layer

To determine the effect that *N.gonorrhoeae* of different multiplicity of infections (MOIs) would have on the epithelial layer, a confluent epithelial layer was infected with different MOIs of *N. gonorrhoeae*.

3.6.1 Trans-epithelial Migration Assay with *N. gonorrhoeae* at Different MOIs

The confluent epithelial layer was prepared on the Transwell inserts as described in section 3.5. The cells were then infected with *N. gonorrhoeae* at MOIs of 100,10, 1, 0.1 and 0.01. In addition, the cells were infected with heat killed *N. gonorrhoeae* at a MOI of 1. At 30 minutes and 24 hours post infection, the basolateral media was collected for colony forming unit (CFU) counts (as described in section 3.3).

3.6.2 Tight Junction Gene Expression Assay for Different *N.gonorrhoeae* MOIs

ME180 cells were prepared as described in section 3.2. The cells were counted and 1 x 10^5 cells were seeded per well in collagen-coated 24 well plates. The cells were monitored daily under an inverted microscope until a fully confluent epithelial layer was obtained. When the cells were fully confluent, the epithelial layer was infected with *N.gonorrhoeae* only at a MOI of 100, 1, 0.1 and 0.01, as well as with heat killed *N. gonorrhoeae* at a MOI of 1, and incubated at 37°C in ahumidied atmosphere with 5% CO_2. At each time point 30 minutes and 24 hours post infection, the cells were washed with PBS and 500 µL of Trisure reagent (Bioline, London, England) was added to each well and pipetted up and down a few times to lyse the cells. The lysed samples were then collected into appropriately labeled microcentrifuge tubes and stored at -80°C for extraction of RNA.

3.6.2.1 RNA Extraction

Total RNA was extracted from the stored samples. The RNA extraction was performed by firstly thawing the frozen samples and then adding 100 µL of chloroform to each of the samples. The samples were incubated at room temperature for 5 minutes, shaken vigorously for 15 seconds and incubated at room temperature for 3 minutes. Thereafter, the samples were centrifuged using a Heraeus Fresco 21 microcentrifuge (ThermoFisher Scientific, Germany) for 15 minutes at 12 000 x g. The colorless upper aqueous phase, which contained the RNA, was carefully transferred to a sterile 15 mL tube. An equal volume of cold 70% ethanol (prepared by adding 70 mL absolute ethanol to 30 mL Diethyl Pyrocarbonate (DEPC)-treated water) was added to the tube. The RNA was then extracted from these samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, the sample was added to the RNeasy spin column and centrifuged at 8000 x g for 15 seconds. The RNA, which was bound to the spin column membrane, was then washed once with Buffer RW1 and thereafter washed twice with

Buffer RPE. The RNA was then eluted in 40 µL of ribonuclease (RNase)-free water. The quantity and purity of the RNA was determined using a Nanodrop spectrophotometer. This was followed by deoxyribonuclease (DNase) treatment of the RNA.

3.6.2.2 DNase Treatment of RNA

One microgram of the extracted RNA was DNase treated using DNase I (ThermoFisher Scientific, Waltham, Massachusetts) according to the manufacturer's instructions. In a 0.2 mL PCR tube, 1 µg of RNA was added to 1 µL of 10 X reaction buffer, 1 µL DNase I and DEPC-treated water to obtain a final volume of 10 µL. The sample was then incubated for 30 minutes at 37°C. One microlitre of 50 mM ethylenediaminetetraacetic acid (EDTA) was added to each tube and further incubated for 10 minutes at 65°C. The RNA was then used to prepare complementary deoxyribonucleic acid (cDNA) and the remaining RNA was stored at -80°C.

3.6.2.3 cDNA Synthesis

The DNase-treated RNA samples were reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription kit (ThermoFisher Scientific, Waltham, Massachusetts) according to the manufacturer's instructions. The cDNA master mix was prepared by adding 2.0 µL 10X RT Buffer, 0.8 µL 25X dNTP Mix (100mM), 2 µL10X RT Random Primers, 1 µL MultiScribe™ Reverse Transcriptase and 1.0 µL RNase Inhibitor per reaction. One microgram of RNA was used in each reaction and the volume of the water was adjusted to obtain a final volume of 20 µL. Each reaction was prepared in PCR tubes and loaded onto a thermal cycler. The PCR was performed at the cycling conditions shown in Table 3.2.

Table 3.2. PCR Cycling Conditions for cDNA Synthesis from Epithelial Cell RNA

Temperature	Time	Number of cycles
25°C	10 minutes	1
37°C	120 minutes	1
85°C	5 minutes	1
4°C	Hold	

The cDNA was then stored at -20°C until required.

3.6.2.4 Real Time PCR

The FastStart Essential DNA Green Master kit was used according to the manufacturer's instructions. This kit uses SYBR Green technology. Each 20 µL reaction contained 10 µL Master Mix, 1µL forward primer (0.25 µM final), 1 µL reverse primer (0.25 µM final), 3 µL PCR grade water and 5 µL cDNA. The tight junction gene primers, as well as GAPDH, can be seen in Table 3.3. Amplification was performed in the ABI 7500 Real Time PCR Instrument (Applied Biosystems, Life Technologies) using a 96-well microtitre reaction plate. The cycling conditions used can be seen in Table 3.4.

Table 3.3. PCR Primer Sequences for Tight Junction Protein Coding Genes

Gene	Primer Sequence	References
Occludin	Forward Primer: CATTGCCATCTTTGCCTGTG Reverse Primer: AGCCATAACCATAGCCATAGC	(Nazli et al. 2010)
Claudin 4	Forward Primer: TCATCGGCAGCAACATTGTC Reverse Primer: GCAGTGCCAGCAGCGAGT	(P. Ye 2012)
Claudin 5	Forward Primer: TCATCGGCAGCAACATTGTC Reverse Primer: GCAGTGCCAGCAGCGAGT	(P. Ye 2012)
GAPDH	Forward Primer: TCTCTGCTCCTCCTGTTC Reverse Primer: CTCCGACCTTCACCTTCC	(Nazli et al. 2010)

Table 3.4. Real Time PCR Cycling Conditions for Gene Expression Assay

Stage	Temperature	Time	Number of cycles
	50°C	2 minutes	1
Pre-incubation	94°C	10 minutes	1
Amplification	94°C	15 seconds	40
	62°C	1 minute	
Melting Curve	95°C	15 seconds	1
	60°C	1 minute	
	95°C	30 seconds	
	60°C	15 seconds	

A melt curve was performed to confirm the specificity of the amplification. Analysis of the PCR data was carried out using the comparative CT method. Briefly, the comparative CT method calculates the fold change ($2^{-\Delta\Delta CT}$) of the tight junction gene in the infected sample compared to the uninfected (control) sample using the following equations:

ΔΔCT = [(CT target – CT reference) sample – (CT target – CT reference) calibrator)]

Expression Fold Change = $2^{-\Delta\Delta CT}$

where: $\mathbf{2^{-\Delta\Delta CT}}$ refers to the fold change, **CT** refers to the threshold cycle, **target** refers to the tight junction gene, **reference** refers to the housekeeping gene and **calibrator** refers to the control or uninfected samples

The methodology from section 3.6.2 was adapted for the gene expression assay. The cells were seeded onto collagen-coated 24 well plates and when the cells were fully confluent, the epithelial layer was infected with *N. gonorrhoeae* only, HIV only or a combination of *N. gonorrhoeae* and HIV at a MOI of 1 and incubated at 37°C in a humidified atmosphere with 5% CO_2. At each time point (i.e 30 minutes, 6 hours and 24 hours) post infection, the cells were washed with PBS and 500 µL of Trisure reagent (Bioline, London, England) was added to each well and pipetted up and down a few times to lyse the cells. The lysed samples were then collected into appropriately labeled

microcentrifuge tubes and stored at -80°C for extraction of RNA. The RNA extraction, DNAse treatment, cDNA synthesis and Real Time PCR was performed as described in sections 3.6.2.1, 3.6.2.2, 3.6.2.3 and 3.6.2.4 respectively. The primer sequences for the tight junction genes, as well as GAPDH can be seen in Table 3.5.

Table 3.5. PCR Primer Sequences for the Tight Junction Protein Coding Genes

Gene	Primer Sequence	References
ZO-1	Forward Primer: AGAAGGATGTTTATCGTCGCATT Reverse Primer: CCAAGAGCCCAGTTTTCCAT	(P. Ye 2012)
Occludin	Forward Primer: CATTGCCATCTTTGCCTGTG Reverse Primer: AGCCATAACCATAGCCATAGC	(Nazli et al. 2010)
Claudin 1	Forward Primer: CAATGCCAGGTACGAATTTGG Reverse Primer: TGGATAGGGCCTTGGTGTTG	(P. Ye 2012)
Claudin 4	Forward Primer: TCATCGGCAGCAACATTGTC Reverse Primer: GCAGTGCCAGCAGCGAGT	(P. Ye 2012)
Claudin 5	Forward Primer: TCATCGGCAGCAACATTGTC Reverse Primer: GCAGTGCCAGCAGCGAGT	(P. Ye 2012)
GAPDH	Forward Primer: TCTCTGCTCCTCCTGTTC Reverse Primer: CTCCGACCTTCACCTTCC	(Nazli et al. 2010)

3.7 Migration of Heat Killed *N. gonorrhoeae*

The heat killed *N. gonorrhoeae* was prepared as described in section 3.3. The confluent epithelial layer was prepared on the Transwell inserts as described in section 3.5 and infected with the heat killed *N. gonorrhoeae*. At 24 hours post infection, the basolateral media was collected, genomic DNA was extracted and conventional pili protein gene PCR was performed.

