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GROOTE SCHUUR
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THE ROLE OF CRUDE SALIVARY MUCUS AND ITS PURIFIED MUCINS MUC5B AND MUC7 IN THE INHIBITION OF THE HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

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To Mum, Dad,

My sisters, Kirsten, Lisa and Hannah

and Gary, with much love

University of Cape Town

PRESENTATIONS

This work has been presented at the following occasions.

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University of Cape Town

DECLARATION

This work was completed under the supervision of Professor A. S. Mall, from 2010 to 2011 in the Division of Surgical Research, University of Cape Town.

I hereby certify that the following study is my own work. I have used the Virology Journal output style for citation and referencing (RefWorks Web Based Bibliographic Management Software employed by the University of Cape Town). Each contribution to, and quotation in, this thesis from the work(s) of other people has been attributed, and has been cited and referenced.

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ABSTRACT

Sub-Saharan Africa is the world's worst HIV-AIDS affected region. More interventions to manage this pandemic are urgently required. Transmission of the virus through an exchange of saliva is rarely known to occur. The role of saliva and its mucus in this inhibition requires clarification.

This project sought to verify the previous findings in our laboratory (Habte *et al.*, 2006; Peacocke *et al.* (manuscript under revision)), that crude saliva from uninfected individuals together with its purified mucin components inhibited HIV-1 in an *in vitro* assay. Mucins from infected saliva did not show inhibition in this assay.

Mucus was extracted in 6M guanidinium hydrochloride and a cocktail of protease inhibitors, pH 6.5. Sepharose CL-4B gel filtration separated MUC5B and MUC7 in saliva. Mucins were purified by density gradient ultra-centrifugation in caesium chloride. Western blotting and amino acid analysis determined the size, charge and identity of the mucins. The inhibitory activity of crude saliva, salivary mucin components MUC5B and MUC7 from HIV-negative and HIV-positive donors, was tested by their incubation in an *in vitro* HIV-neutralisation assay using a pseudovirus of Subtypes A and C, and infection of susceptible epithelial tumour cells (genetically modified TZM-bl cells). Glycosylation analysis by HPLC was also performed on each group.

The presence of MUC5B and MUC7 in saliva was confirmed and it was shown that there was inter-individual variation in their amounts and electrophoretic behaviour between and within groups. Crude HIV-negative and HIV-positive saliva and its purified mucins MUC5B and MUC7 significantly inhibited the infection of HIV-1 in an *in vitro* pseudoviral assay in a dose-response manner. HPLC revealed two glycoforms of salivary MUC5B.

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University of Cape Town

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ABBREVIATIONS

MUC	Mucin
HIV	Human immunodeficiency virus
HIV-AIDS	Human immunodeficiency virus – acquired immune deficiency syndrome
ARV	Antiretroviral
SIV	Simian immunodeficiency virus
SIgA	Salivary immunoglobulin A
AA	Amino acids
GuHCl	Guanidinium hydrochloride
NaCl	Sodium chloride
NaN ₃	Sodium azide
GALNAc	N-acetyl galactosamine
GLCNAc	N-acetyl glucosamine
Tn (Tn antigen)	Structure N-acetylgalactosamine (GalNAc) linked to serine or threonine by a glycosidic bond
Gp120 (or gp160, gp41)	Glycoprotein
SAG	Salivary agglutinin
ER	Endoplasmic reticulum
CD4 (or CD8)	Cluster of differentiation 4
CCR5	C-C chemokine receptor type 5
CXCR4	C-X-C chemokine receptor type 4
HPLC	High performance liquid chromatography
LC ESI-MS	Liquid chromatography electrospray ionisation – mass spectrometry
PBMC	Peripheral blood mononuclear cells
ELISA	Enzyme-linked immunosorbent assay
GCLP	Good clinical laboratory practice
ENV	Envelope

EDTA	Ethylenediaminetetraacetic acid
PMSF	Phenylmethanesulfonylfluoride
RT	Room temperature
CsCl	Caesium chloride
PAS	Periodic acid Schiff's
V_0	Void volume
V_i	Included volume
MWCO	Molecular weight cut-off
TAE	Buffer solution – Tris base, acetic acid and EDTA
SDS	Sodium dodecyl sulphate
SSC	Saline sodium citrate buffer
TBS	Tris-buffered saline
TBST	Tris-buffered saline and Tween-20
PVDF	Polyvinyl difluoride (membrane)
ECL	Enzyme chemiluminescence
DTT	Dithiothreitol
NBT	Nitro-blue tetrazolium chloride
DMF	Dimethylformamide
BCIP	5-Bromo-4-chloro-3'-indolyphosphate p-toluidine salt
AgPAGE	Agarose polyacrylamide gel electrophoresis
PA	Polyacrylamide
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris – hydrogen chloride
TEMED	Tetramethylethylenediamine
ddH ₂ O	Double distilled water
NaOH	Sodium hydroxide
NaBH ₄	Sodium borohydride
NH ₄ HCO ₃	Ammonium bicarbonate
LTR	Long terminal repeat

DEAE	Diethylaminoethyl
RLU	Relative light units
DMEM	Dulbecco's Modified Eagle's Medium
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
FBS	Fetal bovine serum
NEAA	Non-essential amino acids
IC ₅₀	Half-maximal inhibitory concentration
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate buffered saline
HCl	Hydrogen chloride
T.B.	Tuberculosis
HAART	Highly active antiretroviral treatment
ART	Antiretroviral treatment
S	Serine
T	Threonine
P	Proline

1.1 BACKGROUND

Mucus forms a slimy, highly viscous secretion coating the surface of epithelial tissues of the gastrointestinal, respiratory and reproductive tracts of the body. The mucus secretion contributes to innate immunity, providing a protective barrier against infection, dehydration, and physical or chemical injury [1, 2]. The highly elastic and adhesive properties of mucins lend to this effect [3, 4]. It is our ultimate aim to harness the role of salivary mucus and its mucins in the development of a protective mechanism against HIV-transmission. Human salivary mucus functions as an anti-viral, anti-bacterial, and anti-fungal agent by aggregating and removing pathogenic microorganisms from the oral cavity [5, 6]. Saliva contains two mucin glycoproteins identified as MG1 (MUC5B) and MG2 (MUC7) of higher and lower molecular weights respectively [7-9]. Both MUC5B and MUC7 are of the secreted gel-forming type of mucin glycoproteins although MUC7 is more dominant in the soluble phase.

The major MG1 mucin glycoprotein, identified as MUC5B, is expressed in all mucous cells of salivary glands [10]. MG1 subunits extracted from saliva were reactive with antiserum recognizing the MUC5B mucin [8]. Using an anion exchange distribution, followed by Western blots of agarose gels, Thornton *et al.* (1999) showed that the polypeptide underlying the mucins was the product of the MUC5B gene [8]. Amino acid analysis and peptide mapping also confirmed that the MUC5B gene product was the predominant mucin polypeptide present [8]. The MUC5B gene is located on chromosome 11p15.5 together with the genes for MUC2, MUC5AC and MUC6 [11, 12]. Desseyn *et al.* (1998) determined the size of MUC5B to be approximately 600 000Da [11]. The primary amino acid sequence of the MUC5B glycoprotein is

highly similar to the other members of its mucin gene family and particularly MUC5AC, due in part to its high content of cysteine residues [11]. The O-glycosylation profile of MUC5B is complex with substantial inter-individual variation [13, 14]. Additionally, MUC5B is found in the epithelia lining the respiratory airways [15, 16].

MG2 or MUC7 is expressed in the serous cells of all the salivary glands except for the parotid glands [10] and in a different population of mucous cells when compared with MG1 expression. Cohen *et al.* (1990, 1991) also noted differential expression of MUC5B and MUC7 [17, 18]. MG2 was classified by Bobek *et al.* (1993) as a 150 000- 250 000Da monomer encoded by the MUC7 gene [9]. The location of MUC7 is on chromosome 4 (UCSC) [12]. The O-glycosylation of MUC7 is similar between individuals, in contrast with MUC5B [13]. Besides its expression in the oral glands, MUC7 is secreted in the respiratory airways [9, 19].

Through the characterisation of the salivary mucins MUC5B and MUC7, we aim to determine their role in the inhibition of the human immunodeficiency virus. We aim to determine whether this protective action is due to the 'sticky nature' and aggregative properties of mucins alone, or a more specific binding interaction between mucins and invading organisms.

1.2 SOCIAL ASPECTS - SOUTHERN AFRICA

The impact of HIV-AIDS (Human Immunodeficiency Virus – Acquired Immune Deficiency Syndrome) in sub-Saharan Africa is phenomenal. A report compiled in 2007 by the World Health Organisation (WHO) and the United Nations AIDS initiative (UNAIDS) have estimated that adults and children living with HIV in sub-Saharan Africa are roughly two-thirds of the

population that is affected globally. The estimated number of new infections in that year was significantly large in sub-Saharan Africa as were deaths from HIV-AIDS. Of the number of new infections in adults (aged 15 years and older), almost 50% of those infected were women, a reflection of the current social structure in these societies in which important decisions such as those regarding the use of protection in sexual encounters are taken by the male. The focus on the social structure of these communities is imperative when examining the realities of what is required to beat this pandemic. Women face enormous challenges when decisions are made regarding the use of protection during sexual intercourse, because of the unfortunate stigma attached to HIV infection in this region. The dynamics of gender power and the analysis of the covert use of protection were discussed by Sahin-Hodoglugil *et al.* (2009) who highlighted the importance of covert use of protective measures occasionally or continuously by women [20].

1.2.1 THE IMPORTANCE OF MICROBICIDES

Protection against HIV-transmission, mainly by condom use, is compromised by negligence and choice, and influenced by belief and ritual. The use of a microbicide would be appropriate in such circumstances. Microbicides are anti-HIV preparations in a gel-like or cream form applied to the vaginal surface. The criteria thus provide substantial support for the need to develop more numerous mechanisms for the prevention of transmission and particularly those that will encourage the role and participation of women in preventing infection with HIV. Microbicial development is currently limited and although some clinical trials have been successful, there are few pioneering molecular initiatives. The adoption of antiretroviral drugs (ARVs) as microbicial drugs shows great potential, but the possible creation of drug resistance in recurrent use of such drugs is a point that cannot be ignored. What is needed is a microbicide that will be able to create a substantial decrease in transmission of the HI-virus whilst not

interfering with ARVs. As some microbicides are potentially cytotoxic, the drug must be effective in protecting the vaginal mucosa from damage during sexual intercourse.

Our results in this study in which mucins were shown to have an inhibitory effect on the HI-virus led us to contemplate the possibility of the development of a mucus-based microbicide. Mucus gels line the epithelial surfaces of the internal tracts of the body where they protect the mucosal lining of these tracts against the hostile milieu in the lumens of these passages. Mucus gels form unstirred layers on the mucosa and are replenished by the epithelia upon loss through enzymatic activity [21]. The resistance of these gels on epithelial surfaces is highlighted by the hostile milieu in which they are found [1]. Attempts have been made in the past to strengthen these gels with preparations such as sucralfate (sucrose octasulphate) in gastric ulceration, to accelerate re-epithelialisation and healing [22]. Therefore, it is conceivable to investigate ways of strengthening mucus gels that would be able to withstand friction during sexual intercourse when applied to the vaginal surface.

1.3 MUCUS AND TRANSMISSION OF THE VIRUS

The major route of HIV transmission is via exposure at the mucosal interface. However, the interface also provides the first line of defence against the virus and contributes hugely to the low infection rate when compared with level of exposure. A study by Miller *et al.* (2005) highlights the protection afforded by the mucus barrier, which greatly limits the viral infection (Simian Immunodeficiency Virus/SIV) of cervicovaginal epithelial tissues in particular [23]. This suggests an association between the strength of the mucus layer and an individual's susceptibility to infection and disease.

Research has been done to prove the strength and protective nature of the mucus barrier throughout the internal tracts of the body. Van der Sluis *et al.* (2006) identified the characteristics of the protective nature of the mucus barrier [24]. The insolubility and unstirred and adherent nature of the mucus gel layer, as described for gastric epithelium, contributes to its protective role [2, 25]. Mojzis *et al.* (2000) demonstrated the protective role of the mucus layer in the stomach by showing that gastric mucus plays an important role in the protection of gastric mucosa after ischemia/reperfusion-induced gastric mucosal injury in rats [26]. If the mucous barrier has been damaged or weakened, the epithelial tissue will be more susceptible to contact with carcinogenic and other injurious or harmful compounds leading to the damage of the mucosa. It is known that the role of MUC2 is to form part of the protective mucus barrier for the epithelial cells lining the small intestine and colon.

Sufficient evidence has been provided to suggest that the mucous layer has a major role in the protection of exposed epithelia. Furthermore, in our laboratory Habte *et al.* (2007) investigated the anti-viral activity of purified mucin in breast milk of normal individuals and reported its inhibition of the poxvirus, as well as inhibition of HIV in an *in vitro* assay [27]. Kazmi *et al.* (2006) identified inter-individual differences in the inhibitory capabilities of mucosal fluids against HIV [28]. Notably Habte *et al.* (2006, 2010) identified the salivary mucins MUC5B and MUC7 as a key inhibitory component of saliva against infection of HIV-1 through the oral route [29, 30].

1.3.1 SALIVA PROVIDES AN INNATE MUCOSAL IMMUNITY IN HIV TRANSMISSION

Whilst vaginal and rectal mucosae are the predominant sites of viral entry, as well as transmission through breast milk from HIV-positive mother to child, an exposed intact oral mucosa is not. However, transmission of HIV-AIDS rarely occurs during the exchange of oral fluids [31]. Whole saliva is composed of secretions from the parotid, submandibular and sublingual glands. These secretions are mucins, but also consist of electrolytes, inorganic molecules, such as calcium and phosphate, and a buffer of hydrogen carbonate [32]. Other ions such as fluoride, thiocyanate, sodium, potassium, and chloride are systemically present [32]. Organic molecules in saliva include proline-rich proteins, enzymes such as amylase, lipase, peroxidase and lysozyme, lactoferrin, and antibodies (sIgA), histatin proteins and statherin [32]. Blood group substances, sugars, steroid hormones, amino acids, ammonia and urea are also found in saliva [32]. Of these substances, cystatins (inhibitors of cysteine proteases), antibodies and mucins complete the anti-viral component [32]. Wu *et al.* identified another salivary glycoprotein namely salivary agglutinin that exhibited specific anti-HIV properties [33]. Mucins and statherins form the lubricative, viscoelastic portion of the crude mucus in the oral cavity [32]. Mucins in particular have unique rheological properties such as high elasticity, adhesiveness, and low solubility [32, 34]. Because of the rarity of infection by oral transmission, it is possible that there are numerous substances in crude saliva that contribute to the inhibition of HIV infection. This can be safely assumed given a lack of evidence for oral transmission in communities studied throughout the global population, although few definitive studies have been done. The Encyclopedia of Sexually Transmitted Diseases reports a study carried out by The University of California Options Project that showed that the risk associated with oral sex was less than that associated with other unprotected forms of intercourse [35, 36]. This project aims to provide information on the protective capability of oral mucosal fluids, whilst the anti-

viral substances in saliva may function collectively, we however would like to verify, singularly, the mucin contribution to protection against HIV-1.

It has been hypothesized that innate oral protection is due to the inability of the virus to remain viable in the salivary mucus environment [37]. It is possible that there is a mucous barrier between the epithelial membranes and oral cavity. This barrier prevents attachment and entry of the virus into its target cells, not unlike the mucosal barrier of cervicovaginal tissues that greatly limits its infection [23]. The salivary secretions of the mouth have numerous enzymes and include cell debris and products of catabolism, which could play minor roles in viral inhibition. Additionally, saliva has a hypotonic nature that may disrupt both cellular and viral entities and prevent their attachment to each other [37]. Our study, like that of Bergey *et al.* (1994) assumes the presence of infectious, viable HIV in the saliva, which makes the recipient susceptible to infection [5]. Bergey *et al.* (1994) proposed that a specific component in saliva inhibits the transmission of viable HIV-1 from an infected person to the target cells of the mucous epithelial layer lining the mouth cavity of the recipient [5]. Several researchers proposed that there could be a key macromolecular component in saliva that could play a significant role in preventing replication and subsequent infection of receptive cells by HIV-1 [28, 30, 31, 38]. A study by Archibald *et al.* (1990) investigated the *in vitro* inhibitory activities of saliva against the HIV-1 virus and found that whole saliva and specific glandular salivas, except parotid secretions, were inhibitory. They suggested that “complexing of the virus with high molecular weight, submandibular mucins” could play a role in viral inhibition [39]. The mucin component is responsible for the ‘sticky’ nature of salivary mucus and is composed of mucins MUC5B and MUC7. There is sufficient support to suggest that mucus and mucins may play a possible role in the fight against the oral transmission of HIV-1. Mucin, when isolated from saliva, has been shown to inhibit the replication and infection of HIV-1 (the prevalent subtype amongst the study population) [30].

1.4 MUCUS AND MUCINS

Mucins (mucous glycoproteins) are high molecular weight glycoproteins named after the genes that encode their apomucin (protein core) structure. The mucous glycoproteins are found in saliva, mucosal secretions from goblet cells and are particular to glandular epithelial tissue lining the airways, gastrointestinal tract and other mucinous tissues. There are two major types of mucin: the gel-forming secretory type and those that appear on the apical surfaces of glandular cell membranes, the trans-membrane mucins [40]. Transmembrane mucins can be located in most mucosa and their aberrant expression is noted in disease [41-46]. The most well known trans-membrane mucins are MUC1 and MUC4 [40]. Secreted mucins form major components of salivary, tracheal and bronchial secretions, and cervical mucus, to name a few. The major secreted gel-forming mucins are MUC2, MUC5AC, MUC5B and MUC6, found in different regions of the airways and gastrointestinal tract. These mucin genes are localized on chromosome 11p15.5 [40]. MUC7, a salivary mucin (gene located on chromosome 4 (Entrez, Gene)), and together with MUC8 and MUC9, are secreted mucins that are non-gel forming. The gel-forming mucins are relatively cysteine-rich in comparison with the non-gel forming secreted mucins [47] (Genbank, Entrez). MUC2 is a secreted type, which is a gel-forming (polymeric) mucin within the crude mucus found lining the epithelium of the small intestine and colon. Both groups of mucins have tandem repeat sequences within the protein backbone.



Figure 1: An illustration of the mucin genes MUC5B and MUC7 as shown by Dekker *et al.* (2002) [48]. Chromosomal location is noted. The C terminus is at the right. Any known peptide domains present were detected in the sequences using the SMART software [49]. Each type of peptide domain is depicted in a separate colour, indicated in the key. The representations are based on the full-length cDNA sequences. Allelic variation was not taken into account. The scale of the deduced polypeptides is indicated by the bar representing 500 amino acids (aa) [48].

1.4.1 MUC GENES AND THEIR ASSOCIATION WITH DISEASE

There have been 17 mucin genes (MUCs) identified to date. These are for the secreted gel-forming mucins: MUC2, MUC5AC, MUC5B, MUC6, MUC19, secreted, non-gel-forming mucins: MUC7, and transmembrane mucins: MUC1, MUC3a and 3b, MUC4, MUC11, MUC12, MUC13 and MUC16 and cell-surface associated MUC20 [48, 50]. MUC8, MUC15, and MUC17 are additional mucin genes but do not belong to any specific family [48]. There are both similarities and significant differences in structure relating to the specific function of each protein.