3.7.1 DNA Extraction of *N. gonorrhoeae*

DNA was extracted from the basolateral media using the Wizard Genomic DNA Purification Kit (Promega, USA). The basolateral media was centrifuged for 2 minutes at 14 000 x g. The supernatant was then discarded and 600 µL of nuclei lysis solution was

added, mixed gently and incubated for 5 minutes at 80°C. The suspension was then allowed to cool to room temperature and thereafter 3µL of RNase solution was added, mixed and incubated at 37°C for 30 minutes. The suspension was then allowed to cool to room temperature and 200 µL of protein precipitation solution was added, vortexed and incubated on ice for 5 minutes. The suspension was then centrifuged for 3 minutes at 14 000 x g. The supernatant was then transferred to a sterile microcentrifuge tube and 600 µL of isopropanol was added and inverted to mix until thread-like strands of DNA were visible. The tube was then centrifuged for 2 minutes at 14 000 x g. The supernatant was discarded and 600µL of 70 % ethanol was added, inverted to mix and centrifuged for 2 minutes at 14 000 xg. The ethanol was discarded allowed to air dry for 10 to 15 minutes. The DNA pellet was rehydrated in 100 µL of nuclease free water overnight at 4°C. The DNA was then used immediately or stored at -20°C.

3.7.2 Conventional Pili Protein Gene PCR

The DreamTaq PCR Master Mix (2X) kit (ThermoFisher Scientific, Waltham, Massachusetts) was used according to the manufacturer's instructions. Each 50 µL reaction contained 25 µL DreamTaqPCRMaster Mix (2X), 1µL forward primer (0.25 µM final), 1 µL reverse primer (0.25 µM final), 21 µL PCR grade water and 2 µL DNA. The Pili protein gene forward primersequence was 5' GGCTTTCCCCTTTCAATTAGGAG 3' and the reverse primer sequence was 5' GCAGGTGACGGCAGGTGC 3'. Amplification was performed in the ABI 7500 Real Time PCR Instrument (Applied Biosystems, Life Technologies). The cycling conditions used can be seen in Table 3.6.

Table 3.6. PCR Cycling Conditions for Pili Protein Gene PCR

Stage	Temperature	Time	Number of cycles
Initial Denaturation	95°C	4 minutes	1
Amplification	95°C	30 seconds	30
	55°C	30 seconds	
	72°C	1 minute	
Final Extension	72°C	5 minutes	1

3.8 *N. gonorrhoeae* and HIV Migration Assays

To determine the effect that *N. gonorrhoeae* or HIV would have on an intact cervical epithelial layer, a confluent epithelial layer was infected with *N. gonorrhoeae* only, HIV only or a combination of *N. gonorrhoeae* and HIV. At each time point the basolateral media was collected. The *N. gonorrhoeae* count was performed immediately after collection, while the basolateral media collected for the HIV migration assay was stored at -80°C until required.

3.8.1 Transmigration of *N. gonorrhoeae*

The *N. gonorrhoeae* migration assay was performed on the basolateral media collected at time points 30 minutes, 6 hours, 16 hours and 24 hours from the wells infected with either *N. gonorrhoeae* only or with *N. gonorrhoeae* and HIV simultaneously. To determine the number of colonies in the basolateral media, CFU counts were performed as described in section 3.3. The percentage of *N. gonorrhoeae* that had migrated to the basolateral media was determined by dividing the number of *N. gonorrhoeae* calculated from the CFU count by the initial inoculums added to the apical chamber. To determine why there was a high percentage of *N. gonorrhoeae* in the basolateral media at 24 hours post infection, the number of *N. gonorrhoeae* that had migrated to the basolateral media at 30 minutes was calculated and that number of *N. gonorrhoeae* was then suspended in 1mL of McCoy's 5A media in a well of a 24 well tissue culture plate and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 24 hours. Following incubation, CFU counts were performed as described in section 3.3.

3.8.2 HIV Count Post Migration

The HIV migration assay was carried out on the stored basolateral media collected at time points 30 minutes, 6 hours and 24 hours from the wells infected with either HIV only or with *N. gonorrhoeae* and HIV simultaneously. Viral RNA was first isolated from the sample and the RNA was then quantified using the HIV Real-TM Quant kit (Sacace Biotechnologies, Como, Italy).The viral RNA was extracted from the stored basolateral media using the QiaAmp Viral RNA Mini kit (Qiagen, Hilden, Germany). The samples were first allowed to thaw and then 500 µL of sample was transferred to a sterile microcentrifuge tube and centrifuged at 4°C at 21 000 x g for 1 hour. The supernatant was removed from the tube, leaving behind 140 µL of supernatant above the pellet. This step

was done carefully to ensure that the pellet was not disturbed. The pellet was then resuspended in the remaining supernatant. Negative and positive control samples supplied with the HIV Real-TM Quant kit (Sacace Biotechnologies, Como, Italy) were also prepared and the RNA extraction was performed as described in section 3.4.1. Quantification of the viral RNA was carried out as described in section 3.4.2. To determine the percentage of HIV that transmigrated across the epithelial layer to the basolateral side, the number of copies HIV per millitre was divided by the initial number of HIV that was added to the apical chamber of the Transwell insert multiplied by 100.

3.8.3 Blue Dextran Permeability Assay

To determine whether infection with the organisms resulted in damage to the epithelial layer, the Blue Dextran permeability assay was performed. The confluent epithelial layer was prepared as described in section 3.5. The cells were then infected with *N. gonorrhoeae* only, HIV only or a combination of *N. gonorrhoeae* and HIV at a MOI of 1. At the appropriate time points (i.e 30 minutes, 6 hours and 24 hours), the apical media of the Transwell® inserts was removed and the insert was transferred into a well containing fresh media. One hundred microlitres (100 µL) of the blue dextran dye was then added to the upper chamber of the insert and incubated for 1 hour. Following the incubation, 100µL of media from the basolateral chamber was removed and the optical density of the dye was measured at 595nm. The optical density was expressed as a percentage of the dye added to the apical side.

3.9 Tight Junction Protein Staining

ME180 cells were prepared (as described in section 3.2) and seeded onto collagen coated acid-washed glass coverslips placed in 24 well tissue culture plates. The cells were monitored daily until a confluent monolayer was obtained. At this point, the cells were infected with *N.gonorrhoeae* only, HIV only or a combination of *N. gonorrhoeae* and HIV at a MOI of 1 and incubated at 37°C with 5% CO_2. At each time point (i.e 30 minutes, 6 hours and 24 hours) post infection, the cells were washed with PBS (pH 7.3) and fixed in 4% paraformaldehyde for 20 minutes at room temperature. The cells were then rinsed three times with PBS and treated with

0.1% Triton X-100 for 7 minutes at room temperature. This was followed by rinsing the cells three times with PBS and suspending the cells in 3% bovine serum albumin (BSA) in PBS blocking solution for 30 minutes at room temperature. A further three washes were done with PBS (pH 7.3) and the cells were then incubated for 1 hour at room temperature in primary antibody (1mg/mL). The primary antibody was anti-occludin rabbit IgG antibody (Abcam, Cambridge, UK). After the incubation, the cells were once again washed three times with PBS and incubated for 1 hour at room temperature in Alexa Fluor® 488 Goat Anti-Rabbit IgG H&L antibody (2mg/mL) (Abcam, Cambridge, UK). Following this incubation period, the cells were washed three times with PBS. A drop of Invitrogen Prolong Gold Antifade mountant with DAPI (ThermoFisher Scientific) was placed on a microscope slide and the coverslip was carefully placed on the drop of mountant, with the cells facing the slide. The cells were viewed under a fluorescent microscope (Leica DM3000, Wetzlar, Germany) at 100x magnification.

3.10 Statistical Analysis

Statistical analysis was performed by a statistician using Stata statistical software. *N. gonorrhoeae* migration was summarized as the mean and standard deviation of the percentage of *N. gonorrhoeae* that migrated to the basolateral media. At each time point, the migration of *N. gonorrhoeae* for the *N. gonorrhoeae* only and the *N. gonorrhoeae* and HIV infections were compared using Wilcoxon rank sum test. For HIV migration, comparisons between *N. gonorrhoeae* and *N. gonorrhoeae* and HIV infections were made using the Wilcoxon rank sum tests. The fold change in gene expression to the control was calculated for each organism at each time point. The data was summarized as the mean and standard error of the fold change of the fold change and the Wilcoxon sign rank test was used to compare the mean fold change to one (indicating no difference). The Wilcoxon rank sum test was then used to compare the *N. gonorrhoeae* only to the *N. gonorrhoeae* and HIV fold change at 24 hours.

The formula and preparation method of all media and reagents used in section 3 is described in the Appendix.

CHAPTER 4: RESULTS

4.1 Concentration of Microbes

The number of *N. gonorrhoeae* in the single-cell suspension prepared as described in section 3.3 was 4.7 x 10^8cfu/mL. The viral load in the HIV serum determined as described in section 3.4 was 9.4 x 10^{12} copies/mL.

4.2 Epithelial Layer for Transmigration Assay

The ME180 cells were seeded onto Transwell® inserts as mentioned in section 3.5. To determine when the cells had formed a confluent epithelial layer with intact tight junctions, the TEER was monitored daily until a steady state was reached. Although a confluent epithelial layer was visible under the microscope approximately 3 days post seeding, the resistance readings only reached a steady state at 6 days post seeding. Table 4.1 shows the TEER of 2 experiments. To further confirm that the tight junctions were intact, ZO-1 immunofluorescence staining of the epithelial layer at 6 days post seeding was performed, as described in section 3.5. The results showed that the ZO-1 tight junction proteins were intact in the epithelial layer (Figure 4.1). In addition, the blue dextran permeability assay was performed to test the permeability of the epithelial layer when the steady state of electrical resistance was reached. There was no leakage of the dye to the basolateral media, thus confirming that the tight junctions of the epithelial layer were intact.

Table 4.1 Trans-epithelial Resistance Readings of Confluent Epithelial Layer

Days (Post Seeding)	Time (hours)	TEER ($\Omega.cm^2$)	TEER Range ($\Omega.cm^2$)
1	24	3.6	(3.3 – 4)
2	48	4.3	(4 – 4.62)
3	72	7.3	(7.3 – 7.3)
4	96	10.2	(9.9 – 10.6)
5	120	11.9	(11.9 – 11.9)
6	144	12.5	(12.2 – 12.9)
7	168	12.5	(12.5 – 12.5)
8	192	12.5	(12.5 – 12.5)

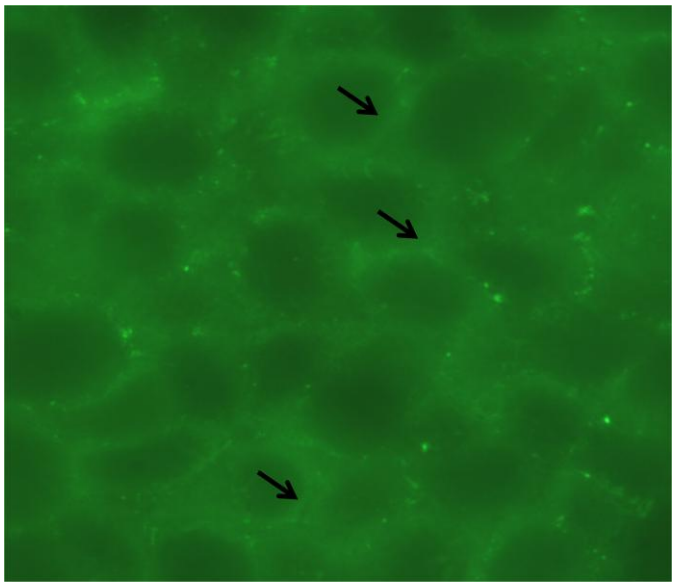


Figure 4.1. ZO-1 staining of the epithelial layer. ZO1 staining of the epithelial layer showed that a confluent epithelial layer formed with intact tight junctions, which are indicated by the arrows.

4.3 Effect of Different *N. gonorrhoeae* MOIs on the Epithelial Layer

4.3.1 Trans-epithelial Migration Assay with *N.gonorrhoeae* at Different MOIs

The confluent epithelial layer was infected with *N.gonorrhoeae* at different MOIs (viz. MOI of 100, MOI of 10, MOI of 1, MOI of 0.1 and MOI of 0.01), as well as with heat killed *N.gonorrhoeae* to determine the effect of the concentration of bacteria on trans-epithelial migration (section 3.6.1). At 30 minutes, the highest migration was observed at a MOI of 1 (Figure 4.2A). At MOIs of 100 and 10, *N.gonorrhoeae* was able to migrate across the epithelial layer, but in number of colonies too low to count reliably (explained in section 3.3). At this time point there was no migration of *N.gonorrhoeae* at a MOI of 0.1, 0.01 nor with the heat killed bacteria. At 24 hours post infection, the number of *N.gonorrhoeae* that was present in the basolateral media was similar at MOI of 100, 10, 1 and 0.1 (Figure 4.2B). The number of migrating organisms was lowest at the MOI of 0.01. There was no heat killed *N.gonorrhoeae* detected in the basolateral media.

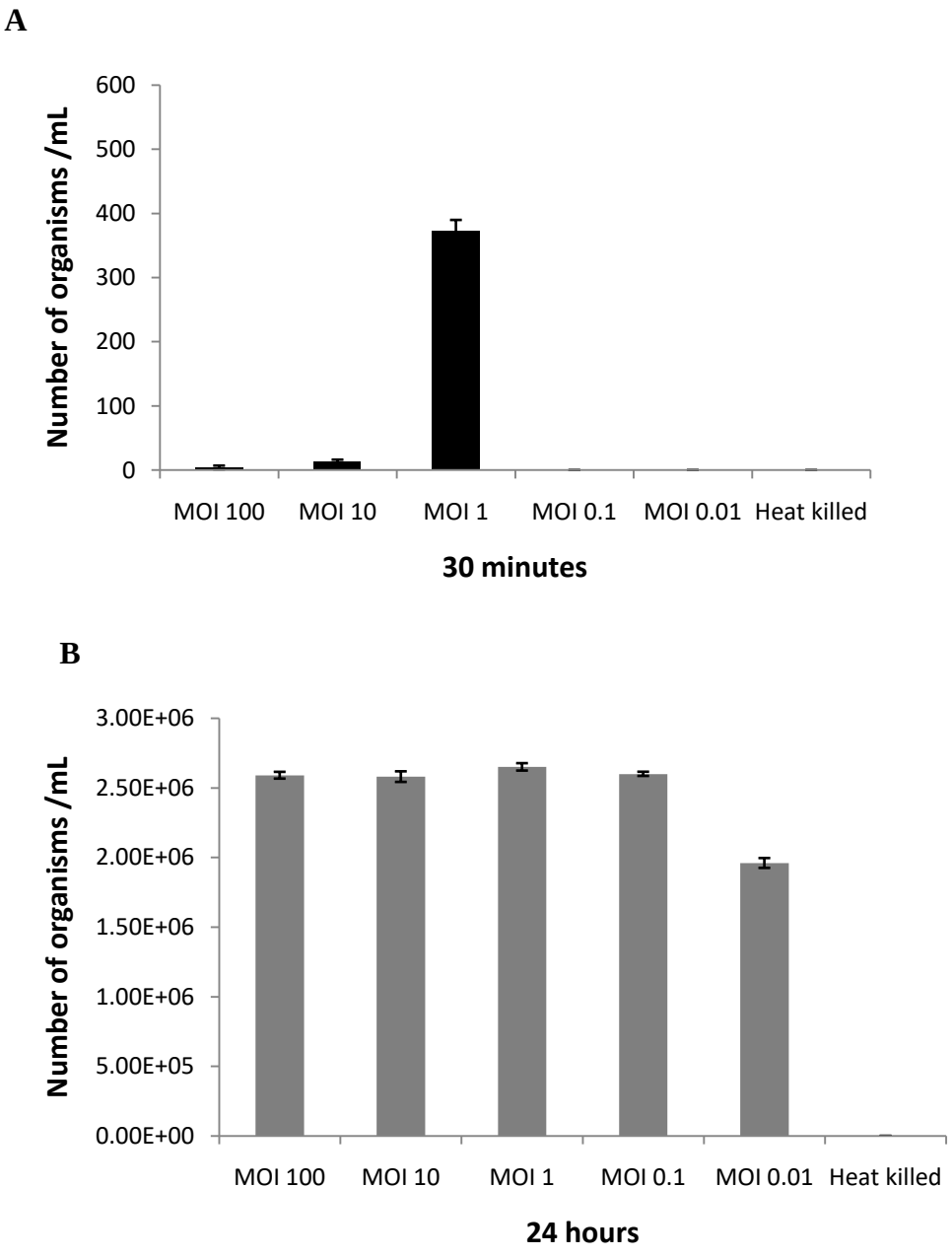
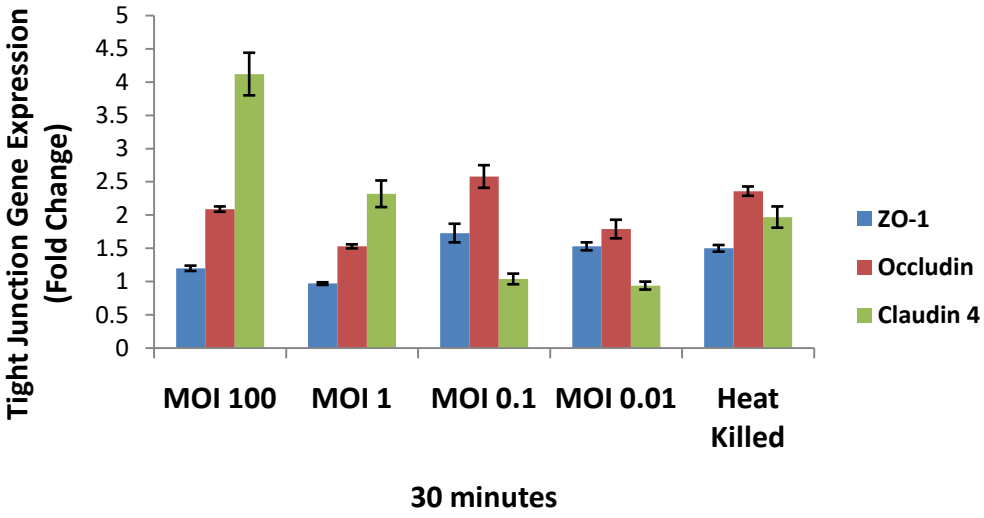


Figure 4.2. *N. gonorrhoeae* Migration for Different MOIs. The graphs show the results of the *N. gonorrhoeae* migration assay using different MOIs of *N. gonorrhoeae* (i.e MOI of 100, MOI of 10, MOI of 1, MOI of 0.1 and MOI of 0.01), as well as heat killed *N. gonorrhoeae*, at 30 minutes (A) and 24 hours (B). The results show that at 30 minutes, *N. gonorrhoeae* was able to migrate across the epithelial layer at a MOI of 1. At 24 hours, the number of *N. gonorrhoeae* that was able to migrate across the epithelial layer was similar for the MOI of 100, 10, 1 and 0.1, however the migration was lower at MOI of 0.01. The heat killed *N. gonorrhoeae* did not move across the epithelial layer. Measurements are the mean values of 9 and the error bars indicate the SEM.

4.3.2 Tight Junction Gene Expression Assay for Different *N. gonorrhoeae* MOIs

In an attempt to determine how *N. gonorrhoeae* at different MOIs would affect the expression of tight junction protein coding genes, confluent epithelial cell layers were infected with *N. gonorrhoeae* at a MOI of 100, 1, 0.1and 0.01, as well as with heat killed *N. gonorrhoeae* (section 3.6.2). The tight junction protein gene expression at 30 minutes post infection and 24 hours post infection can be seen in Figure 4.3 (A) and Figure 4.3 (B), respectively.