Variability in MUC gene structure and protein structure (particularly of the tandem repeat regions) and alterations in the sequence of glycosylation have been shown to play a role in the development and susceptibility to disease and has been particularly described in cancer

development [44, 51-53]. MUC1 gene polymorphisms have been associated with chronic atrophic gastritis and intestinal metaplasia, which are conditions that may precede gastric carcinoma [54]. Mucin alterations have now been described as a common feature of colonic neoplasia and tumour progression [55]. An example of genetic variation of the MUC genes was identified by Kirkbride *et al.* (2001) who described the prevalence of MUC7 alleles and their association with asthma [56]. Additionally, Vinall *et al.* (2000) had previously identified that a MUC2 allele is significant in the protection of atopic, non-asthmatic individuals [57]. However, exceptions have been identified by Swallow *et al.* (1999), who reported that there was no association between allelic length and polymorphism of MUC2 in ulcerative colitis [58]. Polymorphism in MUC genes is significant in its effect on the number of possible glycosylation sites and is likely to have an effect on the functional and physicochemical properties of mucin, relevant in the susceptibility and onset of certain diseases [56].

The MUC5B gene is known to have a considerable polymorphism with respect to its 59-nucleotide tandem repeat region. Desseyn *et al.* (1999) observed five alleles in a sample population of 86 unrelated individuals due to 3-8 direct repeats [59]. MUC7 contains a central tandem repeat region and the most common allele is comprised of 6 tandem repeats each of 69 nucleotides (base pairs) (23 amino acids) [56]. The polymorphisms within MUC5B and MUC7 genes may play a role in the susceptibility to infection by viruses such as HIV. It has been proposed that variable charge on the mucin molecule, due to altered glycosylation patterns, may affect its viral binding properties and the agglutination of the viruses [30, 40]. Less glycosylation may point to a weaker interaction with the glycosylated binding sites on the virus particle. Nagashunmugam *et al.* (1997) observed inter-individual variation, which they could quantify with respect to viral binding properties [60].

Genetic variability of MUC genes also occurs in disease [44, 57]. An investigation of the variation in the structure of mucins MUC5B and MUC7 and their association with susceptibility to infection with HIV was carried out in our laboratory in a pilot study (Peacocke *et al.* manuscript under revision). DNA analysis of tandem repeat regions in the genes of MUC5B and MUC7 from HIV-negative and HIV-positive donors revealed no association of HIV-infection status and gene polymorphisms. Polymorphisms were distributed between both groups. There was no apparent link observed between heterozygosity or homozygosity in either the MUC5B or MUC7 tandem repeat alleles and HIV-infection. This suggests that there is no genetic predisposition in MUC5B and MUC7 for the susceptibility to HIV-infection in an *in vitro* assay, although a wider study with a greater sample number is recommended.

1.4.2 THE STRUCTURE AND CONFORMATION OF MUCINS

The glycoprotein structure of mucins is that of a protein backbone extensively glycosylated with carbohydrate, oligosaccharide side chains attached via O-glycosidic linkages to the residues of the protein backbone. N-glycosidic linkages are few. The apomucin protein core of a mucin subunit has 'naked' regions and glycosylated regions. Digestion with trypsin produces glycosylated subunits known as T-domains [61]. Glycosylation occurs as a post-translational modification at amino acids serine and threonine, which are found throughout the protein backbone of the mucin but mainly in tandem repeat regions. These regions of the apomucin have a repetitive amino acid sequence [62], which is reflected in the nucleotide sequence of the MUC genes. The amino acid content of the protein could therefore reflect the extent of glycosylation of the molecule. Cysteine residues at the 'naked' terminal ends form the disulphide bond between subunits. The disulphide bonds result in the formation of large polymers of gel-forming mucin [63], and in combination with increased glycosylation, the mucin gains properties of a particularly resistant, 'sticky' substance.

The structure and conformation of gel-forming mucins remains controversial. This is in part due to the various methods employed in purifying these glycoproteins from their secreted environment. A windmill model was proposed as the structure of mucin [63], which described the linking of the naked, non-glycosylated regions of four, higher molecular weight protein subunits through a low molecular weight link protein by disulphide bonds in a windmill-shaped conformation. An alternative linear, flexible, random coil model was also proposed, with a disulphide bond linkage between subunits [61, 64]. The major difference between the two models could have been as a result of different procedures for the extraction of the mucus. This differed by the inclusion of 6M guanidinium hydrochloride (GuHCl) in the extraction media to prevent endogenous proteolysis. Pearson *et al.* (1981) used 0.2M sodium azide (NaCl: 0.02% NaN₃) as an extraction media which would ensure an absence of proteolysis [63]. However, guanidinium hydrochloride will decrease the denaturation of all proteins. Mall *et al.* (1998) identified the effects of different extraction and isolation methods on the structure of pig gastric mucins [65]. The subunits in the structures reported by each group were of different sizes and Mall *et al.* (1998) confirmed this effect to be attributed to the presence of guanidinium hydrochloride [65]. Solubilisation of mucus in preparations can be difficult and homogenization by any other procedure than slow stirring overnight may interfere with the integrity of the protein. The pH of solution has an important bearing on the solubility and integrity of the mucus [66]. Non-covalent inter-molecular bonding between charged portions of the mucin glycoprotein may be affected but it is important to question whether disulphide linkages remain intact throughout the process of purification. Most of the results obtained from the structural analysis of mucins were controversial and a consensus model has yet to be found. However, the random coil model has been predominantly well-favoured; this structure being a linear polymeric glycoprotein with subunits joined end-to-end via disulphide bonds. Inter-molecular interactions may be involved between these linear units allowing specific mucins to have either characteristics of a loose lattice network or a more robust barrier layer [67]. The complexity of the conformation of mucins is shown by further revelations in the study of mucin genes [67].

1.4.3 MUCIN GLYCOSYLATION, ANALYSES, AND THE ROLE OF GLYCOSYLATION IN HIV INHIBITION

The monosaccharides found in mucin glycoproteins include N-acetyl galactosamine (GalNAc), N-acetyl glucosamine (GlcNAc), N-acetyl neuraminic acid, fucose and galactose. Terminal residues can be fucose, sialic acid, and sulphated GlcNAc or blood group determinants [47]. Table 1 shows the structures of O-glycan cores and antigenic epitopes found in mucins. The hydrophilic nature of mucins is attributed to the sialic acid and sulphate moieties [4], whilst hydrophobicity could be attributed to fucose [47]. The sequence and extent of glycosylation is specific for mucins from different regions of the body, and can vary in the same mucin between normal and disease states. For example, Tn antigens, formed in O-linked oligosaccharide synthesis have been associated with cancer [68]. Alterations in sialylation and terminal sulphation could be found in the diseased state. Morris and Rees (1978) determined a possible collective effect of glycosylation in inter-chain binding and gelation of the polymers [69]. Thus the question arises as to whether a certain number of the residues have to be present in the sugar composition, or a specific arrangement of the residues is necessary to have an effect on the interaction with the carbohydrate residues of the HI-virus. These hypotheses point to an investigation of the presence of similar charged groups within MUC5B and MUC7, and the possibility of altered glycoforms with infection or susceptibility to an HIV-1 infection. For this study it is necessary for the mucins to be in their native secreted state as collected in saliva. We aimed to investigate the heterogeneity as well as the reproducibility of the glycosylation sequences. Identification of a specific glycoprotein sequence recurrent in the glycoproteins is important, but more so the ratios and relationships between residues present. Heterogeneity of mucin glycosylation has been said to not have any effect on the prevention of gel-forming properties [4], and so it is possible that a collective effect enhances gel-forming properties. Ultimately we aim to look at collective and specific non-covalent interactions between the

glycoproteins of the mucin molecules and the glycoproteins in the capsid structure of gp120 (proposed site due to interaction between SAG (Salivary agglutinin) and gp120 [33]).

O-Glycan	Structure
Tn antigen	GalNAc α Ser/Thr
Sialyl-Tn antigen	Sia α 2-6GalNAc α Ser/Thr
Core 1 or T antigen	Gal β 1-3GalNAc α Ser/Thr
Core 2	GlcNAc β 1-6(Gal β 1-3)GalNAc α Ser/Thr
Core 3	GlcNAc β 1-3GalNAc α Ser/Thr
Core 4	GlcNAc β 1-6(GlcNAc β 1-3)GalNAc α Ser/Thr
Core 5	GalNAc α 1-3GalNAc α Ser/Thr
Core 6	GlcNAc β 1-6GalNAc α Ser/Thr
Core 7	GalNAc α 1-6GalNAc α Ser/Thr
Core 8	Gal α 1-3GalNAc α Ser/Thr
Epitope	
Blood groups O, H	Fuc α 1-2Gal-
Blood group A	GalNAc α 1-3(Fuc α 1-2)Gal-
Blood group B	Gal α 1-3(Fuc α 1-2)Gal-
Linear B	Gal α 1-3Gal-

Blood group i	Gal β 1-4GlcNAc β 1-3Gal-
Blood group I	Gal β 1-4GlcNAc β 1-6(Gal β 1-4GlcNAc β 1-3)Gal-
Blood group Sd(a), Cad	GalNAc β 1-4(Sia α 2-3)Gal-
Blood group Lewis ^a	Gal β 1-3(Fuc α 1-4)GlcNAc-
Blood group Lewis ^x	Gal β 1-4(Fuc α 1-3)GlcNAc-
Blood group sialyl-Lewis ^x	Sia α 2-3Gal β 1-4(Fuc α 1-3)GlcNAc-
Blood group Lewis ^y	Fuc α 1-2Gal β 1-4(Fuc α 1-3)GlcNAc-

Table 1: Structures of O-glycan cores and antigenic epitopes found in mucins [70].

Research has been done to discover whether there are specific properties of the mucin molecules that contribute to defence against disease. For example, Wu *et al.* (2003) identified a similar type of high molecular weight glycoprotein, salivary agglutinin, which specifically inhibits HIV-1 infectivity and binds to the virus at the viral envelope protein gp120 [33]. Malamud *et al.* (1993) did electron microscopy studies, which showed HIV-saliva aggregates, trapped in 0.45 micron pore size nitrocellulose filters and proposed that this could account for the low level of virus detected in oral secretions [71]. A non-covalent interaction between glycosylated regions of the proteins and the glycosylated regions of the viral envelope may explain the protective nature of the mucin molecules and their glycosylated counterparts.

The envelope spike (Env viral protein) gp120 of the HIV-1 virus is that which attaches to binding sites of CD4 and its coreceptors either CCR5 or CXCR4. This viral protein is therefore

the key focus of studies investigating the mechanisms of viral infection and immune invasion. The protein folds and trimerizes in the endoplasmic reticulum (ER) of the host cell, where it obtains ten disulfides and about 30 N-linked glycans depending on the viral isolate [72]. Cellular proteases cleave gp160 into gp120, and a transmembrane subunit, gp41 [72]. The binding between cell-surface receptors and virus occurs at heavily N-glycosylated regions of gp120 suggesting a plausible role of these sugar residues in non-covalent viral interactions. The high N-glycosylation of this viral capsid protein is the focus of much research [73, 74]. Gp120 has the oligomannose glycans on its heavily glycosylated outer domain as described by Zhu *et al.* (2000) [73]. Doores *et al.* (2010) performed mass spectrometry analysis of these viral proteins and discovered a highly conserved virus-specific glycan profile across primary isolates and geographically divergent clades [75]. Doores *et al.* (2010) discovered that the HIV-specific glycosylation pattern of glycans is uniquely conserved across all isolates of HIV-1 from clades A, B and C (A_{G2RW020}, B_{JRCSE}, C_{DU422}) [75]. From this information we can gain perspective on the possible interactions that may exist between mucin O-glycosylation patterns and the unique patterns of glycosylation on gp120 of the HIV-1 virus.

Previous work in our laboratory revealed a noticeable variation in electrophoretic mobility of MUC7 samples isolated from saliva from HIV-positive patients when compared with those samples from HIV-negative donors, which displayed little difference in mobility [29]. This could be the evidence for variable glycosylation between the two groups. Thornton *et al.* (1995) described the separation of mucins by agarose gel electrophoresis that was largely dependent on their charge density [76]. Using the techniques of HPLC, as was done by Thornton *et al.* (1995), we planned to separate individual mucins and apply these to agarose gel electrophoresis based on their elution gradients to compare HIV-negative versus HIV-positive donor saliva [76].

An amino acid analysis of MUC5B and MUC7 provided a profile of the signature amino acids serine, threonine, and proline, which contribute 26% and 28% of the mucins respectively [30]. Our previous work (Peacocke *et al.* manuscript under revision) has determined samples to have the following mean mole percentages of S, T and P of the total amino acids: HIV-negative samples MUC5B – 27.4709%, HIV-positive samples MUC5B – 28.5468%, HIV-negative samples MUC7 – 21.9692%, and HIV-positive samples MUC7 – 23.6117%. Little differences were observed with HIV-status; however, MUC7 has fewer potential glycosylation sites due to the lesser percentages shown. Glycosylation profile differences have been observed between MUC5B and MUC7 [13]. Using amino acid analysis we hoped to investigate these differences in our sample population.

To further investigate the functional behaviour of the salivary mucins, a full sugar analysis and possible structural determination of the glycoprotein species between sample populations will be done. The chosen method of Liquid Chromatography Electrospray Ionization-Mass spectrometry (LC ESI-MS) is a highly sensitive method employing a graphitized carbon column which enables highly specific isomeric separation. The method is well advised for separation and detection of neutral, acidic or permethylated glycans [77]. The method will allow identification of the complete glycan profile of each mucin. Methods of glycosylation analysis can involve lectin or antibody binding to the carbohydrate epitopes. However these methods only give a partial result and the analysis using LC ESI-MS was followed.

It has been postulated that the role of mucin in protection lies in physically trapping the virus. However, we hypothesise that a specific interaction between mucin and virus is possible. This interaction could occur between the sugar sequences of viral capsid proteins and those of the mucus glycoproteins. We aim to characterise the sugar side-chains of salivary MUC5B and MUC7

from HIV-negative and HIV-positive donors. Whether mucin structure and particularly glycosylation, is altered by HIV-infection status is unknown. Sugar analysis of mucins may yield information of the interaction between mucins and the HIV-1 virus as well as reasons for the differences seen in the inhibition of the HIV-1 virus by mucins isolated from HIV-negative individuals compared with HIV-positive individuals. More evidence may be found for the relationship between viral load, in patient blood, and transmission and if this exists in oral transmission.

1.5 PREVIOUS STUDIES IN OUR LABORATORY

It was the definitive work of Habte *et al.* (2006) in our laboratory, who performed a qualitative study where saliva from uninfected individuals together with its purified mucin components MUC5B and MUC7 inhibited the HI-virus in an *in vitro* assay, and provided an explanation for the inhibitory activities of the crude saliva [30]. Purified salivary mucins from HIV-positive individuals did not inhibit the virus [30]. The virus used was an HIV-1 subtype D isolated from a patient at the Faculty of Health Sciences, University of Stellenbosch at Tygerberg Hospital, Cape Town. The reason for the involvement of mucins in the inhibition of the virus has been proposed to be the agglutination property of mucin [30]. The infection status of HIV-positive individuals directly relates to the viral load in the blood of that infected person. The influence of CD4 count may have an effect on mucosal immunity although Habte *et al.* (2010) determined no difference in the inhibitory action, against HIV-1, of mucins MUC5B and MUC7 collected from patients with different CD4 counts [29]. Limitations to these findings by Habte *et al.* (2006, 2007, 2008, 2010) included the fact that they were qualitative in nature, where the pooling of samples to obtain greater yield of mucins occurred, not taking into account inter-individual variation [29, 30, 78, 79]. There was no defined HIV-negative group, except for those who

'declared a disease-free lifestyle'. This project is a quantitative study where the cohort is of individual donor samples of saliva and their purified mucins. Donors will be tested either HIV-negative or HIV-positive individuals.

Further work in our laboratory investigated the role of MUC5B and MUC7 in the inhibition of the HIV-1 virus. The *in vitro* HIV-1 inhibition assay confirmed the inhibitory activities of crude HIV-negative saliva as observed by Habte and Mall *et al.* (2006) [30]. The virus used was an HIV-1, subtype C, also isolated from a patient at Tygerberg Hospital, Cape Town. An additional finding was that crude HIV-positive saliva inhibited the infection of cells by HIV-1 in the *in vitro* assay. Crude saliva also contains a large amount of other protein contaminants that may play a role in the inhibition of the virus, for example the high molecular weight glycoprotein salivary agglutinin [33]. Purified salivary mucins MUC5B and MUC7, from both groups, with dilutions of 10^{-1} , 10^{-3} , 10^{-5} , 10^{-10} , 10^{-20} and 10^{-40} also inhibited the infection of cells in the HIV-1 inhibition assay. Further dilutions of purified mucin sample have been necessary to determine a dilution at which there is 50% inhibition of HIV-1 and establish a dose-response curve. An HIV-1 inhibition assay involving a larger sample size of purified salivary mucins MUC5B and MUC7 from similar donors could reveal any correlation with the results obtained by Habte *et al.* (2006) [30].

1.6 IN VITRO ASSAYS FOR HIV INHIBITION AND INFECTION

The *in vitro* HIV-inhibition assay used previously by Habte *et al.* (2006) was also questioned for its accuracy in describing the sample population, as results remained inconclusive [30]. Polonis *et al.* (2008) formed an analysis of recent advances in HIV-1 neutralization assays [80]. The inhibition and neutralisation assays were applied for standardized evaluation of the antibody

response to infection and vaccination. Polonis *et al.* (2008) discussed a comparison between the HIV-inhibition assay (using live isolated virus, PBMCs (peripheral blood mononuclear cells), and quantified using a p24 antigen ELISA kit), and an HIV-neutralisation assay (using pseudovirus and TZM-bl cells, quantified using a luciferase reporter gene assay) [80]. In vaccine development, it has been necessary to create a standardized platform for reproducible measurement and meaningful comparison of neutralizing antibodies against the virus, as well as an assay that can be used for high throughput in clinical trials [80]. Variable parameters exist in the neutralisation assays. These include: the target cell used and cell density; the host cell used for viral stock propagation; virus dose and antibody dilution/concentration, (virus particle: antibody ratio); the inclusion of complement; volumes of components added; duration of pre-incubation of virus and antibody; duration of infection with or without antibody; cell washing steps to remove unbound antibody and virus; length of culture time; the endpoint measured and other variables [80]. These can be easily controlled. It is important to note that the assay must have physiological relevance and the ability to have value as a replacement for *in vivo* assays [80]. In the viral inhibition assay used by Habte *et al.* (2006) and our previous work (Peacocke *et al.* manuscript under revision), the virus used was a clinical isolate [30]. PBMCs (including CD4+ cells targeted by the HI-virus) were isolated from normal, healthy donors. This assay has a strong physiological relevance. Reproduction of the assay is difficult due to numerous variables which can be used to explain some of the differences observed with the work of Habte *et al.* (2006) where HIV-positive donor mucins did not inhibit the virus, and the more recent work we have done where HIV-positive donor mucins did inhibit the virus (Peacocke *et al.* manuscript under revision). Donor PBMCs are taken from different cohorts of individuals. Host genetic factors (CD8+ cell factors, CD4+ cells and expression of these molecules, genetic polymorphisms in chemokines or chemokine receptors, such as CCR5), as well as the effects of host cell-derived molecules on the viral surface cause variability [80]. Thus, inter-experimental comparison has limited value due to the number of variables that can influence the outcome of the assay.