A



B

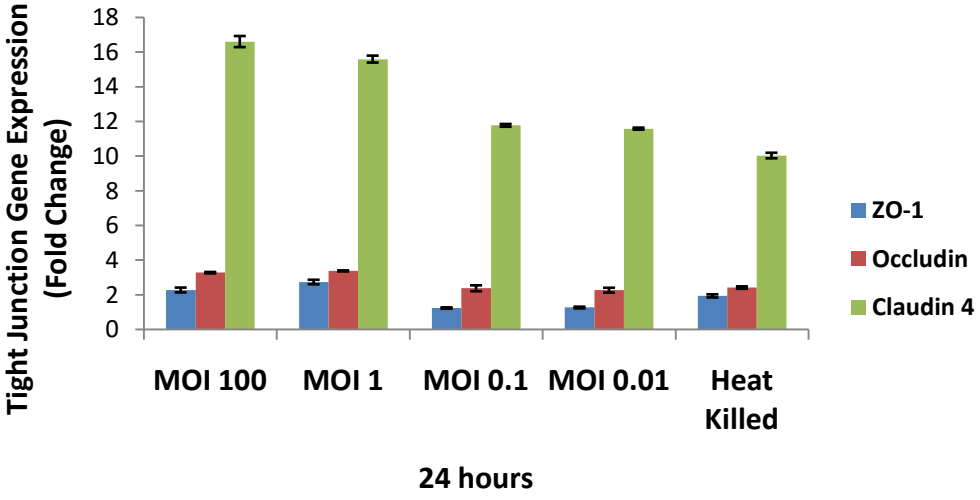


Figure 4.3. Tight Junction Protein Gene Expression Assay for Different *N. gonorrhoeae* MOIs. The graphs show the fold change of the expression of the tight junction genes in the cells infected with *N.gonorrhoeae*at different MOIs (i.e MOI of 100, MOI of 10, MOI of 1, MOI of 0.1

and MOI of 0.01), as well as heat killed *N.gonorrhoeae,* at 30minutes post infection (A) and 24 hours post infection (B). A fold change of one implies that the expression of the target gene in the infected cells is the same as in the uninfected cells. Measurements are the mean values of 9 experiments and the error bars indicate the standard error of the mean (SEM).

4.4 Migration of Heat Killed *N. gonorrhoeae*

The heat killed *N. gonorrhoeae* was not detected in the basolateral media in the trans-epithelial migration assay (as shown in section 4.3.1). Since heat killed *N. gonorrhoeae* would not be viable and would therefore not produce colonies in the colony-count assay, conventional pili protein gene PCR was performed on DNA extracted from the basolateral media to determine if the heat killed *N. gonorrhoeae* was able to migrate across the epithelial layer. The results can be seen in Figure 4.4. This shows that the *N. gonorrhoeae* strain used does express the pili protein gene and it also shows that killed *N. gonorrhoeae* was not able to migrate across the epithelial layer which shows that the migration of *N. gonorrhoeae* is a process that involves live bacterial cells.

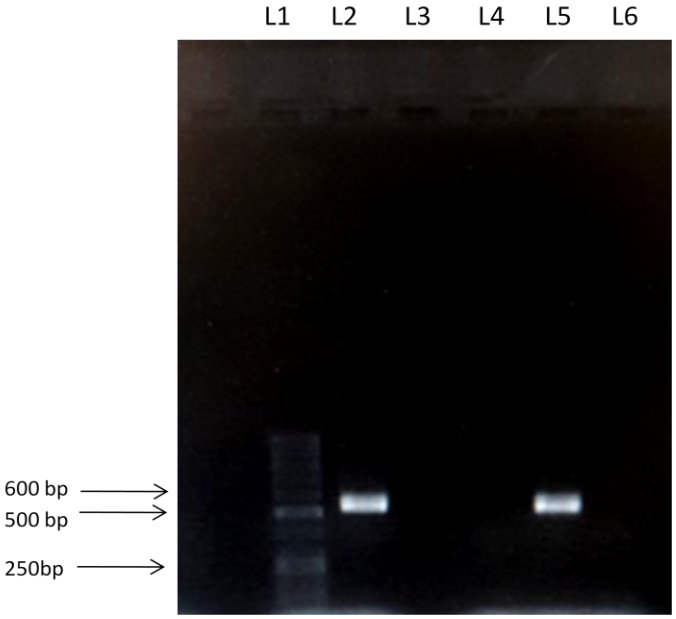


Figure 4.4 PCR Products of the *N. gonorrhoeae* pilin gene. The results of amplification of pilin gene in DNA extracted from *N. gonorrhoeae* (lane 2), heat killed *N. gonorrhoeae* (L5) and heat killed *N.gonorrhoeae* from the basolateral media 24 hours post infection (L6). Lane 1 (L1) contains the 50 bp DNA ladder, lane 4 (L4) contains the negative control and lane 3 (L3) is empty. The PCR product is 516bp.

4.5 *N. gonorrhoeae* and HIV Migration Assays

The confluent epithelial layer was exposed to *N. gonorrhoeae* only, HIV only or a combination of *N. gonorrhoeae* and HIV. At each time point (i.e 30 minutes, 6 hours and 24 hours) post infection, the blue dextran permeability assay was carried out as described in section 3.8.3to determine if exposure to the microorganisms affected the tight junctions. The results showed that at each time point there was no leakage of the blue dextran from the apical to the basolateral compartment, which shows that the tight junctions remained intact when exposed to both microbes individually and together.

The results of the *N. gonorrhoeae* migration assay showed that even though the tight junctions were intact, the organism had started to move across the epithelial layer as early as 30minutes post infection (Table 4.2). There was minimal movement of the microorganism across the epithelial layer until 16 hours post infection. At 16 hours post infection the number of colony forming units in the basolateral media was for *N. gonorrhoeae* only 92 % of the inoculum. At 24 hours post infection for *N. gonorrhoeae* only and from 16 hours post infection for the combination with HIV, the percentage of *N. gonorrhoeae* that was present in the basolateral media had increased above 100% of the inoculum added to the apical compartment. To confirm that the high percentage of *N. gonorrhoeae* that was present in the basolateral media was due to multiplication of the microorganisms that had migrated across the epithelial layer, the same number of *N. gonorrhoeae* that migrated through to the basolateral media at 30 minutes was suspended in media and incubated for 24 hours as described in section 3.8.1. The result obtained confirmed that the high percentage of *N. gonorrhoeae* shown at 24 hours was indeed due to growth of the bacteria in the media. Further migration beyond 6 hours is likely to have taken place in addition to multiplication. How much of the 92 % at 16 hours for *N. gonorrhoeae* only is the result of migration could not be established.

Table 4.2 Results of *N. gonorrhoeae* Migration Assay

	***N.gonorrhoeae*Only**		***N.gonorrhoeae*and HIV**	
	Mean Migration	**SD**	**Mean Migration**	**SD**
30 minutes	0.61 %	0.25	0.63 %	0.09
6 hours	1.02 %	0.14	2.44 %	0.17
16 hours	92 %	7.48	225.67 %	8.98
24 hours	600 %	126.75	3260 %	395.1

At each time point, more colony forming units of *N. gonorrhoeae*were found in the basolateral medium with HIV present as compared to *N. gonorrhoeae* alone. This difference was statistically significant at time points, 6 hours ($p = 0.0039$), 16 hours ($p = 0.0039$) and 24 hours ($p = 0.0039$).

The percentage of HIV that transmigrated across the confluent epithelial layer was determined as described in section 3.8.2. The results obtained for the infection with HIV and *N. gonorrhoeae* simultaneously and HIV only can be seen in Figure 4.5.

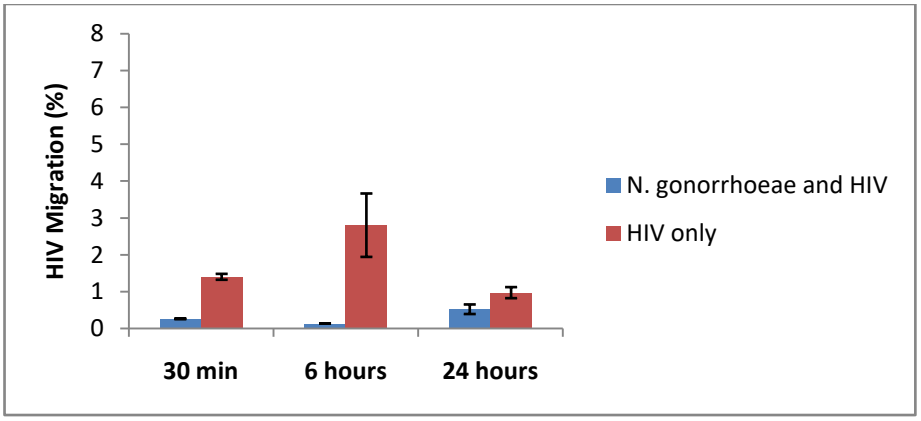


Fig 4.5 Results of HIV Migration Assay. The results of the HIV migration assay showed that there was less than 4% migration of HIV across the epithelial layer.Measurements are the mean values of 3 experiments and the error bars indicate the SEM. The difference between *N. gonorrhoeae* and HIV was not significantly different at 30 minutes ($p = 0.12$), 6 hours ($p = 0.12$) and 24 hours ($p = 0.12$). The statistical method used was the Wilcoxon rank sum test.

The percentage of HIV that was able to migrate across the epithelial layer in each of the experiments was less than 4%. The highest amount of HIV in the basolateral fluid was seen at 6 hours post inoculation in the absence of *N. gonorrhoeae*. No migration of HIV was observed with *N. gonorrhoeae* present.

4.6 Gene Expression Assay

To determine if exposure to HIV only, *N. gonorrhoeae* only or a combination of *N. gonorrhoeae* and HIV altered the expression levels of the tight junction genes, the gene expression assay was performed as described at the end of section 3.6.2. The RNA extracted from the epithelial cells had concentrations that ranged from 95 – 145 ng/µL and the purity ranged from 1.8 to 2.0.The results of the tight junction gene expression assay show the fold change in ZO-1 (Figure 4.6), occludin (Figure 4.7), claudin-1(Figure 4.8), claudin-4 (Figure 4.9) and (Figure 4.10) for *N. gonorrhoeae* only, *N. gonorrhoeae* and HIV simultaneously and HIV only. The results for exposure with HIV only showed that the virus did not seem to alter the expression of the tight junction genes, as compared to the uninfected control cells (Figure 4.11 C). Exposure to *N. gonorrhoeae* only or a combination of *N. gonorrhoeae* and HIV (Figure 4.11 A and 4.11 B, respectively) did not appear to alter the tight junction gene expression at time points 30 minutes and 6 hours. However, 24 hours post exposure there was a downregulation of the claudin 5 gene expression, as well as an upregulation in the claudin 4 and occludin gene expression. This difference was statistically significant for claudin 5 with a p value of 0.00.