The HIV-neutralisation assay used in this study adopts the use of a pseudovirus, a genetically modified form of the HIV-1 virus that is composed of an inactive or dysfunctional env gene, the significance of which is that the virus can only undergo a single round of replication and infect the target cell once. The pseudoviral use has a significant advantage of safety in the laboratory, that is, unless recombination occurs during production of the virus. The pseudoviruses are generated in a 293T cell line by cotransfection of an env-mutated viral backbone with the env clone of choice, and then used to infect an epithelial HeLa-derived transformed cell line expressing the appropriate receptors and holding the luciferase reporter gene [80]. The gene is sensitive to the presence of the HIV Tat protein, which initiates its transcription [80]. This TZM-bl assay is the chosen method in the analysis of neutralizing antibodies in the good clinical laboratory practices (GCLP) as reported by Polonis *et al.* (2008) [80]. Envs used can be derived from multiple clades, enabling high throughput testing with a range of virus type. An advantage is that the assay length of 2-3 days is shorter than the PBMC assay. The pseudoviral assay measures inhibition of cell attachment/entry, whilst the PBMC assay not only measures cell attachment and entry, but cell-to-cell transmission as well. This may potentially distort results when comparing between experiments. The coreceptors used in the PBMC assay have more of a physiological relevance than the pseudoviral assay, which has a greater representation of CCR5 in particular [80]. However, there is discrepancy when the pseudoviral assay data using neutralising antibodies is compared with the PBMC assay as identified by Polonis *et al.* (2008) [80-82]. Through this work an HIV-neutralisation assay has been developed where the effect of mucins on the virus can be quantitatively evaluated. The assay used was primarily developed from that of Montefiori *et al.* (2009) [83]. This research seeks to purify mucin glycoproteins from saliva and identify their role and specific interaction with the HIV-1 virus using a pseudoviral platform.

1.7 RATIONALE AND RESEARCH QUESTION

The inhibition of HIV-1 by mucins provides insight into the body's natural protection mechanisms that hold potential for the first line of defence against spread of disease. Perhaps the answer to understanding and creating resistance to disease is to adopt an approach to enhance those innate protective mechanisms we have in our bodies [84]. Our work on the inhibitory effect of HIV-1 by mucus and mucins offers promise of developing or formulating a mucus based compound that could act as a microbicidal gel or paste for epithelial surfaces, such as in the vaginal and anal mucosae, thus reducing the possibility of viral transmission and also decreasing the risk of injury to mucosae during sexual intercourse through its lubricating effect.

We hypothesise that crude salivary mucus and its purified mucins MUC5B and MUC7 have inhibitory activity against the HIV-1 virus particles and that the extent of inhibition varies between HIV-negative and HIV-positive donors. This hypothesis required further verification for quantification, thus requiring an optimization of the inhibition assay [83]. A credible dose-response curve has been established. The current hypothesis is that there is a specific interaction between salivary mucins MUC5B and MUC7 and the HIV-1 virus. This inhibition of the virus is significantly greater in HIV-negative donor samples compared with those from HIV-positive donors [30]; we propose the extent of inhibition is variable in relation to infection status. Questions have been raised as to the nature of the inhibition, whether it is due to specific sugar interactions or due to aggregatory properties of 'sticky' mucin molecules. Glycosylation analysis was performed to investigate this. The work follows that of Habte *et al.* (2006, 2007, 2008, 2010) and my own previous work in the Department of Surgery as part of a BSc Honours project on the same topic (Peacocke *et al.* manuscript under revision) [29, 30, 78, 79]. This Masters research is an extension of my Honours project, and will include the following

objectives. The sample size will be $n=15$, for HIV-negative and HIV-positive donors, and a volume of approximately 30ml per donor.

University of Cape Town

2.1 ESTABLISHED OBJECTIVES

1. Samples were collected (in 6M GuHCl and protease inhibitors) from n=15 HIV-tested negative individuals and n=15 HIV-positive individuals from the Voluntary Counselling and Testing Drive at UCT and the HIV-AIDS Clinic at Groote Schuur Hospital respectively. Collation of patient information was done directly and via patient folders in order to compare infection status with determined inhibition.
2. A mass analysis was performed to enable a comparison of total protein and mucin content and to investigate the variation in the content of mucin in samples with relation to possible aggregatory potential of mucin in saliva.
3. Separation of mucins, MUC5B and MUC7, was done by Sepharose CL-4B size-exclusion column chromatography. Further purification was performed by caesium chloride density-gradient ultracentrifugation. In order to test for the presence of the particular dominant species of mucin in saliva, MUC5B and MUC7, Western blotting and amino acid analysis were performed. Amino acid analysis was performed on MUC5B and MUC7 samples by HPLC methods, in collaboration with Dr Jerry Rodrigues at the Molecular and Cellular Biology Department, University of Cape Town.
4. An investigation of the potential inhibition of the HIV-1 virus by crude salivary mucus and purified MUC5B and MUC7, including the development of a dose-response curve using an HIV-neutralisation assay, was performed and developed in collaboration with Dr Jeff Dorfman at the IIDMM (Institute of Infectious Disease and

Molecular Medicine), University of Cape Town and the ICGEB (International Centre for Genetic Engineering and Biotechnology).

5. HPLC was performed, to determine the presence of altered glycoforms of MUC5B and MUC7, in association with Dr D. Thornton (Department of Life Sciences, University of Manchester, UK). Further glycosylation (sugar) analysis was performed in collaboration with Dr N. Karlsson (Medical Biochemistry, University of Gothenburg, Sweden). (These visits were made possible by the National Research Foundation of South Africa). The glycosylation analysis was performed on the mucins to investigate the potential inhibitory mechanisms and variability of MUC peptides in the inhibition of the virus.
6. Statistical analysis of results was performed to determine the significance of the results for each group.

2.2 RESEARCH ETHICS

REC REF: 078/2010 23 February 2010

Work done in this project was approved by the Ethics Committee of Research at the Health Sciences Faculty at the University of Cape Town.

University of Cape Town

2.3 SAMPLE COLLECTION

Samples of crude saliva were collected from healthy symptom-free normal female and male volunteers who tested negative for HIV-AIDS at a University of Cape Town HIV voluntary counselling and testing drive. HIV-positive saliva samples were collected from volunteers at the Clinic of Infectious Diseases in Groote Schuur Hospital (Observatory, Cape Town, South Africa). A patient information sheet and consent form is included in the appendix.

Prior to sampling donors were asked to rinse their mouth with water. Secretion of whole saliva was stimulated by chewing on the inert material plastic Parafilm "M" Laboratory Film. Saliva was collected into 6M guanidinium hydrochloride (GuHCl), containing a cocktail of protease inhibitors (10mM EDTA, 1mM PMSF) pH 6.5. Samples were collected and transported on ice and then stored at -20°C until purification.

2.4 SAMPLE PREPARATION

Samples were thawed at 4°C and then solubilised overnight by gentle stirring at 4°C. This step was necessary to ensure solubilisation of the mucus. The mucin component of saliva exists as a gel polymer that is partially separated from the more liquid components. Gentle solubilisation ensures no risk of breaking molecular bonds, as would be the case with high speed homogenisation. Insoluble debris was separated from soluble mucus by centrifugation at 3000rpm for 30min at 4°C in a 'Hitachi HIMAC' centrifuge. An aliquot of the 'crude' supernatant was used for size exclusion chromatography using a Sepharose CL-4B column. Another

solubilised 'crude' sample aliquot was dialysed against three changes of distilled water overnight at 4°C, freeze-dried and stored at -20°C for use in the HIV-neutralisation assay.

2.5 PURIFICATION OF MUCIN GLYCOPROTEINS FROM SALIVA SAMPLES

2.5.1 SIZE EXCLUSION CHROMATOGRAPHY

Size exclusion chromatography allows for the separation of molecules according to size and in this experiment particularly the separation of mucins, MUC5B and MUC7 in the crude saliva. Aliquots of the supernatant were chromatographed on a Sepharose CL-4B gel filtration column (bed volume of 200 ml) (Sigma), eluted with 0.2M NaCl: 0.02% NaN₃ at a flow rate of 60ml/h at room temperature (RT) into 2ml fractions. Glycoprotein was estimated with a periodic acid Schiff's (PAS) assay according to the method of Mantle and Allen (1978) [85], and analysis of protein was performed following the method of Lowry *et al.* (1951) [86], for eluted fractions, in order to identify the void volume (V_0) (MUC5B) and included volume (V_i) (MUC7) fractions. The fractions under each peak were pooled for each mucin, dialysed exhaustively against three changes of distilled water and freeze dried.

2.5.2 DIALYSIS OF SAMPLES

Prior to analysis, it was essential to remove GuHCl, a protein denaturant in which the mucus was collected and solubilised, to prevent endogenous proteolysis. The properties of GuHCl would beneficially render the HI-virus harmless by its denaturing effect on all proteins but its

presence would interfere with reagents in the Lowry assay for protein estimation. The removal of GuHCl was done by dialysing against three changes of distilled water overnight at 4°C with constant stirring using a magnetic stirrer. Dialysis tubing used was Spectra/Por molecular porous membrane tubing MWCO: 12-14 000, flat width: 25mm, diameter: 16mm, volume: 2.0ml/cm, obtained from Argon Laboratory Supplies.

2.5.3 FREEZE DRYING

Freeze drying removes water from the sample leaving it in powder form by the process of lyophilisation. This is a method of drying that will enable concentration of the protein, molecular stabilization for storage or quantitative distribution, and reduction of molecular damage [87]. Freeze drying enabled determination of the total amount of crude or purified sample present in this experiment. Freeze drying was performed using a Christ ALPHA 1-5 freeze drier from Lasec Laboratory and Scientific Equipment.

2.6 PURIFICATION OF MUCIN COMPONENTS

2.6.1 CAESIUM CHLORIDE DENSITY GRADIENT ULTRACENTRIFUGATION

Material from the V_0 and V_i peaks eluted on a Sepharose CL-4B column containing both mucin and contaminant salivary protein components, were purified using caesium chloride (CsCl) density gradient ultracentrifugation according to the method of Creeth and Denborough (1970) [88]. The material from each peak was adjusted to a density of 1.39 to 1.40g/ml by the addition of solid CsCl (Sigma, UK) at a final concentration of 3.5M CsCl. Density gradient

ultracentrifugation was performed using a Beckman L45 ultra-centrifuge for one session of 48h, at 105 000g at 4°C. Mucin-rich fractions were identified using a PAS assay for glycoprotein and Bradford assay for protein estimation (Quickstart, BioRad). This assay for protein quantification was deemed useful because the reagents do not interact with the GuHCl and CsCl used in the density gradient ultracentrifugation method, as would be the case in the Lowry protein assay, and so no dialysis was required prior to analysis. The selected glycoprotein-rich fractions were pooled, dialysed against three changes of distilled water at 4°C, and freeze-dried.

2.7 ANALYTICAL PROCEDURES

2.7.1 AGAROSE GEL ELECTROPHORESIS

A 1% Agarose, Type V (Sigma), in 1X TAE buffer (Tris-acetate buffer (with EDTA)) containing 0.1% SDS (Sodium dodecyl sulphate) gel, was used for Western blotting. A concentration of 1mg/200ul of mucin in reducing loading buffer (2% sodium dodecyl sulphate (SDS), 10% glycerol (British Drug House/BDH), 0.01% bromophenol blue (Merck), 5% mercaptoethanol (BDH)) was used. The sample was then boiled for 1min and 50ul loaded into each well. The gel was run at 65V for 2.5h.

2.7.2 WESTERN BLOTTING

Western blotting was performed in order to prove that V_0 and V_i were indeed MUC5B and MUC7 respectively and to note any differences of these mucins between the HIV-negative (N) and HIV-positive (P) sample groups.

Purified MUC5B (V_0) and MUC7 (V_i) were transferred from the gel onto nitrocellulose membranes (Nitrocellulose, 2.0 micron, obtained from Argon Laboratory Supplies), by vacuum blotting for 1h at a suction pressure of 40mbar using a Pharmacia LKB Vacugene XL and using 4X SSC buffer to transfer the mucin fractions onto the membrane.

In order to block non-specific binding to antibody, the membranes were then incubated for 1h in 5% (m/v) low fat milk powder in TBS (Tris buffered saline), 0.05% Tween-20 (TBST) (Merck). The membranes were then washed with 1X TBST for 3 × 5min and incubated for 2h (or overnight at 4°C) with rabbit anti-MUC5B monoclonal (MUC5Bb EU; location – cys in TR (tandem repeat)) and mouse anti-MUC7 monoclonal (MUC7a EU; location – Nter (N terminal)) primary antibodies (antibodies kindly donated by D. Thornton (Manchester) and D. Swallow (London) from the EU Consortium, respectively), diluted 1 in 2000 (rabbit anti-MUC5B) and 1 in 1000 (mouse anti-MUC7) in 5% (m/v) low fat milk powder in TBST. The membranes were then washed with 1X TBST for 3 × 5min and incubated for 1h with HRPO-linked secondary antibodies goat anti-rabbit (MUC5B) and rabbit anti-mouse (MUC7) (obtained from Dakocytomation) diluted in 5% (m/v) low fat milk powder in TBST at dilutions of 1 in 5000 (goat anti-rabbit) and 1 in 1500 (rabbit anti-mouse). The membranes were then washed in 1X

TBST for 3 × 5min and place in distilled water. Bands were detected using an enzyme-chemiluminescence (ECL), detection kit (obtained from Whitehead Scientific, SA).

2.7.3 AMINO ACID ANALYSIS

The analysis involves acid hydrolysis of peptide bonds between amino acid residues present in the mucin protein molecules and 'chemical derivation of the hydrolysate'. High Performance Liquid Chromatography (HPLC) separation, detection and analysis of the resulting derivatised amino acids allows for quantitative estimation of the amino acid composition. The amino acid analysis allowed for identification of the serine, threonine and proline content of the mucin molecules, which verifies the presence of mucin and provides a source of investigation into the extent of glycosylation of the molecules. The alteration of mucin glycosylation may be related to genetic predisposition and susceptibility to infection with the virus as well as a result of the viral infection.

2.8 GLYCOSYLATION ANALYSIS

2.8.1 HPLC TECHNIQUE

Mucins required reduction prior to HPLC analysis. Mucins were solubilised into urea reduction buffer (6M urea containing 0.1M Tris/5mM EDTA, pH 8.0) and treated with 10mM dithiothreitol (DTT) for 5h at 37°C. Iodoacetamide was added to a final concentration of 25mM and the mixture left in the dark overnight at RT.

This method was according to Thornton *et al.* [89]. Anion-exchange HPLC for mucin subunit fractionation and purification was done using a Resource-Q column. The ion-exchange column used a 2150 LKB titanium-head pump connected to a 2152 LKB controller and a 2040-203 LKB mixing valve. Gradients were formed at the low pressure side and 5ml of mucin-rich sample was loaded with an injector each time. A pressure maximum was set for 5Mpa. The column was eluted at a flow rate of 0.5ml/min, first isocratically for 10min with 10mM piperazine/perchlorate buffer, pH 5.0 (buffer A), then with a linear gradient (60min) to 0.25M lithium perchlorate/10mM piperazine/perchlorate buffer, pH 5.0 (buffer B). Fractions (0.5ml) were analysed for the neutral sugars of the reduced mucin subunits using the PAS assay (Mantle & Allen, 1978) [85], and a slot blotting technique. The positive fractions were then applied to 0.7% agarose gel electrophoresis at 60V for 3h. Proteins were transferred to nitrocellulose (Whatman, ProTran, MOL 3008) by vacuum blotting (50mbar, 1.5h) and detected using antibody. Mucins were detected using EU MUC5B, a monoclonal antibody obtained from cell culture, at a concentration of 1:100, and EU MUC7a (mouse anti-MUC7) obtained from D. Swallow (London) at a concentration of 1:1000. A goat anti-mouse secondary antibody (whole molecule-linked alkaline phosphatase affinity-isolated antigen-specific antibody) (Sigma), was used for MUC5B. Detection was in alkaline phosphatase buffer with NBT (Nitro-blue tetrazolium chloride) (50mg/ml in 70% DMF (dimethylformamide)) and BCIP (5-Bromo-4-chloro-3'-indolyphosphate p-toluidine salt) (50mg/ml in 100% DMF) substrates. A GS800 calibrated densitometer was used to acquire the image and analyse band intensity for both slot blotting and visualising the MUC5B bands. HRPO-linked rabbit anti-mouse (secondary antibody obtained from Dakocytomation) was used for MUC7, diluted at 1:1500. Bands were detected using an enzyme-chemiluminescence (ECL), detection kit (obtained from Amersham Biosciences, Amersham, UK). The fractions positive for each mucin were pooled, dialysed exhaustively against three changes of distilled water and freeze-dried.

2.8.2 LC-MS TECHNIQUE

Liquid chromatography-Mass spectrometry was performed to analyse the O-linked oligosaccharides from the mucin glycoproteins separated by gel electrophoresis. This methodology was according to Schulz *et al.* [77] and was carried out with the assistance of Dr Niclas Karlsson (Medicinaregatan, University of Gothenburg, Gothenburg, Sweden).

Glycoprotein was applied to a composite gel for AgPAGE. This gel electrophoresis allows for glycoprotein separation and relevant protein identification. The gradient was 0-6% polyacrylamide (PA) (BioRad), 0.5%-1% agarose and 0-10% glycerol within the gel, made up in a 0.375M Tris-HCl buffer, pH 8.1. Forty percent ammonium persulphate and TEMED were added to activate the acrylamide. A 5X stock of buffer, 50% glycerol, 40% acrylamide (BioRad), agarose (Sigma, low electroosmotic flow grade) was used to cast the gel. The gel was poured using a BioRad miniprotein system at 50°C and allowed to set for 30min at this temperature followed by additional time at RT. Sample buffer was made up 2X at a concentration of 0.75M Tris-HCl pH 8.1, 20% glycerol, bromophenyl blue, 2% SDS. Unreduced sample was placed in the reducing sample buffer and boiled for 20min. Iodoacetamide was added in excess (25mM) and the solution was incubated in the dark at RT for 20min. Running buffer was 192mM borate, 1mM EDTA, 0.1% SDS and Tris, pH 7.6. Gel electrophoresis was at 40mA for 2-3h. A Kaleidoscope prestained marker was used. These enable observation of problems with polymerisation as the high molecular weight standards will not separate. The electrophoresis was followed by electroblotting of the glycoproteins to PVDF using a semi-dry method and staining with Direct Blue (DB71, Sigma) for the glycoprotein bands. The gel was also stained with Coomassie Brilliant Blue. The identified bands were then removed from the blot.

The bands were wetted with methanol. Oligosaccharide side-chains were separated by reductive alkaline β -elimination of sugar side-chains from the mucin glycoprotein. Samples from gel electrophoresis or purified sample (100ul at a concentration of 1mg/ml in ddH₂O), were incubated in eppendorfs, and sealed to prevent evaporation, at 50°C for 16h in 20ul of 50mM NaOH and 1.5M NaBH₄. The resulting solutions were neutralised by the addition of 1ul of glacial acetic acid, before being desalted with 25ul of AG50WX8 cation-exchange resin (BioRad), laid on top of a reversed-phase *u*-C18 ZipTip (Millipore), and dried in a Savant SpeedVac. Borate was then removed by repeated (5 times) addition and evaporation of 50ul of 1% acetic acid in methanol. The samples were resuspended in 10ul of MilliQ water for liquid chromatography coupled to electrospray mass spectrometry (LC/ESI-MS) analysis.

The desalted oligosaccharide alditols were then analyzed by liquid chromatography coupled to electrospray tandem mass spectrometry (LC/ESI-MS/MS) on a graphitized carbon column (7- μ m Hypercarb particles (Thermo-Hypersil, Runcorn UK) in a 100 \times 0.25mm column) or a 150 \times 0.32mm Hypercarb column (Thermo-Hypersil). A solvent rate through the column of 5ul/min was provided by a Surveyor LC pump (ThermoFinnigan, San Jose, CA) with flow splitting from 100ul/min. Oligosaccharides were eluted with an H₂O/acetonitrile gradient (0-40% acetonitrile in 30 min, followed by a 3min wash with 90% acetonitrile) containing 10mM NH₄HCO₃. Mass spectrometry was performed using an LCQ Deca (ThermoFinnigan) in negative ion mode, with three scan events: full scan with mass range 320-2000 m/z , dependent zoom scan of the most intense ions in each scan, and dependent MS/MS scan after collision-induced fragmentation. The capillary temperature was 180°C, the capillary voltage was 32.0V, and the electrospray voltage was 2.5kV. Dynamic exclusion of ions for zoom scan for 30s was introduced after three selections within 30s. For MS/MS, the normalized collision energy was 35%, with an activation time of 30ms. Oligosaccharide structure and linkage were determined using a combination of

analysis of LC/ESI-MS/MS data and the GlycoSuiteDB sugar database

(<http://www.glycosuite.com>).

2.9 HIV-1 NEUTRALISATION ASSAY

This is a pseudo-viral assay that uses TZM-bl/JC53 cells (a HeLa cell line engineered with CCR5, CXCR4 coreceptors and CD4 receptors). The cells have been transfected with an LTR (long terminal repeat) promoted luciferase and β -galactosidase gene sequence. The assay was initially developed by Montefiori *et al.* [90]. The cell line is adherent therefore requires a trypsin-EDTA digest to create a suspension before use. Trypsin acts by disrupting the cell proteins that adhere to the plate and EDTA chelates the magnesium involved in these bonds preventing re-adhesion. Cells are prepared so as to plate 10^4 cells/well. A cell count was performed using Trypan blue (0.1%). DEAE dextran was added to the cells at a concentration of 7.5 μ g/ml. DEAE dextran (Diethylaminoethyl cellulose) is a positively charged molecule that prevents cell/virus repulsion due to their same negative charge and therefore allows for better binding. Cells were applied to a flat-bottomed 96-well plate on day 1 and left to incubate for 24h to ensure adhesion to the wells.

The virus was made up from plasmid SG3 Δ *env that transcribes all viral genes except for a dysfunctional env (envelope-protein gene), and a plasmid containing env. The functional env can be varied to represent different viral strains. The viral tat gene initiates the LTR (long terminal repeat) promoter and thus luciferase and β -galactosidase production.

The assay is roughly quantitated according to the following table:

RLU – Relative Light Units

<u>Viral infection</u>	<u>Luc (RLU)</u>	<u>β-gal</u>
+	+	+
-	-	-

The experiment is measured as an indication of inhibition of the virus by antibodies for example. In the presence of antibody, the virus does not infect cells. If there is no virus present there is no luciferase production (or only base-line levels). If there is virus present but no inhibitory antibody then the cells have been infected and luciferase production initiated. Therefore, upon addition of a luciferase substrate a relative luminescence is detected.

The virus was made via transfection of 293T cells with SG3Δ*env and the env plasmids. Virus is produced by these cells but does not contain the functional env gene in its genes. The env plasmid is incorporated and transcribed to enable incorporation of the proteins into the viral capsid thus allowing the virus to bud from the cells. However the virus can now only infect cells for one round as the functional env gene is no longer present in the TZM-bl cells described above.

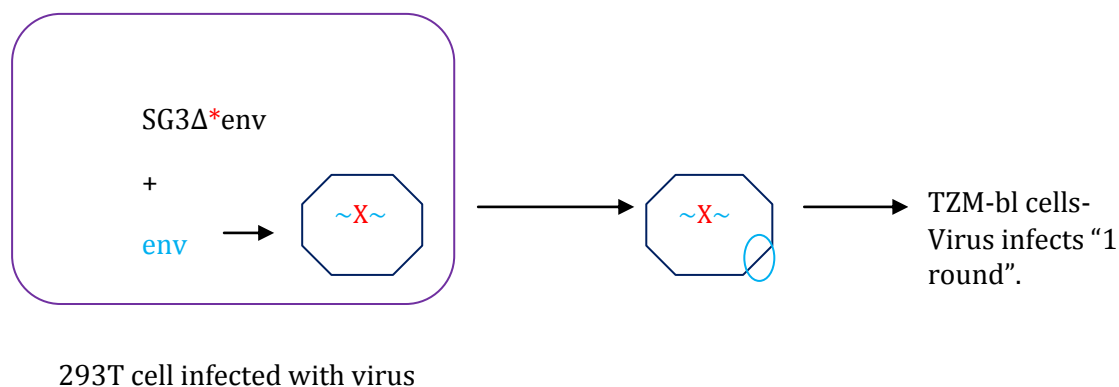


Figure 2: Diagram illustrating virus production using a 293T cell.

A viral titration was performed in order to determine the dilution required for the assay. Only harvest 1 was titrated. The dilution required needs to produce 50 000 RLU when incubated with cells (10^4 cells per well) as a control. Virus was diluted with media for the assay. Growth media was made up from DMEM (Dulbecco's Modified Eagle's Medium, Sigma) with HEPES, 10% fetal bovine serum (FBS) (diluted from a 200X stock to a 1X solution and heat inactivated) (a 5% solution was used for normal cell maintenance), penstrep and gentamycin (50ug/ml), and 1% non-essential amino acid (NEAA) (all from Gibco).

The experiment was carried out in a 96-well plate. Samples were plated into a 96-well plate in duplicate on day 2 of the experiment. Starting dilutions of mucin in growth media were 1mg/ml and two-fold serial dilutions were performed. Virus was added at the pre-determined concentration. Incubation was for 1h at 37°C.

After 1h incubation period, the virus/mucin sample mixture was transferred directly to the plated cells and further incubated for 48h. All wells had a 200ul total volume.

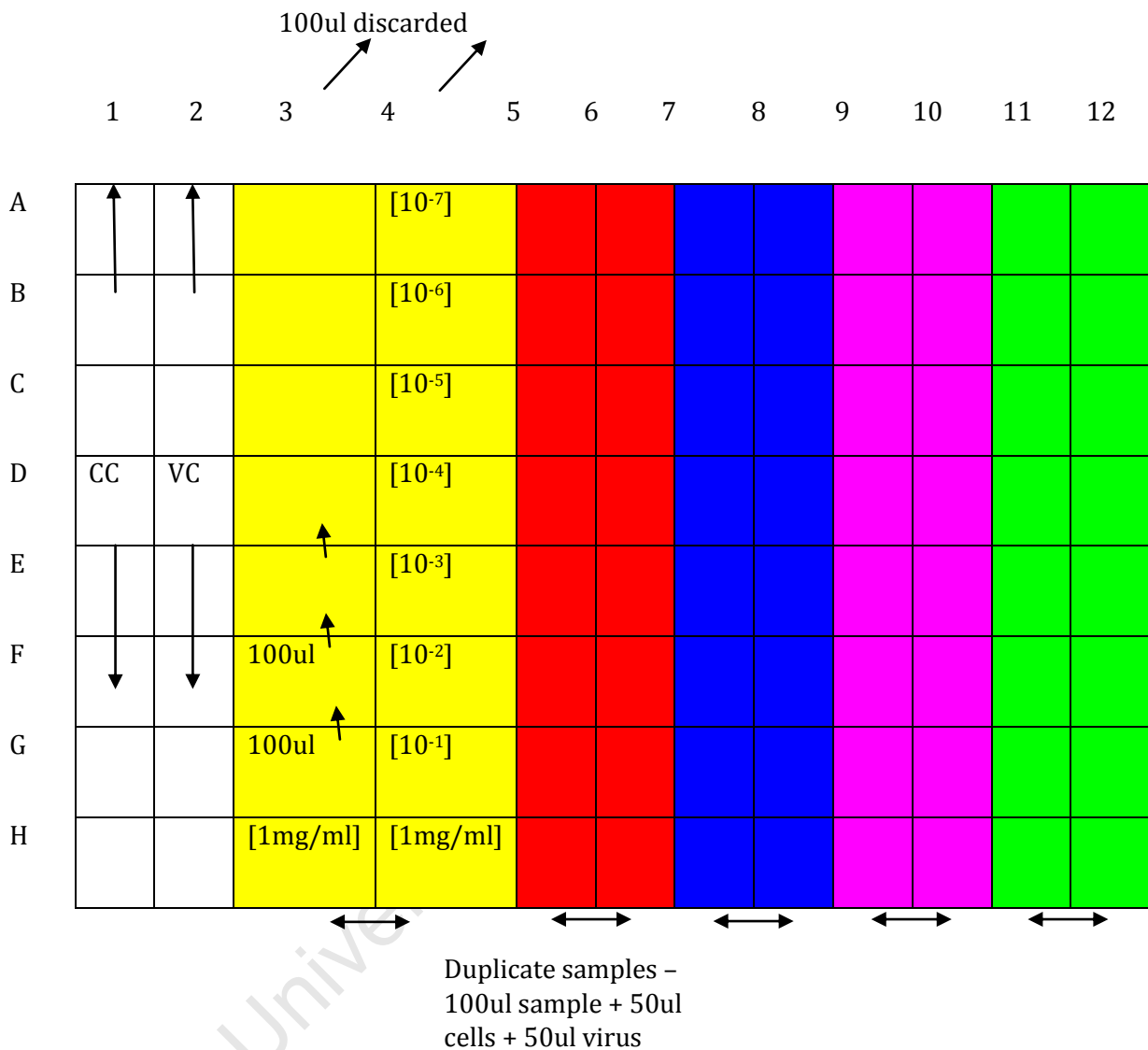


Figure 3: Template of HIV-neutralisation assay showing sample dilution. CC represents the cell control where each well contains 150ul Growth Media (GM) and 50ul cells. VC is the virus control where 100ul of GM, 50ul cells and 50ul of virus was added to each well. The remaining rows 3 – 12 were plated with duplicates of samples. 200ul of sample was plated in the first row and serial diluted 2-fold with GM. After incubation with 50ul of virus, the sample was then added to 50ul of cells.

After 48h incubation, 100ul from each well was discarded and 100ul of luciferase substrate (Promega, Anatech), added thereafter to each well. Following this there was a 2min incubation period in the dark. 100ul from each well was applied to a black plate. Detection was done using a luminometer to measure relative light units (RLU). Percentage neutralisation was calculated from the following formula. RLU of the sample test (average of each duplicate) subtracted from the virus control (VC) RLU, divided by the RLU of the virus control minus the RLU of the cell control (CC) multiplied by 100.

$$\frac{(RLU_{VC} - RLU_{Sample})}{(RLU_{VC} - RLU_{CC})} \times 100 = \% \text{ neutralisation}$$

$$(RLU_{VC} - RLU_{CC})$$

An IC₅₀ was determined for each sample.

2.9.1 MTT ASSAY

In the development of the HIV-neutralisation assay cell death was noted and attributed to practical methods, and therefore it was important to investigate a novel method to eliminate this variable. The MTT (3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) assay is a well-developed system for colorimetric analysis of cell viability and was identified as the most suitable. The assay identifies metabolically active cells from their activity of mitochondrial dehydrogenase enzymes. Water soluble yellow MTT is metabolised to form water insoluble purple formazan. Upon solubilisation, this is quantified by spectrophotometry at 570nm [91].

The method was constructed to closely replicate that of the neutralisation assay. TZM-bl cells were plated with a density of 10^4 cells in 100ul media per well in a flat bottomed, sterile 96-well plate, and incubated for 24h at 37°C (5% CO₂). After 24h, 100ul sample that had been solubilised in growth media was added to the cells and further incubated for 44h. Media was plated to serve as a blank. A positive control of cells incubated without mucin was compared with cells to which mucin had been added. Mucin sample was tested by serial 2-fold titration, and assayed in duplicate. After 44h incubation 20ul MTT solution (Sigma) was added at a concentration of 5mg/ml in PBS and incubated for a further 4h. After this period, 100ul of the well contents was discarded and replaced with 100ul solubilisation agent (10% SDS in 0.01M HCl) and mixed. The cells were further incubated overnight, thereafter a reading taken at 595nm using a spectrophotometer.

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3.1 SAMPLE COLLECTION

Patients 3, 5, 81 and 82 (Table 2) had inconclusive background information especially that of treatment status, out of the HIV-positive donor cohort (n=15).

3.2 PURIFICATION OF MUCIN GLYCOPROTEINS FROM SALIVA SAMPLES

MUC5B and MUC7 were separated by Sepharose CL-4B gel filtration and material under each of the peaks was further purified by density gradient ultracentrifugation in CsCl (caesium chloride) to remove any associated protein contamination. Purified mucins were required for the HIV-neutralisation assay and for further analysis.

3.2.1 SIZE EXCLUSION CHROMATOGRAPHY

The mucins in the V_0 (void volume) and V_i (included volume) of the gel filtration column have previously been identified as MUC5B and MUC7 respectively [8]. Sepharose CL-4B chromatography successfully separated MUC5B and MUC7 and to some extent freed the mucins of their protein contaminants, especially in the case of MUC5B which eluted in the V_0 (void volume) of the gel filtration column whilst MUC7 eluted in the V_i (included volume) of the gel filtration column.

Crude saliva was collected in 6M GuHCl containing protease inhibitors (10mM EDTA, 1mM PMSF) at pH 6.5 and solubilised. Approximately 20ml of sample was applied to the Sepharose CL-4B column (10% of bed volume). The column was eluted with 0.2M NaCl: 0.02% NaN₃ at a flow rate of 1ml/min at RT. An aliquot of each of the eluted fractions was assayed for glycoprotein and protein by the Periodic acid Schiff (PAS) (A₅₅₅) [85], and Lowry assays (A₇₀₀) [86] respectively (Figures 4 and 5). Samples showed inter-individual variation in both the amounts of material eluting under the V₀ and V_i peaks and the shape of the peaks, for both groups. In some instances, there was hardly any MUC5B detectable in both groups, but all samples showed the presence of MUC7. A third peak was observed for some samples eluting in the included volume in between the two original peaks. Fractions from each peak were pooled separately, dialysed and freeze-dried for further analysis.

3.2.2 CAESIUM CHLORIDE DENSITY GRADIENT ULTRACENTRIFUGATION

Caesium chloride density-gradient ultracentrifugation is the method of choice for the purification of mucins and their separation from contaminant protein and nucleic acid [88] (although it has proven difficult to remove the nucleic acid component from mucin samples completely, in certain instances [92]). Figure 6 shows the clear separation of a mucin sample from contaminant protein. This trend in purification was seen for both HIV-negative and HIV-positive samples as well as for MUC5B and MUC7 individually and Figure 6 is a representative profile for the purification of samples carried out in this study. Mucin fractionated as a PAS positive peak, and this was correlated with the curve for densities where mucin is indicated by a density between 1.39 and 1.40g/ml. In this particular case (Figure 6), mucin rich fractions 4 and 5 were pooled, dialysed and freeze dried for further analysis.

The weight of purified mucin glycoprotein was determined after dialysis and lyophilisation of the sample and related to the weight of crude saliva after the same process. Table 3 shows data for the yield of crude salivary material after lyophilisation of 1ml of sample. Yields from some samples in both groups were deemed inaccurate due to practical losses and these were therefore removed from this analysis (data not shown). A mean yield of lyophilised material from 1ml of HIV-negative donor saliva was 11.07 ± 12.29 mg (range 0.72mg – 53.14mg) and for the HIV-positive group, 14.36 ± 15.96 mg (range 1.29mg – 39.07mg). The data shows the mean yield of MUC5B was 0.40 ± 0.25 mg for HIV-negative donors and 0.86 ± 1.69 mg for HIV-positive patients. The mean yield of MUC7 was 1.56 ± 3.51 mg from HIV-negative donors and 0.82 ± 1.71 mg for MUC7 from HIV-positive patients. A much higher yield of 5.35mg and 5.37mg for MUC5B and MUC7 respectively was noted for one HIV-positive patient.

Table 4 shows the percentage yield of purified mucin from crude salivary protein (a comparison of lyophilised weight). For MUC5B purified from HIV-negative donors the percentage yield was in the range of 0.78% - 4.88%. For HIV-positive patients, yields of MUC5B were in the range of 0.17% - 37.27%. For MUC7, HIV-negative samples gave yields from 0.29% - 92.21% and HIV-positive samples gave yields from 0.00% - 37.41%. When individual samples were examined here it was noted that an HIV-negative sample (Donor 4) had no MUC7 present and an HIV-positive sample (Patient 11) also had no yield of the mucin. A comparatively high yield of MUC5B of 92.21% was noted for an HIV-negative sample (Donor 29). A comparatively high yield of both MUC5B and MUC7 was noted for an HIV-positive sample (Patient 4) with values of 37.27% and 37.41% respectively.

3.3 DISCUSSION

The “Kidzpositive” HIV clinic at Grootte Schuur Hospital in Cape Town South Africa runs an initiative in which HIV-positive mothers and their babies enjoy state-sponsored treatment and support with respect to their day-to-day requirements in a context of poverty and unemployment. Mother-to-child transmission through breast-feeding is the most common route of infection in babies in sub-Saharan Africa [93]. Saliva for this study was obtained from HIV-positive mothers, with a mean age of 34.5 years (range 28-50 years). At least half of these donors were non-smokers with two confirmed smokers (n=7). Patients were of Xhosa ethnicity, the predominant black African ethnic group in the Western Cape region of South Africa.

A huge limitation of this study was the procurement of sample. Besides the resistance of some patients to donate sample, others provided volumes that were far from adequate for the number of investigations planned for this study. Since our objective was to compare the biochemical characteristics of crude saliva and salivary mucin in HIV-negative and positive groups, sample volume was an important consideration and pooling of samples to obtain higher yield of mucin, like in the previous study by Habte *et al.* (2006), was avoided in this study [30]. Planned future studies will include a far larger cohort of sample donors for both groups and individual donors will be approached on more than one occasion to ensure adequate amounts of sample are obtained from each patient.

There were HIV-positive donors such as numbers 3, 5, 81 and 82 (Table 2), who had inconclusive background information, especially that of treatment status. This was due to missing folders in hospital records and transfers between hospitals. Some of the donors were

HIV-positive mothers or care-givers themselves who were not receiving treatment at this HIV-clinic and so were without folders. The HIV-positive mothers were accompanying their children to the clinic and were willing to be part of this study, by donating saliva. Some of the patients had travelled great distances to attend the clinic as they do on a 3-monthly basis. This made it difficult to determine when the patients would return, and whether they returned to the same clinic at all. Most patients' spoke their native tongue and this was a shortcoming in the verbal exchange between patient and investigator.

Whilst each of the donors had a medical history over and above their HIV-status, we were, quite realistically, unable to study the full impact of disease on the mucins and their specific anti-HIV activity for each individual. At least 5 out of 15 patients were confirmed to have previously been infected with Tuberculosis (T.B.). Six patients were taking first regimen antiretrovirals (ARVs), 4 patients were taking second regimen ARVs and one patient was not taking any treatment, but the infection was suppressed (CD4 613cells/ul). For 4 patients the treatment was unknown. The treatment status, CD4 counts and viral loads of the patients were important considerations because opportunistic infections are always a risk. These may include oral lesions, herpes, candidiasis and salivary gland disorders (lymphoepithelial cysts) which improve with topical or systemic treatments and with antiretroviral therapy. Bacterial pneumonia and T.B. are also common infections. Treatment status is therefore a critical factor for consideration of whether individuals within each group are compromised with respect to producing 'typical' mucins. The South African HIV Clinicians Society stipulates that any patient with a CD4 count < 350 cells/ul should receive HAART (Highly Active Anti-Retroviral Therapy). This is the threshold above which patients are considered to be clinically stable. The patients in the HIV-positive group were clinically well, suggesting the success of the ART, except for three patients with associated infections or side effects of treatment, presenting with diarrhoea and peripheral neuropathy. One patient was suffering from hay-fever, an allergic reaction which can increase mucus

production. CD4 counts for clinically well patients were greater than 350cells/ul. One patient however, had a CD4 count of 178cells/ul, and another had treatment adherence problems and associated nausea and diarrhoea, a CD4 count of 92cells/ul and viral load of 110 000copies/ul (high viral load >100 000).

After size-exclusion chromatography (representation of which is shown in Figures 4 and 5), samples showed interindividual variation in both the amounts of material eluting under the V_0 and V_1 peaks and the shape of the peaks, for both groups. In some instances, there was hardly any MUC5B detectable in both groups, but all samples showed the presence of MUC7. A third peak was observed for some samples from both groups, eluting in between the V_0 and V_1 (Figures 4 and 5). MUC5B levels seem to have varied greatly within both groups. The reasons for these observations are unknown. Varying levels of specific mucins in saliva could be due to the state of HIV infection, at least in the HIV-positive group. However, these differences in the gel filtration profiles point to interindividual differences in both groups rather than a distinct difference between each group.