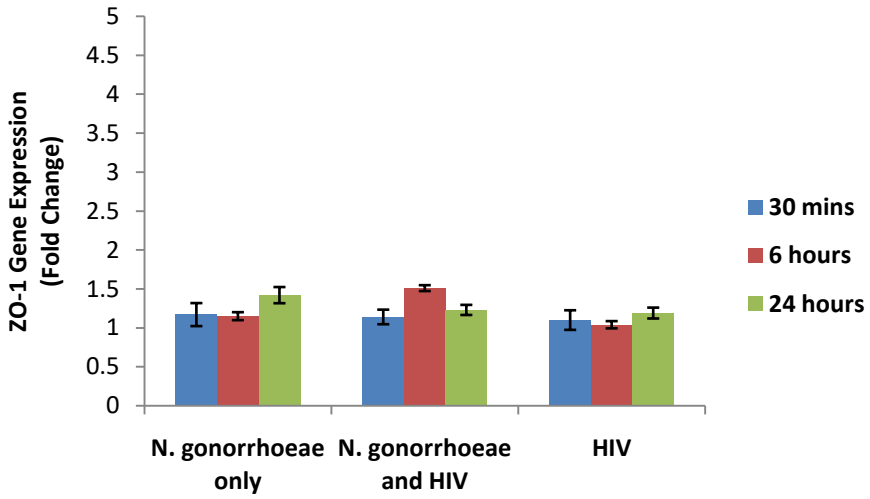


Fig 4.6 Graph showing fold change of ZO-1 gene expression following exposure to *N. gonorrhoeae, N. gonorrhoeae* and HIV simultaneously and HIV only. This graph shows the fold change of the expression of the ZO-1 tight junction gene in the ME180 cells infected with *N. gonorrhoeae, N. gonorrhoeae* and HIV simultaneously and HIV only, as compared to the uninfected cells, at time points 30 minutes, 6 hours and 24 hours. A fold change of one implies that the expression of the target gene in the infected cells is the same as in the uninfected cells. Measurements are the mean values of six to nine experiments and the error bars indicate the standard error of the mean (SEM). The fold change between *N. gonorrhoeae* only and the uninfected cells was not significantly different at 30 minutes ($p = 0.4$), but was significantly different at 6 hours ($p = 0.02$) and 24 hours ($p = 0.02$). The fold change between *N. gonorrhoeae* and HIV simultaneously and the uninfected cells was not significantly different at 30 minutes ($p = 0.3$), but was significantly different at 6 hours ($p = 0.02$) and 24 hours ($p = 0.02$). The fold change between HIV only and the uninfected cells was not significantly different at 30 minutes ($p = 0.6$) and 6 hours ($p = 0.5$), but was significantly different at 24 hours ($p = 0.04$). The statistical analysis was done using a Wilcoxon sign rank test.

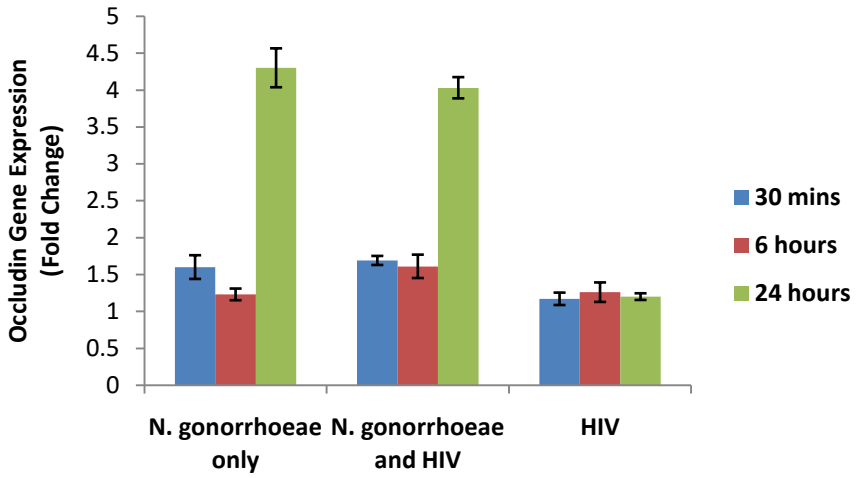


Fig 4.7 Graph showing fold change of occludin gene expression following exposure to *N. gonorrhoeae, N. gonorrhoeae* and HIV simultaneously and HIV only. This graph shows the fold change of the expression of the occludin tight junction gene in the ME180 cells infected with *N. gonorrhoeae, N. gonorrhoeae* and HIV simultaneously and HIV only, as compared to the uninfected cells, at time points 30 minutes, 6 hours and 24 hours. A fold change of one implies that the expression of the target gene in the infected cells is the same as in the uninfected cells. Measurements are the mean values of six to nine experiments and the error bars indicate the SEM. The fold change between *N. gonorrhoeae* only and the uninfected cells was significantly different at 30 minutes ($p = 0.009$), 6 hours ($p = 0.04$) and 24 hours ($p = 0.03$). The fold change between *N. gonorrhoeae* and HIV simultaneously and the uninfected cells was significantly different at 30 minutes ($p = 0.01$), 6 hours ($p = 0.009$) and 24 hours ($p = 0.008$). The fold change between HIVonly and the uninfected cells was not significantly different at 30 minutes ($p = 0.1$) and 6 hours ($p = 0.09$), but was significantly different at 24 hours ($p = 0.01$). The statistical analysis was done using a Wilcoxon sign rank test.

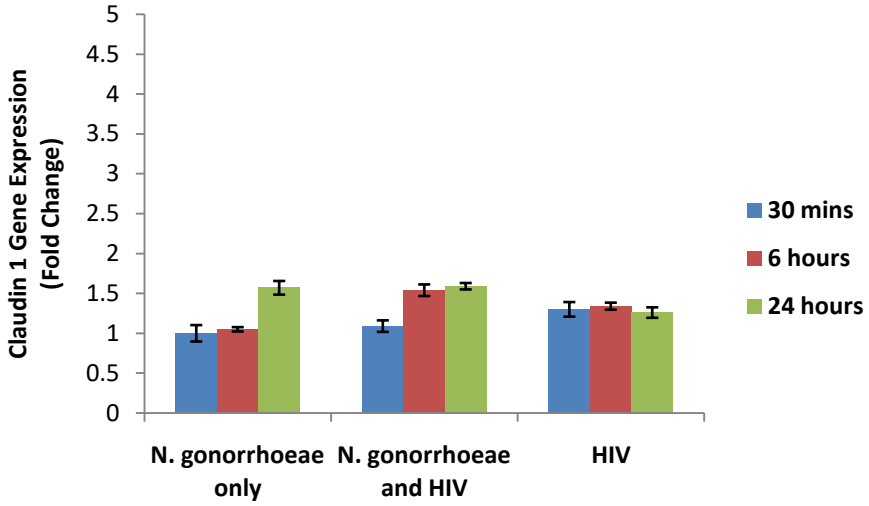


Fig 4.8 Graph showing fold change of Claudin1 gene expression following exposure to *N. gonorrhoeae*, *N. gonorrhoeae* and HIV simultaneously and HIV only. This graph shows the fold change of the expression of the claudin-1 tight junction gene in the ME180 cells infected with *N. gonorrhoeae*, *N. gonorrhoeae* and HIV simultaneously and HIV only, as compared to the uninfected cells, at time points 30 minutes, 6 hours and 24 hours. A fold change of one implies that the expression of the target gene in the infected cells is the same as in the uninfected cells. Measurements are the mean values of six to nine experiments and the error bars indicate the SEM. The fold change between *N. gonorrhoeae* only and the uninfected cells was not significantly different at 30 minutes ($p = 0.09$) and 6 hours ($p = 0.01$) but was significantly different at 24 hours ($p = 0.008$). The fold change between *N. gonorrhoeae* and HIV simultaneously and the uninfected cells was not significantly different at 30 minutes ($p = 0.4$) but was significantly difference at 6 hours ($p = 0.008$) and 24 hours ($p = 0.008$). The fold change between HIV only and the uninfected cells was significantly different at 30 minutes ($p = 0.04$), 6 hours (0.02) and 24 hours ($p = 0.01$). The statistical analysis was done using a Wilcoxon sign rank test.

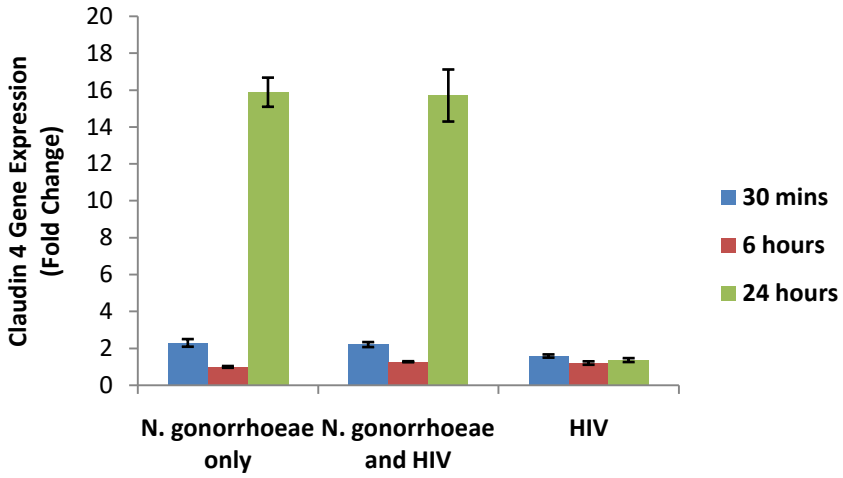


Fig 4.9 Graph showing fold change of Claudin4 gene expression following exposure to *N. gonorrhoeae, N. gonorrhoeae* and HIV simultaneously and HIV only. This graph shows the fold change of the expression of the claudin4 tight junction gene in the ME180 cells infected with *N. gonorrhoeae, N. gonorrhoeae* and HIV simultaneously and HIV only, as compared to the uninfected cells, at time points 30 minutes, 6 hours and 24 hours. A fold change of one implies that the expression of the target gene in the infected cells is the same as in the uninfected cells. Measurements are the mean values of six to nine experiments and the error bars indicate the SEM. The fold change between *N. gonorrhoeae* only and the uninfected cells was significantly different at 30 minutes ($p = 0.008$) and 24 hours ($p = 0.008$) but was not significantly different at 6 hours ($p = 0.7$). The fold change between *N. gonorrhoeae* and HIV simultaneously and the uninfected cells was significantly different at 30 minutes ($p = 0.008$), 6 hours ($p = 0.008$) and 24 hours ($p = 0.008$). The fold change between HIV only and the uninfected cells was significantly different at 30 minutes ($p = 0.008$) and 6 hours ($p = 0.02$) and 24 hours ($p = 0.01$). The statistical analysis was done using a Wilcoxon sign rank test.