HIV-negative samples were obtained from a Student Health clinic, which provides voluntary and free testing at the University of Cape Town as part of an HIV awareness campaign amongst young sexually active people at educational institutions in South Africa. However the general health status of any of these donors was unknown to the investigator, thus leaving us unable to speculate on any association between levels of MUC5B, MUC7 and any underlying pathology in these individuals.

Purification by CsCl density-gradient ultracentrifugation showed a clear separation of mucins from contaminant proteins (Figure 6). A representative profile of purification in both groups is shown here. Fractions 4 and 5 were pooled in this case, in both HIV negative and positive groups, at a density at which mucin is expected to fractionate in CsCl/GuHCl [94].

It was necessary to review the comparison between the yield of crude lyophilized material from saliva and that of purified mucin, given the difficulty in measuring and quantifying total protein by the Bradford assay, and mucin by the PAS assay. Challenges are faced when comparing the concentration of these secretions between donors as this has been found to vary considerably and is determined by numerous variables. Both the protein (Bradford) and glycoprotein (PAS) assays are limited in their lack of specificity in the presence of other proteins and glycoproteins respectively. An ELISA assay using mucin antibodies would probably be the best method of choice for a future study to eradicate these variables. Tables 3 and 4 include the yield measurements and assess the relative contribution of mucins to the amount of total protein in crude saliva. The data in Table 3 shows the mean yield of MUC7 to be 1.56 ± 3.51 mg from HIV-negative donors, which was considerably greater when compared with the mean yield of 0.82 ± 1.71 mg from HIV-positive patients. Yield of MUC5B from HIV-negative donors was less than that from HIV-positive patients with mean values of 0.40 ± 0.25 mg and 0.86 ± 1.69 mg respectively. It may appear that MUC5B production has increased with HIV-positive status. Although the implications of such a finding could suggest that MUC5B production increased in HIV-positive saliva as a protective mechanism against the virus, we have no evidence for such a statement. Noticeably large yields of MUC5B were noted in individual HIV-negative samples too (Donors 11 and 23 and 29), where the sample from donor 29 also had a considerably high yield of MUC7. Again when examining individual samples (Table 4) it was noted that an HIV-negative sample from donor 4 and an HIV-positive sample from donor 11 had no MUC7. Thus the mean

values of these samples may be skewed due to the considerably greater yield of a few samples in the group.

Table 4 shows the percentage yield of purified mucin in relation to crude salivary protein. These values were calculated by comparing the dry weight of purified mucin with the dry weight of lyophilised saliva. The values were based on the assumption that practical losses of mucin during purification of saliva were low. Thus inter-individual variation in the amount of crude salivary material and purified mucin was present in both HIV-negative and HIV-positive groups.

We attempted to include background information of patients to detect if this had any bearing on the mucin production in their saliva. Unfortunately, despite having limited information, we are unable to comment on any associations between mucin production and the information available to us. In a larger study, this would require the input of a clinician and perhaps a medical HIV-AIDS specialist.

HIV-negative donor 4, who had no MUC7 present in saliva, was a black male of 26 years, originating from Botswana and a non-smoker. HIV-negative donor 29, who had 92.21% yield of MUC7 from the saliva, was a coloured male of 22 years, originating from the Western Cape, South Africa, and a non-smoker. When compared with information from the other HIV-negative donors, the unusual yields of mucin could not be attributed to any traits of the donor and perhaps due to the limited information available. In the HIV-positive group it was known that all patients except patient 3 were taking antiretroviral therapy at the time of collection. Patients 9 and 11 (HIV-positive group) were known to be smokers. Patient 11 exhibited no production of MUC7 in the saliva collected. Patients 11, 6 and 7 had previously been infected with T.B. No

trends were noticed when comparing the percentage yield of mucin with viral load or CD4 count at the time of collection. CD4 counts (and HIV-status) have been shown to affect the size, charge and immunoreactivity of each mucin [29]. This will be investigated in the next chapter.

Although a study of this nature has not, as far as we know, been done, where saliva and salivary mucin is compared between HIV-negative and positive groups, the limitations we faced here provide us with crucial information with respect to planning a larger study in the future, taking into account the variables listed above. Important considerations would be to categorize those who are on treatment and those who are not, whether the individual patient has been diagnosed with T.B. or not, the general medical history of each individual and the CD4 counts of each individual. It is also important to select patients who are likely to use Groote Schuur Hospital as their choice of hospital, thus making follow-up easier. This would then provide information on the effect of other medical conditions and opportunistic infections on the mucin profile of the patient and the capacity of these mucins to inhibit the virus.

HIV-Positive donor	Time of collection	Gender	Birth date	Smoking	Ethnic group	Origin	TB-status	CD4 Count cells/ul at time of collection	Viral Load copies/ml at time of collection	Treatment at time of collection	Status at time of collection
2	April-10	F	1982.06.20	N	Black	Xhosa	Watched for symptoms	18.06.10 CD4 246 ALT 42	860; 2.93 (Log ₁₀)	ARV 26.03.08; 20.08.08, A, 3, N; 24.03.10 HAART, AZT, 3TC, NVP – Primary regimen	Trial amitryptiline, restart Bactrim
3	April-10	F	1973.06	N							
4	April-10	F	1980.04.06	N	Black					Defaulted HAART 11.2007; Restarted 07.07.2008; Vit. B, Starudine, Lamivudine, Nevirapine – Primary regimen	
5	April-10	F	1960.11.05	N		Port Elizabeth					
6	April-10	F	1976.05.05	N	Black	Xhosa, Cape Town	Still on double dose alivia despite stopping T.B. Rx, chest clear	24.03.10 CD4 92; 07.04.10 CD4 92	24.03.10 110 000 5.04 (Log ₁₀); 07.04.10 110 000	ARV 4.04.03; 25.10.06 AZT, ddi, Kaletra; 07.04.10 on ART – Secondary Regimen	Adherence problems, nausea, vomiting, diarrhoea, otherwise well, no complaints, good general condition
7	April-10	F	1975.07.27	N	Black	English, Eastern Cape	T.B. Finished treatment	13.01.10 CD4 145 ALT 22, 13.07.10 CD4 308	LDL (Lower than Detectable Limit)	ARV 09.06.09; 21.07.10; 13.03.10 d4T, EfV – Primary regimen	Clinically well, viral suppressed
8	April-10	F	1976.03.24	N						On ART since 2009; 05.2009 - D4T, 3TC, NVP – Primary regimen	
9	April-10	F	1982.05	Y	Black					Since 2005	

11	April-10	F	1982.12.11	Y		English	T.B. 12.08.08. Finished treatment. T.B. Neg. 27.05.10	CD4 178 ALT 40	2.7 (Log ₁₀)	ARV 3.12.08; 30.09.09 restarted; 31.03.10	No symptoms, good, chest clear
50	Sept-07	F	1965.08.08		Black	Xhosa	10.06.10 T.B. Neg. (Prev. T.B. Pos.)	30.11.07 CD4 517 ALT 43; 06.07 CD4 411 ALT 32	30.11.07 LDL 06.07 VLLDL	ARV + HAART - began 28.06.06; Regimen 2 16.04.08 HCT2, Elanapril at time of collection - Secondary regimen	Feeling well, clinically stable
52	Sept-07	F	1975.01.14			Xhosa	Clinically clear	no record before 11.06.09 CD4 466; 9.12.09 CD4 613; 10.11.10, CD4 371		Not on ARV	Stable
80	Sept-07	F	1980.07.22			Xhosa		7.02.07 CD4 381	27.09.07 - 14.02.07 LDL	ARV 02.08.06 d4T, 3TC, NVP; 14.03.07 HAART; 18.07.07 - Primary regimen	14.03.07 sneezing, blocked nose, watering eyes, but getting better today - cold/hayfever, good adherence
81											
82	Sept-07	F	1963.06.14								
89	Sept-07	F	1969.07.12					18.07.07 CD4 512; 30.11.08 239 25.0	LDL	HAART, ARV 20.01.04; 15.02.06, AZT, ddi, Kaletra; 08.01.07 HAART - Secondary regimen	Mild diarrhoea, required antifungicide

Table 2: Demographic and health information of HIV-positive donors recruited for the study from the HIV-clinic at Groote Schuur Hospital, Cape Town.

Yield /mg from 1ml Crude lyophilised Saliva				Yield /mg Purified Salivary Mucins from 1ml after Column chromatography and CsCl centrifugation					
HIV-Negative		HIV-Positive		HIV-Negative			HIV-Positive		
Sample No.		Sample No.		Sample No.	MUC5B	MUC7	Sample No.	MUC5B	MUC7
4	10.47	2	25.39	4	0.45		2	0.30	0.44
8	10.20	3	1.44	8	0.20	0.32	3	0.03	0.03
11	12.92	5	1.58	11	0.54	0.03	4	5.35	5.37
15	11.26	6	1.29	12	0.20	0.63	5	0.30	0.40
20	16.07	32	27.89	15	0.09	0.03	6	0.43	0.05
22	6.00	84	39.07	22	0.70	0.50	7	0.56	0.49
23	7.63	86	3.82	23	0.50	0.74	8	0.37	0.30
27	53.14			27	0.18	0.04	9	0.30	0.35
63	1.38			29	0.78	10.21	11	0.11	0.00
69	1.29								
70	11.83								
71	0.72								
89	7.57								
93	15.13								
95	1.06								
99	10.50								
Mean	11.07	Mean	14.36	Mean	0.40	1.56	Mean	0.86	0.82
S.D.	±12.29	S.D.	±15.96	S.D.	±0.25	±3.51	S.D.	±1.69	±1.71

Table 3: Yield of crude material obtained from lyophilisation of 1ml crude saliva compared with the yield of purified salivary mucins from 1ml saliva, from both HIV-negative and HIV-positive donors. S.D. is the standard deviation.

% of Purified Mucin in Crude Salivary Protein					
HIV-Negative			HIV-Positive		
Sample No.	MUC5B	MUC7	Sample No.	MUC5B	MUC7
4	4.05%		2	2.06%	3.07%
8	1.78%	2.85%	3	0.17%	0.17%
11	4.88%	0.29%	4	37.27%	37.41%
12	1.83%	5.70%	5	2.06%	2.80%
15	0.78%	0.30%	6	3.02%	0.38%
22	6.36%	4.56%	7	3.89%	3.38%
23	4.53%	6.65%	8	2.54%	2.06%
27	1.63%	0.34%	9	2.09%	2.44%
29	7.05%	92.21%	11	0.77%	0.00%
Mean	3.66%	14.11%	Mean	5.99%	5.74%
S.D.	±0.02	±0.32	S.D.	±0.12	±0.12

Table 4: Percentage of purified mucin in crude saliva taken from HIV-negative and HIV-positive donors. (This was calculated by comparing mass of the yield of purified mucin in relation with mass of lyophilized crude saliva from 1ml sample). S.D. is the standard deviation.

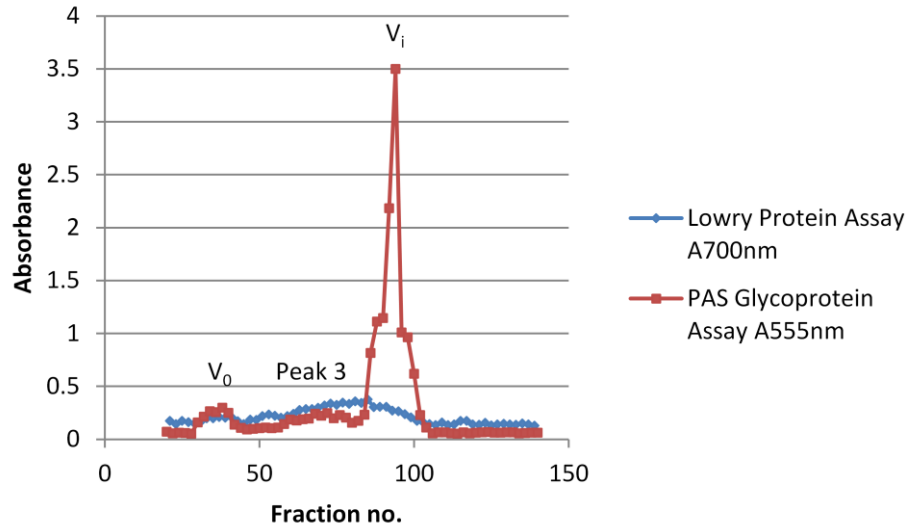


Figure 4: The gel filtration profile of crude saliva from an HIV-negative donor that was chromatographed on a Sepharose CL-4B gel filtration column. The column was eluted at a flow rate of 1ml/min at RT. An aliquot of each fraction was assayed by the PAS and Lowry methods. Fractions from each peak were pooled separately, dialysed against three changes of distilled water overnight at 4°C, and freeze-dried.

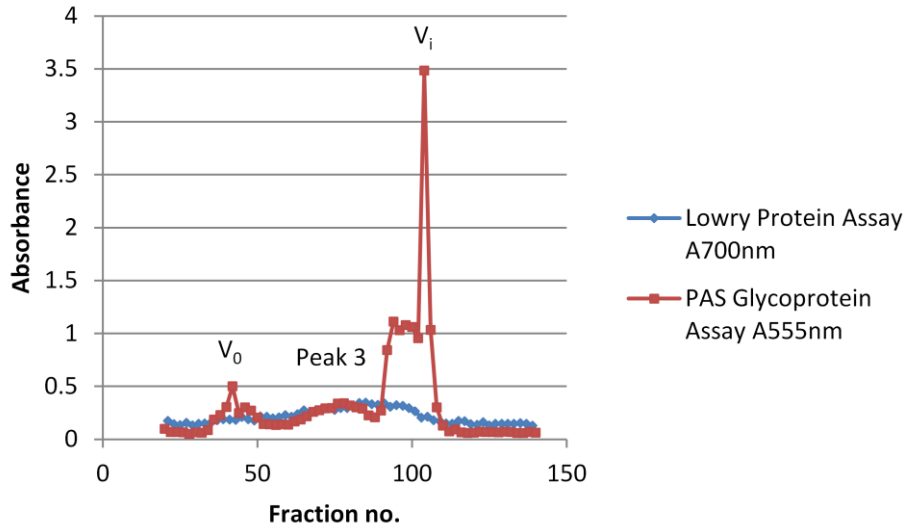


Figure 5: The gel filtration profile of crude saliva from an HIV-positive donor that was chromatographed on a Sepharose CL-4B gel filtration column. The column was eluted at a flow rate of 1ml/min at RT. An aliquot of each fraction was assayed by the PAS and Lowry methods. Fractions from each peak were pooled separately, dialysed against three changes of distilled water overnight at 4°C, and freeze-dried.

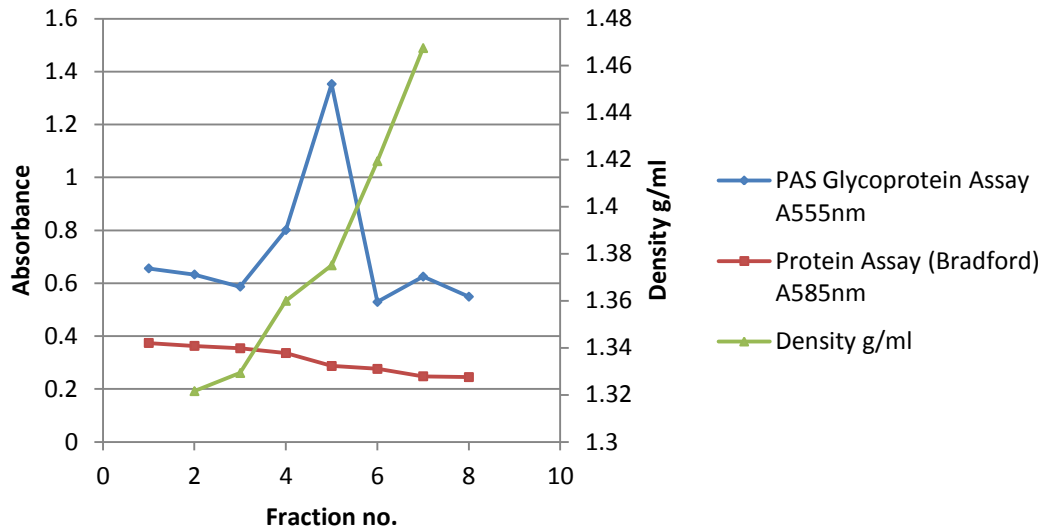


Figure 6: Caesium chloride density-gradient ultracentrifugation purification profile of MUC7 material from an HIV-positive patient. The material containing MUC7 eluted as the V_i peak of the Sepharose CL-4B gel filtration column (Figure 5). Freeze-dried material was dissolved in 4M GuHCl containing protease inhibitors, and adjusted to a density of 1.39 to 1.40g/ml with solid caesium chloride. Density gradient centrifugation was performed in a Beckman L45 ultracentrifuge for 48h at 40 000 rpm, at 4°C. Mucin eluted as a PAS positive peak at a density of 1.38g/ml (1.36 - 1.42g/ml). Fractions 4 and 5 were pooled in this case, dialysed against 3 changes of distilled water overnight at 4°C and freeze-dried.

Agarose gel electrophoresis has been shown to separate mucins according to their charge, an intrinsic variation that characterizes individual mucins more accurately than size variation [95]. SDS-PAGE has been found to be unsuitable to separate mucins because of their large size and the extent of their glycosylation. Because of the extensive glycosylation of the mucins, their mobility in the polyacrylamide gel is affected disproportionately [95]. The large size of MUC5B in particular prevents its entry into the SDS polyacrylamide gels. Therefore reduction of mucins with DTT, followed by agarose gel electrophoresis is the more accurate method of choice for separation of mucins and to allow their identification using Western blotting as confirmed by Thornton *et al.* (1995) [76].

The specific presence of amino acids serine, threonine and proline in mucins allows for further confirmation of their identity and even purity.

4.1 AGAROSE GEL ELECTROPHORESIS AND WESTERN BLOTTING

Western blotting was performed in order to confirm that the identity of the mucins eluting from the V_0 and V_i of the Sepharose CL-4B column were indeed MUC5B and MUC7 respectively and to note any differences of these mucins between the HIV-negative and HIV-positive sample groups. The technique was also necessary to identify the glycoprotein present in the third peak observed (Figures 4 and 5).

Purified MUC5B (V_0) and MUC7 (V_i) were transferred from a 1% agarose gel onto nitrocellulose membranes. The mucins were detected using rabbit anti-MUC5B monoclonal and mouse anti-MUC7 monoclonal primary antibodies. Inter-individual variation was observed in both groups with the detection and amount of MUC5B and MUC7 in each peak (Figures 7 and 8). V_0 was confirmed to be MUC5B in both HIV-negative and HIV-positive samples. Peak 3 had some concentration of MUC5B but less in comparison with V_0 for most samples. The example reflects the observation that there was no difference in the detection of mucin between HIV-negative and HIV-positive samples of saliva except that for some samples MUC5B was distributed over 2 peaks and the detection of each revealed charge differences. Concentrations and charge of mucin varied between samples from both groups.

4.2 AMINO ACID ANALYSIS

Acid hydrolysis of peptide bonds between amino acid residues present in the mucin protein molecules, and 'chemical derivation of the hydrolysate' by HPLC (High Performance Liquid Chromatography) followed by separation, detection and analysis of the resulting derivatised amino acids, allowed for the quantitative estimation of the amino acid composition. 1.0mg of each sample was hydrolysed in the gas phase at 110°C for 24h. The amino acid analysis allowed for identification of the amount of serine, threonine and proline content as a percentage and in relation to the total amino acid content of the purified mucin sample. This was carried out for each of two purified samples of MUC5B from HIV-negative donor saliva where the mean percentages of S, T and P were 35.06% and 36.62%, and one sample of MUC7 where the mean S, T and P was 37.18% (Table 5). One sample of MUC5B and one sample of MUC7 were processed from HIV-positive donor saliva with mean percentages of 26.54% and 29.24% S, T and P for MUC5B and MUC7 respectively (Table 5).