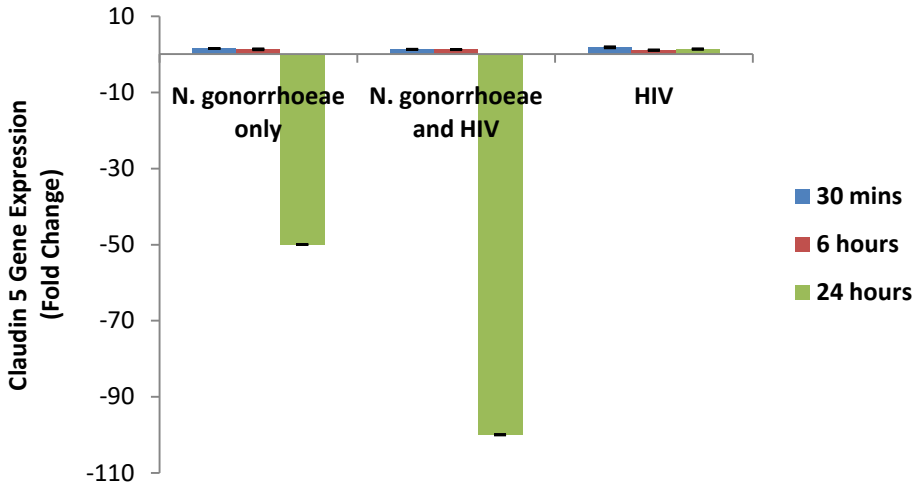


Fig 4.10 Graph showing fold change of Claudin5 gene expression following exposure to *N. gonorrhoeae, N. gonorrhoeae* and HIV simultaneously and HIV only. This graph shows the fold change of the expression of the claudin 5 tight junction gene in theME180 cells infected with *N. gonorrhoeae, N. gonorrhoeae* and HIV simultaneously and HIV only, as compared to the uninfected cells, at time points 30 minutes, 6 hours and 24 hours. A fold change of one implies that the expression of the target gene in the infected cells is the same as in the uninfected cells. Measurements are the mean values of six to nine experiments and the error bars indicate the SEM. The fold change between *N. gonorrhoeae* only and the uninfected cells was significantly different at 30 minutes ($p = 0.01$) and 24 hours ($p = 0.01$) but was not significantly different at 6 hours ($p = 0.07$). The fold change between *N. gonorrhoeae* and HIV simultaneously and the uninfected cells was significantly different at 30 minutes ($p = 0.008$), 6 hours ($p = 0.008$) and 24 hours ($p = 0.003$). The fold change between HIV only and the uninfected cells was significantly different at 30 minutes ($p = 0.008$) and 24 hours (0.02) but not significantly different at 6 hours ($p = 0.6$). The statistical analysis was done using a Wilcoxon sign rank test.

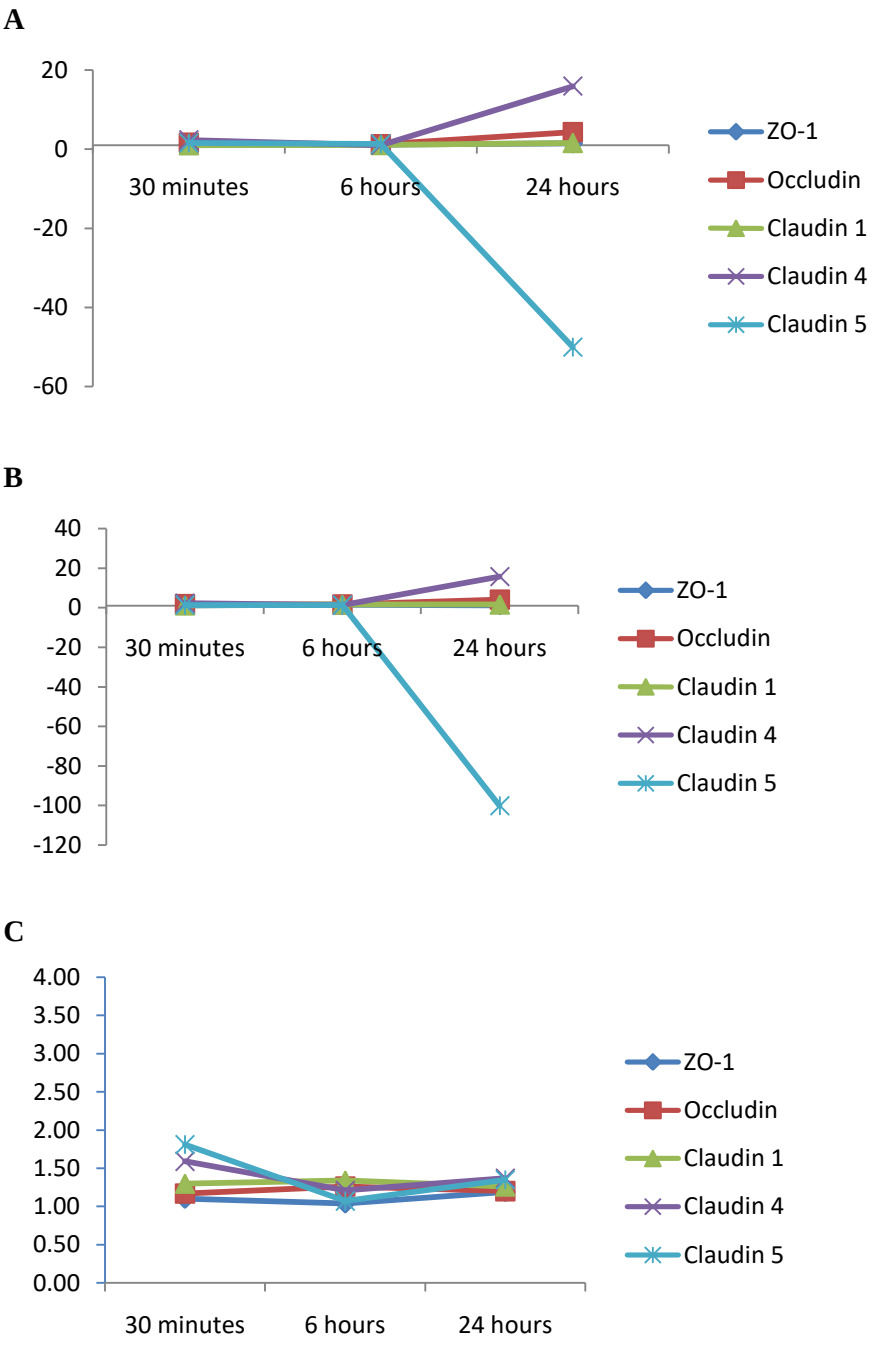


Fig 4.11 Graph showing fold change of the tight junction gene expression following exposure to *N. gonorrhoeae, N. gonorrhoeae* and HIV simultaneously and HIV only. The graphs show the fold change of the expression of the tight junction genes in theME180 cells infected with *N. gonorrhoeae* only (A), *N. gonorrhoeae* and HIV simultaneously (B) and HIV only (C), as compared to the uninfected cells. A fold change of one implies that the expression of the target gene in the infected cells is the same as in the uninfected cells.

4.7 Immunofluorescent Staining

Immunofluorescence staining of occludin was performed as described in section 3.10. The results showed that occludin remained intact with exposure to HIV only, *N. gonorrhoeae* only or a combination of *N. gonorrhoeae* and HIV at each time point (as shown in Figure 4.12).