4.3 DISCUSSION

These results showed that there was inter-individual variation in the quantity, size and charge of samples of mucin from both HIV-negative and HIV-positive groups, using gel filtration and agarose gel electrophoresis followed by Western blotting. The amino acid composition analysis revealed differences in the mean percentage amounts of S, T and P, which were lower in mucins purified from the HIV-positive group when compared with those from the HIV-negative group.

From the Western blotting analysis, it was observed that peak 3 had some amount of MUC5B for most samples, but less MUC5B in comparison to that in the V_0 from gel filtration (Figures 7 and 8). The distribution of MUC5B in each of the peaks varied between samples and this variation was not limited to either HIV-negative donor saliva (N) or that from HIV-positive patients (P) in the samples. It was observed that the portion of MUC5B present in V_0 clearly demonstrated a different charge (more positive/less negative), in one HIV-negative sample (N63) and in three of the HIV-positive samples (P50, P80 and P89), when compared to the MUC5B found in Peak 3 that had travelled further during gel electrophoresis. This difference in electrophoretic mobility between peaks may illustrate two different glycoforms of MUC5B: a more positive group found in V_0 and a less positive group in peak 3. Figure 8, lane 2, shows that the second glycoform for MUC5B is of similar charge to the MUC7 found in the second peak (V_i). It was also observed that although the size of the V_0 peak was relatively small in comparison to the V_i peak when using PAS for detection, the antibodies detected a relatively large amount of MUC5B in V_0 compared with the mucins detected in V_i .

We are quite at loss to explain the appearance of this middle peak and its significance as well as the fact that a PAS-staining peak from Sepharose CL-4B gel filtration (that is either the middle peak or V_0 for some samples), is not necessarily detected by the antibody for MUC7 that was employed here. As far as we know, it has not been shown in this manner before. Glycoforms of MUC5B have been shown in sputa and saliva using anion exchange chromatography [8, 89]. At this stage, it is not possible to argue that this extra peak eluting on Sepharose CL-4B gel filtration, in between the V_0 and V_i is a specific characteristic of saliva in patients infected with the HI-virus since it is present in some individuals from the HIV-negative group too. Our investigation as to its nature and significance is ongoing.

V_i was confirmed to contain MUC7 in both HIV-negative and HIV-positive samples with inter-individual variation in concentration between samples (Figures 7 and 8). MUC7 was detected at different concentrations in V_i for most samples. MUC7 was confirmed to occur in high concentrations for peak 3 for some samples (N63, N71, N89, N99, P82 and P80), but some samples had MUC7 distributed evenly between V_i peak 3 (N63, N71, N89, P82). P80 was distinctly dominant for MUC7 in peak 3. Concentrations of mucin thus varied between samples from both groups (Figures 7 and 8).

Mucins are identifiable by the mean percentage S, T and P in relation to the total amino acid content of the purified mucin. The value was given to be between 13 and 20%; however, guanidinium hydrochloride preparation results in these values being slightly increased due to the retention of material during denaturation and extraction [96]. The amino acid analysis showed variation in the percentage S, T and P between groups. From the analysis described in Table 5, the two HIV-negative samples of MUC5B have similar amounts of S, T and P as would be hypothesised. The amount of proline detected in these samples is greater than that for serine and threonine. The means for these samples of S, T and P are 35.06% and 36.62%. When

comparing the 16.03% and 16.52% proline for HIV-negative MUC5B with the 7.57% proline for HIV-positive MUC5B, we can observe that there is less than half of the percentage of proline in the HIV-positive sample.

For MUC7, the mean percentages of serine, threonine and proline for samples from the HIV-negative and positive groups were 37.18% and 29.24% respectively. These values differ in that the proline in the HIV-positive sample (16.14%) is roughly half of that for the HIV-negative sample (30.53%) as was observed for MUC5B. The HIV-positive sample of MUC7 however, has more threonine present (5.45%) than the HIV-negative sample (1.64%).

It must be noted that the amount of proline detected in MUC7 is also roughly twice that detected in MUC5B for both HIV-negative and HIV-positive samples. More threonine is detected in samples of MUC5B than MUC7 from both groups. The amount of serine is similar between samples and groups, however MUC7 has slightly less serine present than MUC5B. Cysteine and methionine were not found in the samples except methionine which was present for only one HIV-positive sample of MUC7 (0.73%). Aspartic acid is also present in this sample in higher amount (12.04%) compared with the HIV-negative sample of MUC7 (8.59%). Aspartic acid was present in HIV-negative samples of MUC5B at 5.57% and 5.96% and only slightly increased in the HIV-positive sample of MUC5B to 7.17%. Histidine was observed in higher amounts for MUC5B than MUC7 whereas glutamic acid was present in greater concentration for MUC7 than MUC5B.

Whilst the percentage content of amino acids S, T and P in the apomucin is indicative of the specific type of mucin, the significance of the differences observed lies in the potential to form

site-specific O-glycosidic bonds with the different functional groups of the amino acids, and suggesting that variation occurs in mucin structure. O-glycosidic bonds occur at S and T. The presence or absence of amino acids other than S and T and their charge effects, as well as the significance of their increased amount, are not within the scope of this project. Future work will involve a larger sample size and thus a more in-depth analysis of the significant differences in amino acid content between groups.

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HIV-status of donor	MUC	Amino acid	Raw amount /nmol	Total /nmol	% Amino acid	Mean % S+T+P
Negative	5B	S	3.517523	43.31286	8.12%	35.06%
		T	4.722697		10.90%	
		P	6.944472		16.03%	
Negative	5B	S	8.085716	97.00636	8.34%	36.62%
		T	11.40906		11.76%	
		P	16.02448		16.52%	
Negative	7	S	4.196789	83.56336	5.02%	37.18%
		T	1.366596		1.64%	
		P	25.50827		30.53%	
Positive	5B	S	8.100612	94.12948	8.61%	26.54%
		T	9.756009		10.36%	
		P	7.122727		7.57%	
Positive	7	S	7.6317	99.63636	7.66%	29.24%
		T	5.426645		5.45%	
		P	16.07715		16.14%	

Table 5: Amounts of Serine (S), Threonine (T) and Proline (P) that were detected in 1.0mg of pure mucin from HIV-negative and HIV-positive donors are tabulated above. Percentage S, T and P was calculated and the mean content calculated between samples. For HIV-negative samples of MUC5B mean percentages of S, T and P were 35.06% and 36.62%. For the HIV-positive sample of MUC5B the mean percentage S, T and P was 26.54%. The mean percentages of S, T and P for MUC7 were 37.18% and 29.24% from HIV-negative and HIV-positive samples respectively.

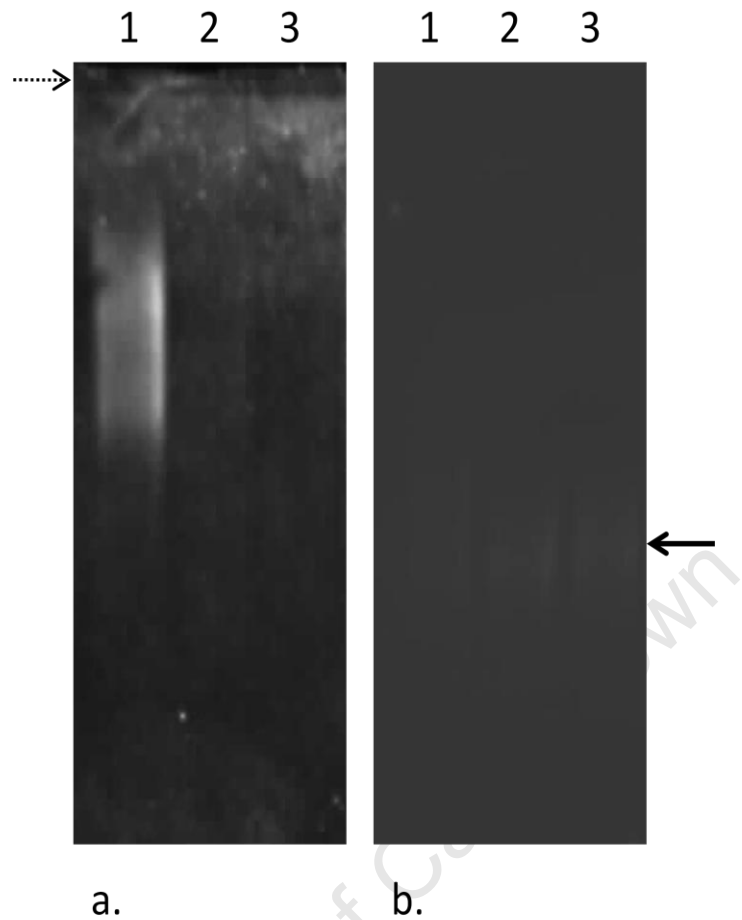


Figure 7: Western blots of samples from V_0 (lane 1), V_i (lane 3), and a third peak (located between the V_0 and V_i peaks) (lane 2), which were detected using the PAS glycoprotein assay, from Sepharose CL-4B size exclusion chromatography of a sample of HIV-negative crude saliva. The membrane was incubated with rabbit anti-MUC5B monoclonal (1:2000) primary antibody (a.) and mouse anti-MUC7 monoclonal (1:1000) primary antibody (b.). The bands were detected using an HRPO-linked secondary antibody and an enzyme-chemiluminescence (ECL), detection kit. V_0 was confirmed to be MUC5B. Peak 3 had some concentration of MUC5B but less in comparison with V_0 for most samples. V_i and peak 3 were confirmed to contain MUC7 (indicated by the arrow) but at varying concentrations between samples. The dashed arrow indicates sample loading.

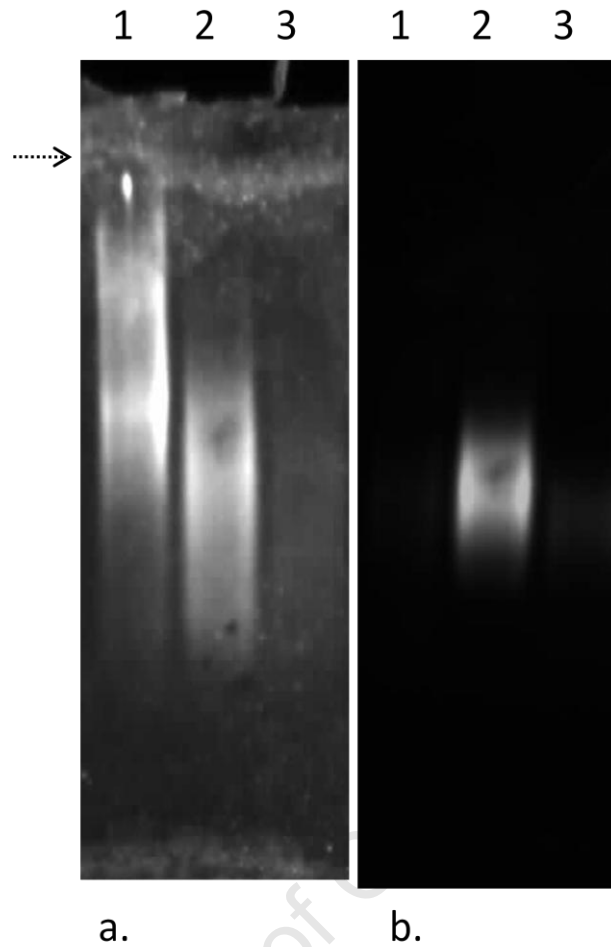


Figure 8: Western blots of samples from V_0 (lane 1), V_i (lane 3), and a third peak (located between the V_0 and V_i peaks) (lane 2), which were detected using the PAS glycoprotein assay, from Sepharose CL-4B size exclusion chromatography of a sample of HIV-positive crude saliva. The membrane was incubated with rabbit anti-MUC5B polyclonal (1:2000) primary antibody (a.) and mouse anti-MUC7 monoclonal (1:1000) primary antibody (b.). The bands were detected with an HRPO-linked secondary antibody and an enzyme-chemiluminescence (ECL), detection kit. V_0 was confirmed to contain MUC5B. V_i was confirmed to contain MUC7 in HIV-positive samples, with inter-individual variation in concentration. Peak 3 (b. lane 2) contained MUC7 at high concentrations as observed in this sample, but also high concentrations of MUC5B. Charge differences were noted between the two detections of MUC5B. The sample travelled further into the gel for peak 3. Concentrations of mucin varied between samples. The dashed arrow indicates sample loading.

We proposed that the mechanism employed by salivary MUC5B and MUC7 in the inhibition of HIV-Type 1 in an *in vitro* assay either involves non-specific interactions whereby mucus physically traps the virus, or a specific interaction between mucin and virus. These interactions could occur between the sugar sequences of viral capsid proteins and those of the mucus glycoproteins. Therefore, a description of glycosylation of both salivary mucins and the comparison of results between individuals was required.

Thornton *et al.* (1995) described the separation of mucins by agarose gel electrophoresis that was largely dependent on their charge density [76]. The negative groups attached to oligosaccharide side chains (for example sialic acid and sulphate groups) contribute to the charge density of mucins. Therefore, samples from both HIV-negative and HIV-positive groups were applied to HPLC in order to investigate the populations of mucin glycoforms, which are thus separated according to their charges. The product was applied to agarose gel electrophoresis based on the elution gradients. The method was according to Thornton *et al.* (1996) and was performed in the laboratory of Dr Dave Thornton and with the assistance of Karine Rousseau at the Department of Life Sciences, University of Manchester, United Kingdom [89].

In addition, a full sugar analysis by LC-ESI MS was done in the laboratory of Dr Niclas Karlsson, University of Gothenburg, Sweden. The methodology was according to Schulz *et al.* (2002) who described the highly sensitive technique, involving liquid chromatography coupled to negative ion electrospray mass spectrometry, employed in the analysis of O-linked oligosaccharides

released from glycoproteins by β -elimination [77]. Tandem mass spectrometry was also performed to yield more specific information about the oligosaccharide structures.

5.1 HPLC TECHNIQUE

Sample mucins were reduced prior to HPLC analysis. Anion-exchange HPLC was performed for mucin subunit fractionation and purification, using a Resource-Q column. The column was eluted first isocratically, then with a linear gradient. Fractions (0.5ml) were analysed for the neutral sugars of the reduced mucin subunits using the PAS assay (Mantle & Allen, 1978) [85], and a slot blotting technique (Figure 9). The positive fractions were then applied to 0.7% agarose gel electrophoresis at 60V for 3h. Proteins were transferred to nitrocellulose by vacuum blotting. Mucins were detected using EU MUC5B (1:100). A goat anti-mouse secondary antibody (whole molecule-linked alkaline phosphatase affinity-isolated antigen-specific antibody) was used for MUC5B. Detection was in alkaline phosphatase buffer with NBT and BCIP substrates. A GS800 calibrated densitometer was used to acquire the image and analyse band intensity for both slot blotting and visualising the MUC5B bands. The analysis of MUC5B was successful whereas MUC7 concentrations were too low to enable detection at suitable levels for comparison.

Figures 10 and 11 illustrate the presence of at least two differentially charged populations of MUC5B that have migrated different distances in the gel electrophoresis and from both an HIV-negative (Figure 10) and HIV-positive (Figure 11) individual.

5.2 LC-MS TECHNIQUE

Liquid chromatography-Mass spectrometry was performed to analyse the O-linked oligosaccharides from the mucin glycoproteins separated by gel electrophoresis.

Oligosaccharide side-chains were separated by reductive alkaline β -elimination of sugar side-chains from the mucin glycoprotein. The desalted oligosaccharide alditols were then analyzed by liquid chromatography coupled to electrospray tandem mass spectrometry (LC/ESI-MS/MS) on a graphitized carbon column or a Hypercarb column. Oligosaccharides were eluted with an H₂O/acetonitrile gradient containing 10mM NH₄HCO₃. Mass spectrometry was performed using an LCQ Deca in negative ion mode, with three scan events: full scan with mass range 320-2000m/z, dependent zoom scan of the most intense ions in each scan, and dependent MS/MS scan after collision-induced fragmentation. This was completed in the laboratory of Dr N. Karlsson (Medicinaregatan, University of Gothenburg, Gothenburg, Sweden) and the MS/MS profiles have been documented successfully.

Oligosaccharide structure and linkage are determined using a combination of analysis of LC/ESI-MS/MS data and the GlycoSuiteDB sugar database (<http://www.glycosuite.com>). This work is currently in progress, and has not yet been wholly completed in the time frame due to the in-depth analysis and specialist attention required.

5.3 DISCUSSION

Figures 10 and 11 illustrate the presence of at least two differentially charged glycoforms in the samples of MUC5B, from HIV-negative and HIV-positive individuals respectively, processed by the HPLC method. Figure 10 (HIV-negative sample of MUC5B) shows fractions 8-30 where a significant portion of a low charged glycoform that has not moved through much of the gel. Fractions 10-14 show a more concentrated portion of this lower-charged glycoform. A higher-charged glycoform is observed in fractions 16-26 as the glycoprotein has travelled further down the gel, and with increasing charge from fractions 16 to 24. In figure 11 (HIV-positive sample of MUC5B), a similar pattern of charge distribution is observed. Fractions 10-24 contained significant amounts of MUC5B. Fractions 10-14 show a lower charged glycoform in higher concentration, given that this glycoform is present in fractions 10-24. The higher charged glycoform is observed significantly in fractions 22-24. (Pale bands were observed in figure 11 due to a lower concentration of protein loading).

The presence of the two differentially charged glycoforms for MUC5B substantiates that observed by Thornton *et al.* (1999) [8]. Further work shall involve specific identification of each glycoform by immunoreactivity with different monoclonal antibodies to glycan epitopes as was done in previous studies [8]. The separation of these glycoforms creates specific glycan groups that may be tested in the pseudoviral neutralisation assay for effectivity against HIV-1.

I was fortunate to have been able to visit these laboratories mentioned above and to have some access to a KISC award as part of the National Research Foundation fund given to my project Supervisor, Professor A.S. Mall. We are in the process of creating an HPLC facility in our

laboratory in order to process a greater sample population and improving up our analysis and interpretation of the results in both cases.

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Figure 9: Slot blot of the fractions for an HIV-negative sample of MUC5B after application to HPLC. Glycoprotein was detected using the PAS assay (although the image is presented in black and white). The arrow indicates where the first fraction was applied to the blot (in this case fraction 3).

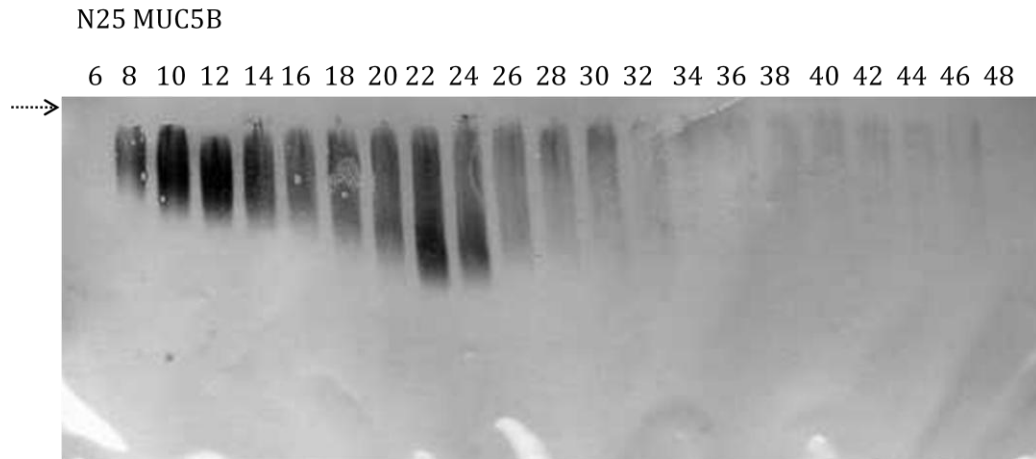


Figure 10: The product fractions (0.5ml) from anion-exchange HPLC of a reduced sample of MUC5B from an HIV-negative donor (N) analysed for the presence of MUC5B. PAS glycoprotein positive fractions (as determined by slot blotting) were applied to 0.7% agarose gel electrophoresis at 60V for 3h. Proteins were transferred to nitrocellulose by vacuum blotting (50mbar, 1.5h) and detected using antibody. Mucins were detected using EU MUC5B (1:100). Detection was in alkaline phosphatase buffer with NBT (50mg/ml in 70% DMF) and BCIP (50mg/ml in 100% DMF) substrates. Fractions 8-30 contained significant amounts of MUC5B. Fractions 8-30 show a significant portion of a low charged glycoform that has not moved through much of the gel. Fractions 10-14 show a more concentrated portion of this lower-charged glycoform. A higher-charged glycoform is observed in fractions 16-26 as the glycoprotein has travelled further down the gel, and with increasing charge from fractions 16 to 24. The dashed arrow indicates sample loading.



Figure 11: The product fractions (0.5ml) from anion-exchange HPLC of a reduced sample of MUC5B from an HIV-positive donor (P) from anion-exchange HPLC of reduced MUC5B (0.5ml) were analysed for the presence of MUC5B. PAS glycoprotein positive fractions (as determined by slot blotting) were applied to 0.7% agarose gel electrophoresis at 60V for 3h. Proteins were transferred to nitrocellulose by vacuum blotting (50mbar, 1.5h) and detected using antibody. Mucins were detected using EU MUC5B (1:100). Detection was in alkaline phosphatase buffer with NBT (50mg/ml in 70% DMF) and BCIP (50mg/ml in 100% DMF) substrates. Fractions 10-24 contained significant amounts of MUC5B. Fractions 10-14 show a lower charged glycoform in higher concentration, given that this glycoform is present in fractions 10-24. The higher charged glycoform is observed significantly in fractions 22-24. The dashed arrow indicates sample loading.

6.1 MTT ASSAY

TZM-bl cells were plated with a density of 10^4 cells in 100ul media per well in a flat bottomed, sterile 96-well plate, and incubated for 24h at 37°C (5% CO₂). After 24h, 100ul sample that had been solubilised in growth media were added to the cells and further incubated for 44h. Media was plated to serve as a blank. The positive control was cells incubated without mucin and this was compared with cells to which mucin had been added. Mucin sample was tested by serial 2-fold titration, and assayed in duplicate. After 44h incubation 20ul MTT solution was added at a concentration of 5mg/ml in PBS and incubated for a further 4h. After this period, 100ul of the well contents was discarded and replaced with 100ul solubilisation agent (10% SDS in 0.01M HCl) and mixed to allow for visualisation of the purple formazan metabolised from yellow MTT in the mitochondria of viable cells. The cells were further incubated overnight, thereafter a reading taken at 595nm using a spectrophotometer.

Data was collected in triplicate. Percentage viability was calculated from the average absorbance from the triplicate minus the average of the blank, divided by the MTT cell control minus the average of the blank. From this it was observed that percentage viability increased when adopting the novel method (plating TZM-bl cells 24h prior to addition of mucin and virus to the wells) rather than the initial procedure (adding cells to the mucin-virus mixture after its incubation period). Using the novel method, average percentage viability greater than 75% was achieved for most samples, when compared with percentage viability using the initial method, which for some samples was less than 50%. Viability using the original method was greatly decreased at higher concentrations of mucin, for example, 26.83% at 1.0mg/ml of purified

MUC5B compared with 54.59% viability at a lower concentration of 7.8×10^{-3} mg/ml (using 2-fold dilutions), suggesting that the mucin had an effect on the cell viability. The results of increased viability with the novel method were observed for both crude lyophilized saliva samples and purified salivary mucin samples.

6.2 HIV-1 NEUTRALISATION ASSAY

Briefly, this assay allowed for detection of the anti-HIV-1 activities of crude saliva and purified salivary mucins MUC5B and MUC7 from HIV-negative and HIV-positive individuals. TZM-bl cells were plated with a density of 10^4 cells in 100ul media per well in a flat bottomed, sterile 96-well plate, and incubated for 24h at 37°C (5% CO₂). Samples of crude and purified mucin isolated from HIV-negative and HIV-positive groups were incubated with HIV-1 pseudovirus for 1h, and then incubated with susceptible TZM-bl cells for a further 48h. The cell control was cells incubated without virus and mucin sample. The virus control was of cells incubated with virus and no mucin sample. Mucin samples solubilised in media were tested by serial 2-fold titration, and assayed in duplicate. After 48h, 100ul of the culture supernatant was discarded and replaced with 100ul luciferase substrate. After 2min incubation in the dark, detection was done in a black plate. Results were determined using a luminometer to measure relative light units (RLU). Viral infection of cells is associated with the initiation of luciferase production and therefore metabolism of the substrate. Prevention of viral infection is indicated by base-line luciferase activity. Percentage neutralisation was calculated from the following formula.

(RLU VC – RLU Sample) X 100

(RLU VC – RLU CC)

VC – virus control

CC – cell control

An IC₅₀ (the concentration of test sample at which 50% neutralisation of the pseudovirus occurred) was determined for each sample. Two subtype C viruses were chosen for the analysis, CAP45 and DU422.1 from KwaZulu Natal (KZN) and Durban, South Africa, respectively, and Q168a.2, a subtype A isolate from Kenya, chosen for its different geographical origin and subtype [97, 98]. Figure 12 illustrates the practical steps involved in making pseudovirus and the neutralisation assay.

Results showed an increase in neutralisation potency with increasing concentration of sample for most samples. A preliminary analysis was performed (Tables 7, 8 and 9) where the number of samples that displayed neutralisation activity i.e. the sample neutralised virus >50% for at least one dilution. This was determined for samples of MUC5B, MUC7 and crude saliva and compared between HIV-negative and HIV-positive donor groups and the viruses with which they were tested – CAP45, DU422.1 and Q168a.2.

From the HIV-negative donor group, 10 samples of crude saliva were tested in the pseudoviral neutralisation assay. Out of the cohort 8 samples neutralised virus and 2 exhibited poor/no neutralisation. Six samples neutralised DU422.1 (subtype C). One sample did not neutralise another subtype C pseudovirus, CAP45. Another sample did not neutralise virus Q168a.2, a subtype A pseudovirus. It was observed that all samples tested with DU422.1 neutralised the virus and 2 out of 3 samples neutralised Q168a.2.

The crude saliva taken from HIV-positive individuals exhibited some neutralisation of the pseudovirus. Four samples were tested here, where 2 samples neutralised pseudovirus DU422.1 and the 2 samples that did not neutralise virus were tested with Q168a.2.

Eleven samples of MUC5B isolated from HIV-negative donors were analysed for their potential to neutralize the pseudovirus. Ten samples neutralised pseudovirus and one sample did not. Of the samples that did neutralise virus, 4 neutralised DU422.1 and 4 neutralised CAP45. Two samples of MUC5B were tested against pseudovirus Q168a.2 and both neutralised the virus. In fact, two of the samples of MUC5B isolated from HIV-negative individuals (Donors 11 and 15) neutralised more than one virus. Sample 15 neutralised both DU422.1 and CAP45. Sample 11 neutralised both CAP45 and Q168a.2. This data highlights the potential for salivary MUC5B to neutralise not only subtype C viruses, but across clade including a subtype A virus.

Sixteen samples of MUC5B taken from HIV-positive donors were tested in the pseudoviral assay. Of the cohort, 12 samples neutralized the virus and 4 samples exhibited no neutralisation. Three samples neutralized DU422.1 and 3 samples showed no neutralisation capability for DU422.1 therefore half of the samples tested against this virus neutralised and half did not. Four samples neutralized CAP45 which was all the samples tested against this virus in the group. Two samples neutralised Q168a.2 but one other sample did not neutralise this virus.

MUC7 isolated from HIV-negative individuals was tested against the pseudovirus. Seven samples were tested where only one sample neutralised the virus and 6 samples did not exhibit neutralisation. For DU422.1 only one out of 5 samples tested neutralised the virus.

MUC7 isolated from HIV-positive patient saliva was applied to the assay. Fifteen samples were tested where 8 samples neutralised the virus and 7 samples did not neutralise the virus. For DU422.1, 4 samples neutralised the virus and 5 samples did not neutralise the virus. For CAP45, all 4 samples neutralised the virus. For Q168a.2 both samples tested did not neutralise the virus.

A representation of the dose-response curve is given in Figure 12 for an HIV-negative sample of MUC5B and Figure 13 for an HIV-positive sample of MUC5B. Figure 14 shows the neutralisation capability of crude saliva from both groups. The geometric means of IC_{50} values were calculated for samples that displayed accurate 95% confidence intervals (CI) as calculated using Graphpad Prism, a statistical programme and are shown in Figure 10. If the values for the IC_{50} /higher CI and the IC_{50} /lower CI were equal (or less than 2) then the data, as processed using the programme, was deemed fit to proceed with calculating the geometric means of the IC_{50} .

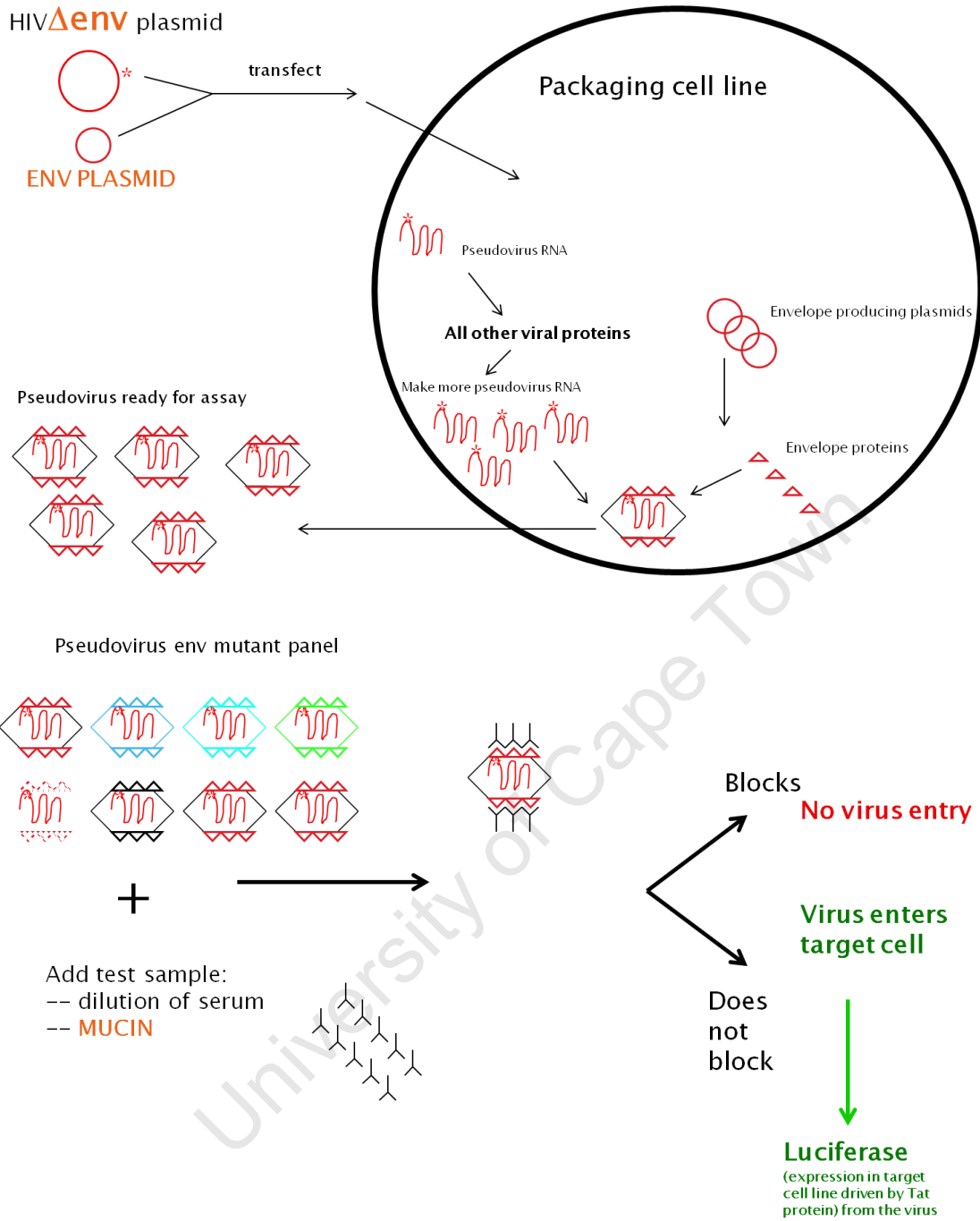


Figure 12: The practical steps involved in making pseudovirus and the neutralisation assay (Dr J. Dorfman).

6.3 DISCUSSION

The MTT assay successfully identified metabolically active cells in the manipulation and refinement of the neutralisation assay. The assay was identified as the appropriate measure for cell viability due to its high throughput and cheaper cost. Whilst the assay is not carried out in the same plate that is measured for luciferase production in the pseudoviral neutralisation assay, (it was deemed impossible to identify a cell viability assay that would not affect or add variables to the results), the MTT assay was carried out under the same conditions and concurrently with the pseudoviral assay using the same samples.

It was observed that cell viability was affected by the concentration of mucin but was deemed sufficient to proceed with the novel method (plating TZM-bl cells 24h prior to addition of mucin and virus to the wells) and because viability was greatly increased when compared with the initial method (adding cells to the wells after incubation of mucin and virus).

The neutralisation capability of crude saliva from both HIV-negative and HIV-positive donors (Table 9) is consistent with the result of the previous study (Peacocke *et al.* manuscript under revision) where inhibition of the virus was seen in both groups. However, the decreased neutralisation potential in the HIV-positive group shows some concordance with the results of Habte *et al.* (2006) who described a lack of inhibition in the group [30]. This highlights the necessity for a larger sample cohort to be tested in order to clarify these differences. Evidence for the inhibition of HIV-1 by the HIV-positive group is that the donor crude saliva may contain antibodies against the virus and in particular IgA. The crude saliva was taken from South African individuals most probably infected with a subtype C virus. Thus it is expected that the complement in their saliva would exhibit neutralisation capability against a pseudovirus of the

same subtype, but less so with a virus from a different subtype and geographical origin [99]. Further experimentation will validate this argument.

This result observed when HIV-negative and HIV-positive salivary MUC5B was tested against the subtype C viruses CAP45 and DU422.1 (Table 7) was expected as it had been observed that salivary MUC5B inhibited a subtype C virus in a live virus assay from our previous work (Peacocke *et al.* manuscript under revision). It appears that the potential for MUC7 to neutralise the virus in both HIV-negative and HIV-positive groups (Table 8) was less than that for MUC5B. Comparable value was added to the data because the viruses are both of subtype C and vary only in the location from which they are derived. Samples tested against the subtype A virus showed potential for salivary mucins to neutralise more than one subtype. However, due to the small sample size tested here significant information could not be extrapolated from this neutralisation data.

A Fisher's exact test and t-test for two sample groups with unequal variances was used to calculate whether any significance could be observed between results, for example between virus and samples. However for all these results $p > 0.05$ and the sample size was deemed too small to yield significance.

HIV-Positive donor	CD4 Count at time of collection	Viral Load at time of collection	Treatment at time of collection	Status at time of collection	Neutralisation of DU422.1	Neutralisation of CAP45	Neutralisation of Q168a.2
2	18.06.10 CD4 246 ALT 42	860; 2.93	ARV 26.03.08; 20.08.08, A, 3, N; 24.03.10 HAART, AZT, 3TC, NVP - Primary regimen	Trial amitriptyline, restart Bactrim	MUC5B did not neutralise DU422.1	MUC5B and MUC7	
3						MUC5B	MUC5B but not MUC7 or crude saliva neutralised Q168a.2
4			Defaulted HAART 11.2007; Restarted 07.07.2008; Vit. B, Starudine, Lamivudine, Nevirapine - Primary regimen		MUC7 and crude saliva neutralised DU422.1 but not MUC5B		
5					MUC7 did not neutralise DU422.1		
6	24.03.10 CD4 92; 07.04.10 CD4 92	24.03.10 110 000 Log 5.04; 07.04.10 110 000	ARV 4.04.03; 25.10.06 AZT, ddi, Kaletra; 07.04.10 on ART - Secondary regimen	Adherence problems, nausea, vomiting, diarrhoea, otherwise well, no complaints, good general condition			The virus was not neutralised by either MUC5B or crude saliva
7	13.01.10 CD4 145 ALT 22, 13.07.10 CD4 308	LDL	ARV 09.06.09; 21.07.10; 13.03.10 d4T, EfV - Primary regimen	Clinically well, viral suppressed	DU422.1 was neutralised by MUC5B but not MUC7		
8			On ART since 2009; 05.2009 - D4T, 3TC, NVP - Primary regimen		DU422.1 was neutralised by MUC5B and crude saliva but not MUC7		
9			Since 2005 - Primary regimen		MUC7 but not MUC5B neutralised DU422.1	CAP45 was neutralised by MUC5B	

11	CD4 178 ALT 40	2.7	ARV 3.12.08; 30.09.09 restarted; 31.03.10 - Primary regimen	No symptoms, good, chest clear		CAP45 was neutralised by MUC5B	Crude saliva did not neutralise the virus
52	No record before 11.06.09 CD4 466; 9.12.09 CD4 613; 10.11.10, CD4 371		Not on ARV	Stable		CAP45 was neutralised by MUC5B and MUC7	
80	7.02.07 CD4 381	27.09.07 - 14.02.07 lower than detectable limit	ARV 02.08.06 d4T, 3TC, NVP; 14.03.07 HAART; 18.07.07 - Primary regimen	14.03.07 sneezing, blocked nose, watering eyes, but getting better today - cold/hayfever, good adherence	Both glycoforms of MUC5B neutralised the virus as well as MUC7	CAP45 was neutralised by MUC5B and MUC7	

Table 6: Relationship between HIV-positive patient data and the properties of the donor crude saliva and its purified mucins in neutralisation of the pseudovirus. It is important to note that this data is qualitative due to the small sample size.

MUC5B				
	HIV status of donor			
Neutralisation of virus	Positive		Negative	
CAP45				
Yes	6	100%	4	100%
No	0	0%	0	0%
Total	6		4	
DU422.1				
Yes	4	57%	4	80%
No	3	43%	1	20%
Total	7		5	
Q168a.2				
Yes	2	67%	2	100%
No	1	33%	0	0%
Total	3		2	
All virus				
Yes	12	75%	10	91%
No	4	25%	1	9%
Total	16		11	

Table 7: Probability of MUC5B samples in neutralising each pseudovirus. CAP45 was neutralised by all HIV-negative and HIV-positive donor samples. DU422.1 and Q168a.2 were neutralised by MUC5B in varying proportions between groups. $p=0.782$ by Fisher's exact test.

MUC7				
	HIV status of donor			
Neutralisation of virus	Positive		Negative	
CAP45				
Yes	4	100%	0	0%
No	0	0%	0	0%
Total	4		0	
DU422.1				
Yes	4	44%	1	20%
No	5	56%	4	80%
Total	9		5	
Q168a.2				
Yes	0	0%	0	0%
No	2	100%	2	100%
Total	2		2	
All virus				
Yes	8	62%	1	14%
No	5	38%	6	86%
Total	13		7	

Table 8: Probability of MUC7 samples in neutralising the pseudovirus. Four samples of MUC7 neutralised CAP45. A more informative comparison could only be drawn from the neutralisation of DU422.1 where most samples did not neutralise the virus. The samples tested with Q168a.2 did not neutralise the virus. $p=0.371$ by Fisher's exact test.