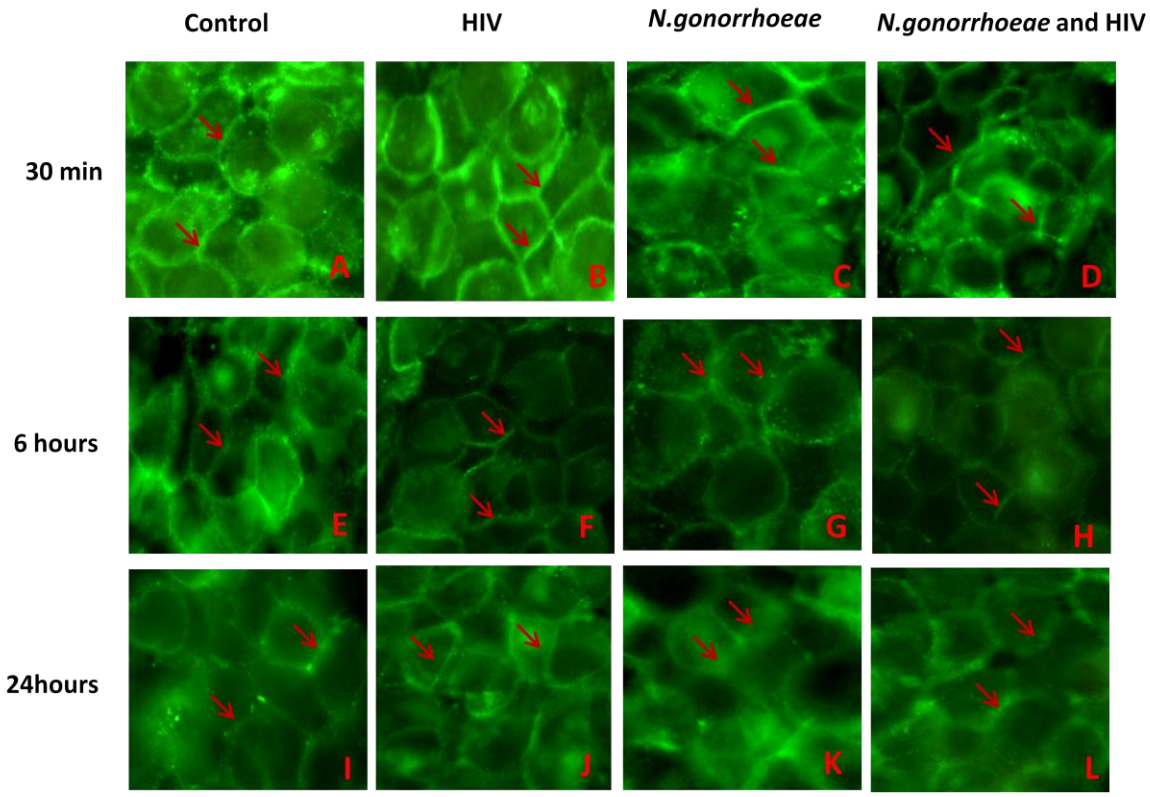


Fig 4.12 Occludin Immunofluorescence Staining. The epithelial layer was exposed to HIV only, *N. gonorrhoeae* only or a combination of *N. gonorrhoeae* at time points 30 minutes, 6 hours and 24 hours. Immunofluorescence staining of occludin in the uninfected control can be seen for 30 minutes (A), 6 hours (E) and 24 hours (I). B,F and J shows the immunoflurescence staining of occludin following exposure to HIV only at 30 minutes, 6 hours and 24 hours, respectively. C,G and K shows the immunoflurescence staining of occludin following exposure to *N. gonorrhoeae* only at 30 minutes, 6 hours and 24 hours, respectively. D, H and L shows the immunoflurescence staining of occludin following exposure to *N. gonorrhoeae* only at 30 minutes, 6 hours and 24 hours, respectively. The occludin remained intact (as indicated by red arrows).

CHAPTER 5: DISCUSSION AND CONCLUSION

This study aimed to investigate the effect of *N. gonorrhoeae* and HIV on the tight junctions of a cervical epithelial cell layer to determine whether HIV disrupts the tight junctions of cervical epithelial cells, thereby allowing migration of *N. gonorrhoeae* and HIV across the epithelial layer. Such a loosening of tight junctions of mucosal epithelial cells by HIV to facilitate microbial translocation was first reported in 2010 by Nazli et al. An endo-cervical epithelial cell line was used since the endocervix is one of the initial sites of infection by *N. gonorrhoeae*. A confluent epithelial layer with intact tight junctions was established once the transepithelial electrical resistance readings reached a steady state. The formation of a confluent epithelial layer with intact tight junctions was further confirmed by ZO-1 immunostaining. In addition, the permeability of the confluent epithelial layer was tested using the blue dextran permeability assay, which showed that there was no leakage of the blue dextran dye from the apical to the basolateral media at the time of infection. The effect of *N. gonorrhoeae* and HIV on the tight junctions of the epithelial layer was determined by infecting the epithelial layer with *N. gonorrhoeae* only, HIV only and a combination of *N. gonorrhoeae* and HIV.

To determine what effect *N. gonorrhoeae* of different multiplicity of infection (MOI) would have on the epithelial layer, the confluent epithelial layer was infected with *N. gonorrhoeae* at MOI 100, 10, 1, 0,1 and 0.01, as well as with heat killed *N. gonorrhoeae*. At 30 minutes, with a MOI of 100 and 10, *N. gonorrhoeae* was able to migrate across the epithelial layer, however the number of colonies were too low to count reliably. This could be due to too many *N. gonorrhoeae* binding to the receptors on the epithelial cells resulting in a decreased migration of the *N. gonorrhoeae*. The highest migration of *N. gonorrhoeae* occurred at a MOI of 1. The MOI of 0.1 and 0.01 did not result in migration across the epithelial layer. At 24 hours post infection, the number of *N. gonorrhoeae* that was present in the basolateral media was similar at MOI of 100, 10, 1 and 0.1. The lowest number of migrating microbes was at a MOI of 0.01. The results show that a MOI of 1 was an appropriate MOI for the infection assays. The trans-epithelial migration assay was also performed with heat killed *N. gonorrhoeae* at a MOI of 1. The heat killed organisms were not detected in the basolateral media at 30 minutes and at 24 hours post infection (Figure 4.2). However, heat killed *N. gonorrheae* would not be viable and would therefore not produce colonies in the colony-count assay. Therefore, to establish if heat killed *N. gonorrhoeae* was present in the basolateral media, conventional pili protein gene PCR was performed on DNA extracted from the

basolateral media. The results (Figure 4.4) showed that *N. gonorrhoeae* was not present in the basolateral media, which showed that migration of the *N. gonorrhoeae* is a process that involves live bacterial cells. To establish how *N. gonorrhoeae* at different MOIs would affect the expression of tight junction protein coding genes, the confluent epithelial cell layers were infected with *N. gonorrhoeae* at a MOI of 100, 1, 0.1and 0.01, as well as with heat killed *N. gonorrhoeae*. At 30 minutes and 24 hours post infection, none of the different amountsof *N. gonorrhoeae* affected the tight junction gene expression.

The *N. gonorrhoeae* migration assay (section 3.8.1) showed that when the epithelial layer was infected with *N. gonorrhoeae*alone or with *N. gonorrhoeae* and HIV simultaneously, a small percentage of *N. gonorrhoeae* was able to move across the epithelial layer into the basolateral media as early as 30 minutes post infection and continued to move across until at least 16 hours post infection. The percentage of migrated *N. gonorrhoeae* at 24 hours post infection was found to increase above 100% but this was shown to be due to multiplication of the organism in the basolateral media. It is likely that the transmigration did not stop at 16 hours. Therefore a combination of migration and multiplication must be responsible for the percentage above 100.In addition, some multiplication may already have occurred before 16 hrs. At each time point, more colony forming units (CFUs) of *N. gonorrhoeae* were found in the basolateral media when HIV was present as compared to when only *N. gonorrhoeae* was present. The difference was statistically significant at time points, 6 hours ($p < 0.05$), 16 hours ($p < 0.05$) and 24 hours ($p < 0.05$). The blue dextran assay was performed on the epithelial layers at each point of measurement. The results showed that there was no leakage of the blue dextran dye from the apical to the basolateral media at any of the points of measurement. This showed that the tight junctions of the epithelial layers remained intact thus preventing the movement of blue dextran through the paracellular pathway. Blue dextran has a hydrodynamic diameter of approximately 70nm, whereas *N. gonorrhoeae* has a diameter of 0.6 – 1 µm (Hagel 2011; Morse 1996). Since the intact epithelial layer prevented the movement of blue dextran through the paracellular pathway, it would also have prevented the migration of *N. gonorrhoeae* via the paracellular pathway. Therefore, *N. gonorrhoeae* that migrated to the basolateral media would have had to use the transcellular pathway. A study by Rodriguez-Tirado et al. (2012) showed that even though *N. gonorrhoeae* did not cause disruption of the tight junction proteins ZO-1 or occludin, it was shown to disrupt the adherens junction proteins, E-cadherin and β-catenin. They suggested that this possibly resulted in the disruption to the cell junction complexes of endometrial and fallopian tube epithelial cells(Rodriguez-Tirado et al. 2012). Another study by Edwards et al. (2013) showed that exposure to *N. gonorrhoeae* resulted in

disruption of the tight junction proteins ZO-1 and occludin, as well as the adherens junction proteins, E-cadherin and β-cateninin monolayers of endometrial and colorectal cell lines. Their study showed that even though there was disruption to those apical junction proteins, it did not affect the paracellular permeability. Paracellular permeability was determined by the movement of Lucifer yellow or fluorescein from the apical media to the basolateral media. They also showed that exposure to *N. gonorrhoeae* did not affect the movement of Lucifer yellow or fluorescein to the basolateral media as compared to the uninfected controls. Their study therefore suggested that *N. gonorrhoeae* was not able to disrupt the "gate" function which regulates paracellular permeability, but it was able to affect the "fence" function of the epithelial cells(V. L. Edwards et al. 2013).A study by Wang et al. (2017) showed that *N. gonorrhoeae* can move to the subepithelium by causing the shedding of the columnar epithelial cells. Their study differed from the here presented study in that they used a human cervical tissue explant model and a human colorectal carcinoma cell line.

In the here presented study, the tight junction gene expression assay for the epithelial layers infected with *N. gonorrhoeae* alone or with *N. gonorrhoeae* and HIV simultaneously, showed a similar pattern of expression (Figure 4.11 A and 4.11 B, respectively). At time points 30 minutes and 6 hours, the expression of the tight junction genes in the infected cells were expressed similarly to the uninfected cells. At 24 hours post infection, there was a substantial downregulation of claudin 5 and upregulation of claudin 4 and occludin. This seemed to occur due to the presence of *N. gonorrhoeae*, since this did not happen with HIV only (Figure 4.11 C). Although claudin 5 was downregulated, it did not seem to affect the integrity of the epithelial layer, as shown by the blue dextran permeability assay. Claudin 5 functions as a molecular sieve preventing the paracellular movement of ions smaller than approximately 0.8 kDa(Gunzel and Yu 2013). Downregulation of the claudin 5 gene would therefore not increase the movement of blue dextran and *N. gonorrhoeae* through the paracellular pathway. Claudin 4 is a pore-sealing claudin and increased expression of this claudin results in an increase in the tightness of the epithelial layer (Khan and Asif 2015). The function of occludin is to control the paracellular permeability to small hydrophilic ions. Therefore upregulation of claudin 4 and occludin should increase the tightness of the epithelial layer. Immunofluorescence staining of occludin following infection with *N. gonorrhoeae* alone or with *N. gonorrhoeae* and HIV simultaneously (Figure 4.12) showed that there was no disruption to the occludin protein.