Crude saliva				
Neutralisation of virus	HIV status of donor			
	Positive		Negative	
CAP45				
Yes	0	0%	1	100%
No	0	0%	0	0%
Total	0		1	
DU422.1				
Yes	2	100%	6	100%
No	0	0%	0	0%
Total	2		6	
Q168a.2				
Yes	0	0%	2	67%
No	2	100%	1	33%
Total	2		3	
All virus				
Yes	2	50%	9	90%
No	2	50%	1	10%
Total	4		10	

Table 9: Neutralisation capability of crude saliva. It was observed in particular for the HIV-negative group and especially due to the greater sample size of this group. Ninety percent of samples neutralised the pseudovirus. $p=0.141$ by Fisher's exact test.

HIV-negative Donor Saliva			
Mucin	MUC5B	MUC7	Crude Saliva
Virus	DU422.1		
Mean IC₅₀ ug/ml	15.4	26.78	80.35
HIV-positive Donor Saliva			
Mucin	MUC5B	MUC7	Crude Saliva
Virus	DU422.1		
Mean IC₅₀ ug/ml	14.55	N.D.	92.25

Table 10: Data for the geometric mean IC₅₀ for each mucin sample and crude saliva tested against pseudovirus DU422.1. The mean IC₅₀ for crude saliva for both groups was much greater than that of the purified mucins as would be expected given the increased concentration of the mucin relative to that present in crude saliva and tested in the assay. For the HIV-negative sample, the mean IC₅₀ for MUC7 was greater than that for MUC5B.

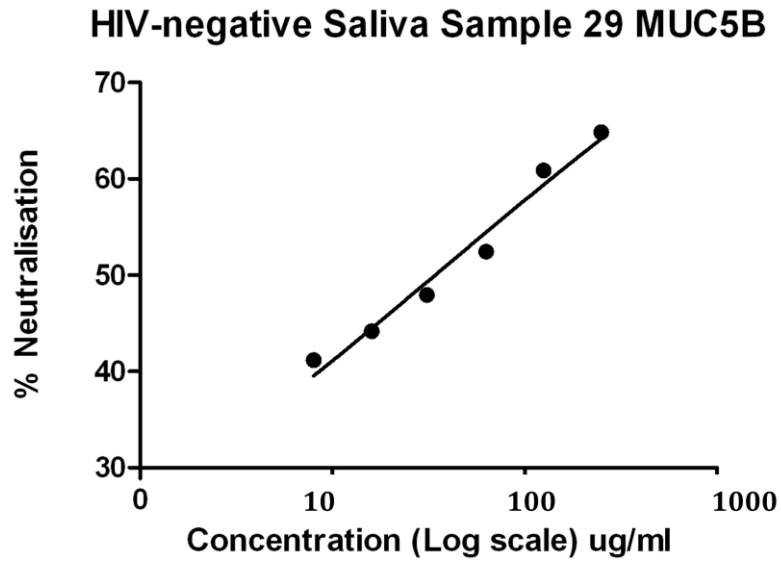


Figure 13: Graph of percentage neutralisation with increasing concentration (Log scale) of an HIV-negative sample of purified MUC5B. 50% neutralisation was observed at a concentration of 34.15ug/ml (IC_{50}). The 95% Confidence Intervals for the IC_{50} were 25.97 to 44.90ug/ml. The virus used was a subtype C pseudovirus, DU422.1.

HIV-positive Saliva Sample 80 from Peak 2

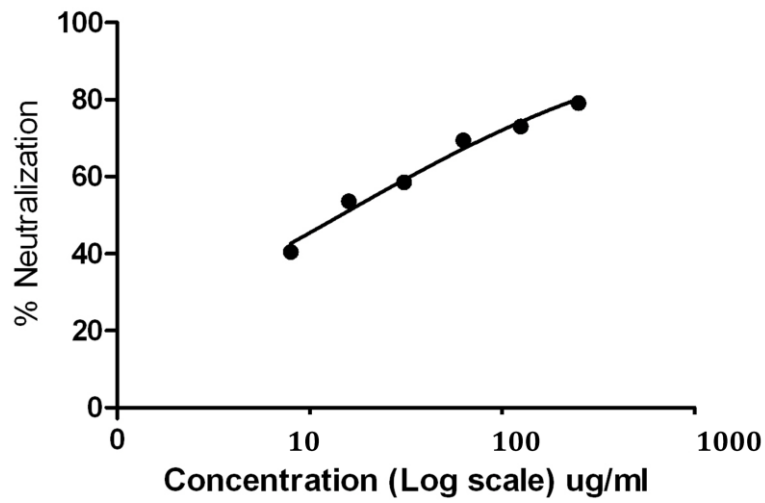


Figure 14: Graph of percentage neutralisation with increasing concentration (Log scale) of an HIV-positive sample of purified MUC5B (purified from peak 3 for this sample after column chromatography). 50% neutralisation was observed at a concentration of 14.55ug/ml (IC_{50}). The 95% Confidence Intervals for the IC_{50} were 10.99 to 19.25ug/ml. The virus used was a subtype C pseudovirus, DU422.1.

Neutralisation assay results with crude saliva

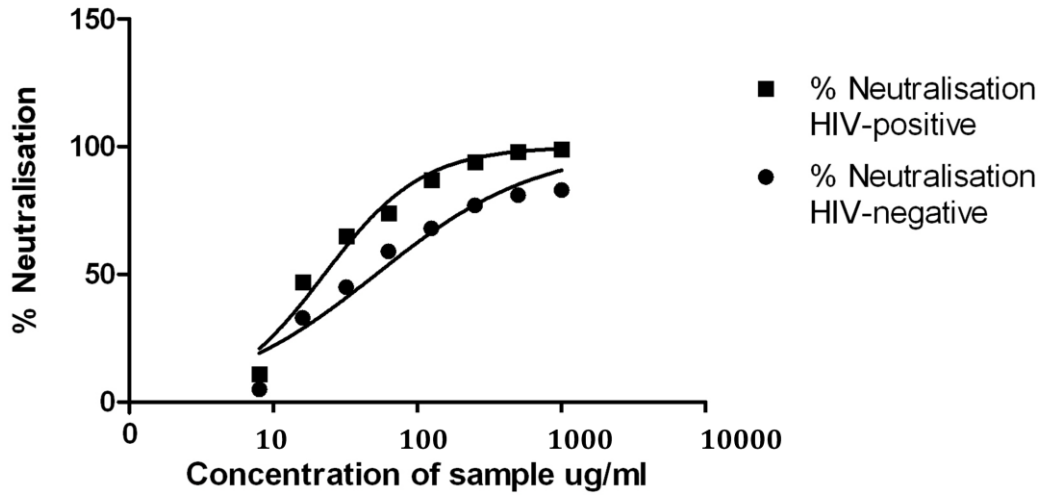


Figure 15: Graph of percentage neutralisation with increasing concentration (Log scale) of a HIV-negative and HIV-positive crude saliva samples. 50% neutralisation was observed at a concentration of 51.44ug/ml and 22.54ug/ml (IC_{50}) for the HIV-negative and HIV-positive samples respectively. The virus used was a subtype C pseudovirus, CAP45.

This work has detailed the development in describing the role of crude saliva and its purified mucins MUC5B and MUC7 in the inhibition of HIV-1. Saliva samples were purified and analysed using previously described methodology [30, 78]. The inhibition of HIV-1 by mucus and mucins required statistical verification and the establishment of a dose-response curve and therefore the development of a suitable platform – a pseudovirus neutralisation assay. The assay enabled high-throughput and reproducibility, and has been engineered to allow for accurate, reproducible results. Using the Graphpad Prism data analysis programme, the IC_{50} and dose-response nature of the inhibition has been described. The hypothesis was that there is a specific interaction between salivary mucins MUC5B and MUC7 and the HIV-1 virus.

This project has shown that salivary MUC5B neutralises HIV-1 pseudoviruses CAP45 (KZN) and DU422.1 (Durban) of subtype C, and Q168a.2 (Kenya) of subtype A, when purified from HIV-negative and HIV-positive individuals. The neutralisation capability of MUC5B (IC_{50} 15.40ug/ml) appears greater than MUC7 (IC_{50} 26.78ug/ml) for the HIV-negative group. Due to the limited information gathered from the HIV-positive donors, it was impossible to draw any statistical relevance from their background, treatment status, and general health at the time of collection and comparison of these with the concentration and inhibitory action of their salivary mucins. Further work will involve a larger sample size, a greater panel of pseudoviruses and a comparison with some of the significant proteins in saliva (sialoglycoprotein, lactoferrin and lysozyme) and a non-salivary glycoprotein such as α -1-acid glycoprotein to be able to include a suitable control. Studies amenable to identifying a peptide or short polypeptide with neutralisation activity, which could be potentially manufactured into a microbicide, are in progress.

Sepharose CL-4B chromatography has proven to be a highly successful method of separating differentially sized mucins, MUC5B and MUC7, as well as purifying the mucins from some protein contaminants. The chosen bed volume may play a role in the elution of fractions. Smaller size differences can be observed as shown in the data (Figures 4 and 5), when compared with previous results using a smaller bed volume of 50ml where only two definitive peaks were eluted. The identity of the mucin in the void (V_0) and included volume (V_i) peaks had been previously determined as MUC5B and MUC7 respectively [8]. This was confirmed using Western blotting (Figures 7 and 8). Samples showed inter-individual variation in both the amounts of glycoprotein material eluting under the V_0 and V_i peaks and the shape of the peaks, for both groups. This is expected as there are great interindividual differences in salivary flow rates as reviewed by Humphrey and Williamson [100]. In order to minimize differences due to flow rate, secretions were collected at similar times of day between 11.00 am and 1.00 pm and a large volume of at least 20ml whole saliva was collected in each case. For some samples there was hardly any MUC5B detectable but all showed the presence of MUC7 and this was consistent with earlier studies in our laboratory (Peacocke *et al.* manuscript under revision). However, a third peak was observed for some samples, which to our knowledge has not previously been described. Western blotting confirmed the peak to contain either MUC5B for some samples. For an HIV-positive sample P80 in particular, this revealed a size similarity between MUC5B and MUC7 as both mucins eluted as this peak in higher concentration than V_0 and V_i respectively. These could be altered glycoforms of each that elute either slightly later than the predominant MUC5B peak due to smaller molecular size due to some degradation or interference with intramolecular organisation. MUC7, as a quaternary folded protein, could have greater portions of intramolecular and intermolecular interactions, which explains its larger size, causing it to elute earlier than in the usual second included volume peak. It has been previously shown that the process of collection in guanidinium hydrochloride, solubilisation, and purification can alter the intermolecular properties of mucins [96]. With greater elution capability it is clear that

there are many factors that influence the integrity and presence of structural properties of mucins.

Caesium chloride density-gradient ultracentrifugation was successful in the separation of mucin sample from contaminant protein and nucleic acids. Where protein contamination was large, the method was repeated. However, the yield of purified mucin after this method was low.

The weight of lyophilised purified mucin glycoprotein was compared with the weight of crude saliva after the same process. It was necessary to review this comparison given the difficulty in measuring and quantifying total protein by the Bradford assay, and mucin by the PAS assay. Challenges are faced when comparing the concentration of these secretions between donors as this has been found to vary considerably and is influenced by numerous variables. The relative contribution of mucins to the amount of total protein in crude saliva was able to be quantitated by comparison of the dry weights yielded from specific volume. Much interindividual variation was noted here as was observed in the results of size-exclusion chromatography (Figures 4 and 5). This analysis was necessary when collating the data from HIV-neutralisation assays as the innate concentration of mucin in crude saliva could be compared with the concentrations of crude and purified mucus used, and their relative inhibition in the assay. Future analysis of the contribution of the concentration of purified mucin in crude saliva will be done using an ELISA. This method, however, is not without limitation as quantitative comparisons are difficult when using specific mucin antibodies due to the variable glycosylation of the mucins and thus antibody affinities.

Salivary mucins afford a universal protection against HIV infection, irrespective of the sub-type of the virus. This project has allowed for such observations in the inhibitory activity of salivary

MUC5B. The interaction between virus and mucin could well be a non-specific interaction of cumulative charge that lends an aggregatory activity to the mucin, inhibiting the virus from binding to susceptible cells. It has been postulated that the role of mucin in protection lies in physically trapping the virus.

However, we hypothesise that a specific interaction between mucin and virus is possible. This interaction could occur between the sugar sequences of viral capsid proteins and those of the mucus glycoproteins. It has been proposed that altered patterns of glycosylation of mucins with a consequent variation of charge would influence the viral binding properties of the mucin [30, 101]. Altered properties of mucins due to aberrant O-glycosylation has been observed using SDS-PAGE and in particular for MUC7 purified from the saliva of HIV-positive donors (Peacocke *et al.* manuscript under revision). From this we postulate altered glycosylation patterns in the mucin structure of those isolated from HIV-positive individuals. If this glycosylation can be described it may provide an explanation for the variable results achieved by Habte *et al.* (2006) and this work. Habte *et al.* pooled only a small group of HIV-positive samples, therefore the assumption that they did not inhibit virus could indeed be skewed. A greater sample size analysed by the methods employed in this project could go a long way to resolve some of these uncertainties. Investigating the mechanism by which HIV-1 infects its target cells and drawing similarities between the structure of the HIV-1 capsid and mucin glycoproteins may allow for the development of further hypotheses into the role of salivary mucins and their inhibition of the virus.

The infection status of HIV-positive individuals directly relates to the viral load in the blood of that infected person. The influence of CD4 count may have an effect on mucosal immunity although Habte *et al.* (2010) determined no difference in the inhibitory action, against HIV-1, of

mucins MUC5B and MUC7 collected from patients with different CD4 counts [29]. Whether mucin structure and particularly glycosylation, is altered by HIV-infection status is unknown. Previous work in our laboratory has shown that HIV-infection does not influence a genetic difference in MUC gene structure, and thus protein sequence and possible affected structural changes (Peacocke *et al.* manuscript under revision). Therefore sugar analysis of mucous glycoproteins may rather yield information of the interaction between mucins and the HIV-1 virus as well as yield reasons for the differences seen in the inhibition of the HIV-1 virus by mucins isolated from HIV-positive individuals compared with HIV-negative individuals. More evidence may be found for the relationship between viral load, in patient blood, and transmission and if this exists in oral transmission.

To further investigate glycosylation patterns of mucin glycoproteins and the effects of such variation, treatment of samples with reducing agents such as β -mercaptoethanol/DTT, and enzymes to form peptidic digests can be done and the resultant peptides applied in the HIV-neutralisation assay. This will allow for the investigation of the possible mechanisms of inhibition by the mucin glycoproteins. LC-MS has been employed to analyse the glycosylation patterns between the HIV-negative and HIV-positive donor purified mucins. Our aim is to fully characterise the sugar side-chains of salivary MUC5B and MUC7 from HIV-negative and HIV-positive donors. The work done in this project in identifying altered glycoforms by HPLC, and our continuing work describing glycan structures in salivary mucins using LC-MS, will enable the identification of specific glycan groups that may have affectivity against HIV-1 and their possible application in the pseudoviral neutralisation assay. We are in communication with Dr Karlsson whose valuable input will help us interpret the data we obtained during a visit to his laboratory.

The results in this study are intriguing in that they differ from a previous study in our laboratory by Habte *et al.* (2006) [30] firstly where 'normal' saliva and its purified mucins inhibited the virus whilst HIV-positive salivary mucins did not inhibit the virus, with the added advantage of comparing a well-defined group of HIV-negative and HIV-positive samples, and by showing that inhibition of the virus occurred in both groups with crude saliva. Whilst such a study requires more development in terms of numbers and size and a more in-depth investigation of the specific role of saliva in the inhibition of the virus, the implications of our study need to be considered.

Secreted mucins are known for their gel-forming properties and contribution to forming unstirred crude gel layers on epithelial surfaces to which they afford protection in the most hostile of environments [2]. Considering that mucins inhibit the HI virus in an *in vitro* assay not only in saliva but breast milk and cervical secretions too, we ask whether mucins could be used as a basis for a formula that could be used as a vaginal and anal microbicide to prevent transmission of the virus through sexual intercourse [78]. By investigating the particular interactions between mucin and virus, we hope to harness the inhibitory mechanism as a strategy for preventing the transmission of HIV-1. We aim to pursue studies amenable to identifying a peptide or short polypeptide with activity that could be manufactured into a microbicide.

Future work in our laboratory will be guided by such considerations as outlined above, whilst we continue to investigate in greater depth the characteristics of mucus and mucins from the saliva, breast-milk and gastrointestinal and cervical tracts [78].

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APPENDIX

SAMPLE COLLECTION

PATIENT INFORMATION SHEET

University of Cape Town

Department of Surgery

Information Sheet

16/02/10

Dear patient

We are doing a study on the 'sticky' mucus portion of your saliva. We are interested in whether the mucous glycoproteins in your saliva act against the virus that causes HIV-AIDS. We also wish to find out whether some people get HIV infection more easily than others by examining some of your mucus genes.

You will be expected to spit into a jar after chewing a piece of plastic parafilm. You will be asked to rinse your mouth prior to the procedure, to prevent food contamination of the saliva. A buccal swab will be taken to get some DNA from your inner cheek. It requires a little 'tickle' on the inside of the cheek and is not painful.

Please note the following:

1. We will need to take your folder number (NOT necessarily your name) from which we will get details of your age, gender and origin. All the information will be kept absolutely confidential and we promise not to use your sample for any other purpose but that which we have described.
2. You have a right to say that you do not wish to take part in this study and be rest assured that it will not affect the treatment you receive in hospital.
3. If you agree to participate in this research project now and then decide at a later time that you do not wish to continue, that is also fine. That decision will once again not affect your right to treatment and care. We will NOT test for HIV/AIDS.

4. It is possible that we will publish the findings of this study but your name will not be mentioned.

If there are any queries, please refer them to your clinician. If you think of any queries at a later date, please do not hesitate to contact me at the telephone number below.

Professor A. Mall

Tel: 021 406 6232/6227

I have read and understand the above document.

Name:

Signature:

University of Cape Town

PATIENT/DONOR CONSENT FORM

REQUEST FOR SALIVA SAMPLE AND BUCCAL SWAB FOR DNA

2010/2011

Research Laboratory

Division of General Surgery

OMB Groote Schuur Hospital

UCT Medical School, Observatory 7925

Tel: (021) 406 6168/6227 Fax: (021) 448 6461

Please fill in all the information requested:

Surname:

First Name(s):

Sex: M F

Date of Birth Year: _____ Month: _____ Day: _____

Ethnic Origin: _____

Contact Address: Hospital/Clinic where samples are taken

Town: _____ Fax: _____

Tel: _____

Reason for referral (clinical diagnosis):

Additional disorders (apparent or previously treated):

.....

Smoker / Non-smoker

Folder no.:

For Laboratory use only:

DNA number

Date Received: YY ____ DD ____ Computer Index No.: ____

CONSENT FOR DNA ANALYSIS AND STORAGE

- The Human Research Ethics Committee is contactable at 021-4066338
- Please note that you are **entitled to withdraw** from the study at any time that you so wish to. This will in no way jeopardize your care as a patient
- You may not wish your samples to be re-used for analysis. If so, please sign in the space provided below

I hereby declare that I do not wish my saliva and DNA to be stored for re-use in the future for analysis

Signature.....

Date:

1. I, _____ give permission that saliva and a buccal smear be taken for research purposes in the investigation of mucus (slime) in HIV-AIDS.

2. I give permission that a portion of the sample be stored indefinitely for:
 - a) possible re-analysis;
 - b) research purposes, subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research will remain confidential.

3. I authorize / do not authorize my doctor(s) (DELETE WHERE NOT APPLICABLE) to provide relevant clinical details to the researchers.

ALL OF THE ABOVE HAS BEEN FULLY EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED

Please note that your HIV status is **not** recorded on this form.