Some studies have shown that HIV causes disruption to the tight junctions of epithelial layers, thereby causing the paracellular space between cells to open, which facilitates the movement of other microbes across the epithelial layer (Nazli et al. 2010; Sufiawati and Tugizov 2014). Nazli et al. (2010) demonstrated that exposure of intestinal and primary genital epithelial cells to HIV resulted in the downregulation of tight junction genes, as well as in the increased permeability of these epithelial layers (Nazli et al. 2010). Similar findings were reported in a study which showed that exposure of oral epithelial cells to HIV caused the disruption of the tight junctions, thereby enabling HSV-1 to move across the oral epithelial layer (Sufiawati and Tugizov 2014). The results of this study are in contrast to their findings as it showed that HIV did not affect the permeability of the epithelial layer. The results of the gene expression assay showed that the infection of the epithelial layer with HIV (Figure 4.11C) did not affect the tight junction gene expression. Furthermore, the blue dextran permeability assay showed that the tight junctions of the epithelial layer were intact as the blue dextran was unable to move from the apical to the basolateral media.

The HIV migration assay showed that a small percentage of HIV was able to migrate across the epithelial layer to the basolateral side when the epithelial layer was infected with HIV alone or with *N. gonorrhoeae* and HIV simultaneously. Since blue dextran is smaller than HIV (which is approximately 100 nm in diameter), it is highly unlikely that the small percentage of HIV that had migrated across the epithelial layer had migrated via the paracellular pathway. The virus could possibly have moved across the epithelial layer via transcytosis. This is in keeping with other studies that have shown that HIV moved across the epithelial layer through transcytosis. A study by Bobardt et al. (2007) demonstrated that cell-free HIV was able to transcytose through primary genital epithelial cells. However, the efficiency of HIV transcytosis was very poor as only less than 0.02% of the original inoculum passed through (Bobardt et al. 2007). Similarly, Kinlocket al. (2014) showed that HIV-1 was able to transcytose across vaginal epithelial cells using the endocytic recycling pathway (Kinlock et al. 2014). However, their study was performed using cell-associated virus. Hocini and Bomsel (1999) showed that HIV can cross the epithelial cell layer via transcytosis of endocytosed HIV. The study also reported that cell-associated HIV transcytosis occurs more efficiently than cell-free virus (Hocini and Bomsel 1999). Real et al. (2018) demonstrated the process of endoctyosis of cell-associated HIV by using live imaging. They were able to show that when HIV infected cells come into contact with the epithelial layer, the HIV infected cells cause budding of the HIV towards the epithelium. The budding HIV is then endocytosed by the epithelial cells and is then transported across the epithelial layer, where they are

able to infect susceptible cells. Their study also showed that transcytosis of cell-associated virus occurred more efficiently than cell-free virus (Real et al. 2018).

The study by Nazli et al. (2010) was performed on an intestinal cell line and on primary epithelial cells that were obtained from the female genital tract. Interestingly, even though there were differences in the results obtained for the gene expression and blue dextran permeability assay for the here presented study and the study by Nazli et al. (2010), their HIV translocation assay (which was performed on the intestinal cell line) showed that at six hours post HIV infection, less than 0.04 % of the initial inoculum translocated to the basolateral media and this increased to less than 0.06% at 24 hours post HIV infection. Moreover, there was no bacterial translocation of *Escherichia coli* after infection of the intestinal epithelial cell layer with HIV and *E. coli* for six hours, while exposure of the cell layer to HIV for 24 hours and *E. coli* for six hours, resulted in approximately 3 x 10^5cfu/ml *E. coli* translocating to the basolateral media. Since 1 x 10^8cfu/ml *E. coli* was added to the apical layer, this implies that approximately 0.3% of the initial inoculum translocated to the basolateral media. It is therefore possible that even though Nazli et al. (2010) was able to show disruption to the tight junction gene expression and leakage of the blue dextran dye across the epithelial layer, the translocation of HIV and *E. coli* (which is approximately 0.25 to 1 µm in diameter) was not through the paracellular pathway, as the results of the HIV translocation assay is similar to the results by Bobardtet al. (2007) and the results of the study presented here.

When considering the bidirectional relationship existing between HIV and "classic" STDs, this study adds to a better understanding of the relationship between *N. gonorrhoeae* and HIV during co-infection. This study suggests that when there is co-infection, *N. gonorrhoeae* seems to result in the HIV moving across the epithelial layer less efficiently, which could mean that other factors may be responsible for the increased susceptibility to HIV during co-infection. This study also shows that infection with *N. gonorrhoeae* only, *N. gonorrhoeae* and HIV simultaneously and HIV only does not disrupt the tight junctions of the epithelial layer, which means that these organisms are not disrupting the epithelial layer to expose areas for microbial entry across the epithelial barrier. In addition, the results presented in this study show that *N. gonorrhoeae* and HIV could possibly move across the epithelial layer through transcytosis of the microbes, which means that preventative measures could involve targeting the transcytosis of the microbes. In the absence of other effective means to prevent this transcytosis, preventing exposure of the epithelial lining of the female genital tract by means of condoms remains the best option.

A limitation of this study was that only one isolate of *N. gonorrhoeae* was used. Previous clinical observations have shown that certain phenotypes of *N. gonorrhoeae* are more invasive than others (Chan and Wiseman 1975). In addition, the drastic increase in *N. gonorrhoeae* from time point 6 hours to 24 hours does not indicate the percentage of *N. gonorrhoeae* that migrated across the epithelial layer during this period as it is unclear at which time point the multiplication of *N. gonorrhoeae* surpassed the *N. gonorrhoeae* migrating through. Another limitation of this study was that the epithelial layer was not infected with cell-associated HIV. Therefore, future research should involve infecting the epithelial layer with cell-associated virus, infecting the cells using different HIV clades, as well as with different pheno- and genotypes of *N. gonorrhoeae*. Furthermore, the limitation of using the transwell model is that even though it provides an idea of the in vivo response to pathogens, the model does not mimic all characteristics of human infection.

In conclusion, the results of this study showed that exposure of the cervical epithelial layer to *N. gonorrhoeae* alone, HIV alone or to *N. gonorrhoeae* and HIV simultaneously did not affect the paracellular permeability of the epithelial layer. The results showed that a small percentage of *N. gonorrhoeae* was able to migrate across the epithelial layer and that the presence of HIV did not seem to influence the migration of *N. gonorrhoeae*. In addition, a small percentage of HIV was able to migrate across the epithelial layer, however the presence of *N. gonorrhoeae* seemed to cause the HIV to pass through the epithelial layer less efficiently than with exposure to HIV only. The overall results suggest that the migration was possibly through transcytosis of the microbes. The overall results from this study therefore does not support the hypothesis that HIV disrupts the tight junctions of the epithelial cells, thereby allowing *N. gonorrhoeae* to move together with the virus across the epithelium by means of the paracellular pathway.

CHAPTER 6: REFERENCES

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CHAPTER 7:APPENDIX

1. **Phosphate Buffered Saline (PBS)**

   PBS was prepared by dissolving 1 PBS tablet, pH 7.3 (Oxoid, Hampshire, England) in 100 mL of triple distilled water. The PBS solution was then autoclaved at 121°C for 15 minutes. The solution was then stored at 2 – 8°C.

2. **ME180 Cell Freezing Fluid**

   To prepare 50 mL of ME180 cell freezing fluid, 5 mL of dimethyl sulfoxide (DMSO)and 5 mL of FBS(BioWhittaker, Walkerville, USA) was added to 40 mL of McCoy's 5A media (Gibco, Paisley, United Kingdom). The solution was filter-sterilised through a 0.22 µm filter (Millipore, Burlington, Massachusetts).

3. **Enriched GC Agar plates**

   To prepare the NYC plates, 18g GC agar base (Oxoid, Hampshire, England) was dissolved in 485 ml triple distilled water and the autoclaved at 121°C for 15 minutes. The solution was allowed to cool to 55°C in a waterbath. Fifteen millilitres of autoclaved water was added to a vial of Yeast Autolysate Supplement (Oxoid, Hampshire, England). When the solution had reached 55°C, the Yeast Autolysate Supplement was added and the solution was immediately poured into petri dishes. To prepare 90 mm plates, 20 mL of the solution was added to each plate and to prepare 60 mm plates, 10 mL of the solution was added to each plate.

4. **Brain Heart Infusion (BHI) Broth**

   The brain heart infusion broth was prepared by adding 3.7g BHI powder (Oxoid, Hampshire, England) and 5g yeast extract (Oxoid, Hampshire, England) to a sterile bottle. Triple distilled water was then added to the bottle to obtain a final volume of 100ml. The broth was thoroughly mixed and autoclaved at 121°C for 15 minutes. When the broth was cool, 200 µL of 5% hemin(Sigma, St Louis, USA)and 200 µL of 10% vitamin K(Sigma, St Louis, USA)was added.

5. **5% Hemin**

   Add 2.5mg hemin(Sigma, St Louis, USA) to 1 ml of sodium hydroxide (NaOH), vortex and filter-sterilise using a 0.22µm filter (Millipore, Burlington, Massachusetts). The solution was stored at 4 °C.

6. **10% Vitamin K**

   Add 5mg vitamin K (Sigma, St Louis, USA) to 1 mL of absolute ethanol, vortex and filter-sterilise using a 0.22 µm filter (Millipore, Burlington, Massachusetts). The solution was stored at 4°C.

7. ***N. gonorrhoeae* Storage Media**

   The storage media was prepared by adding 3.7 g of BHI powder to 80 ml of triple distilled water. The solution was autoclaved at 121 °C for 15 minutes and then allowed to cool. Twenty milliliters of filter-sterilised glycerol was added and the media was stored at 4°C.

8. **4% Paraformaldehyde**

   Four percent paraformaldehyde was prepared by dissolving 4 g of paraformaldehyde powder (Sigma, St Louis, USA) in a final volume of 100 mL of autoclaved water.

9. **0.1% Triton X-100**

   To prepare 0.1% Triton X-100, 100 µL of Triton X-100 (Sigma, St Louis, USA) was added to 99.9mL of PBS.

10. **3% Bovine Serum Albumin (BSA)**

    Three percent BSA was made up by adding 3 g of BSA powder(Sigma, St Louis, USA) to a final volume of 100 mL of PBS.