

**THE ASSESSMENT OF HUMORAL
IMMUNITY IN THE VAGINAL
MUCOSA OF PREGNANT AND NON-
PREGNANT WOMEN.**

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ABSTRACT

Mucosal surfaces are prominent in the gastrointestinal, urogenital, and respiratory tracts and provide portals of entry for pathogens. The mucosal immune system consists of molecules, cells, and organised lymphoid structures intended to provide immunity to pathogens that impinge upon mucosal surfaces. The aim of this study was to assess humoral immunity in the vaginal mucosa and compare this immune response to a systemic response.

The use of commercially available tampons provided a self-administered, pain free method for the collection of vaginal secretions. To standardise specimens, a total protein determination was performed on vaginal secretions and on sera. All subjects were screened for sexually transmitted infections (STIs) using conventional and deoxyribonucleic acid (DNA) amplification tests. Immunoglobulin levels in vaginal secretions and in sera were quantitated using a quantitative sandwich enzyme-linked immunosorbent assay (ELISA).

The immunoglobulin levels quantitated were analysed on the basis of pregnancy status and the presence or absence of an STI. Immunoglobulin results for serum showed a significant increase in IgG and IgA in women with an STI regardless of pregnancy ($p < 0.001$). This study showed a decrease in vaginal IgG and IgA in women with an STI. Non-pregnant women with an STI had significantly lower levels of IgG and IgA in the cervico-vaginal secretions as compared to the controls ($p=0.002$ and $p=0.0002$ respectively). This was also observed in pregnant women ($p=0.03$ and $p < 0.001$ respectively). IgM levels were mostly too low to be detectable but showed a tendency to increase in vaginal secretions of women with an STI. Pregnancy did not have an effect on immunoglobulin levels except for IgA.

The effects observed were due to the presence of an STI. All the STI pathogens studied displayed a similar effect on immunoglobulin levels. Bacterial vaginosis, however, appears to exert an effect specifically on lowering IgG ($p=0.008$) in vaginal fluid and increasing IgG levels ($p=0.008$) in serum.

Once a more complete understanding of the mechanisms associated with the host defence of the vaginal mucosa is obtained, specific immunotherapeutic strategies can be developed. A greater knowledge of host defence factors specific to the vagina will provide insights into understanding susceptibility to opportunistic infections and STIs.

PREFACE

This study represents the original work by the author. Where the work of others has been used, it has been duly acknowledged in the text. The research described in this thesis was carried out in the Department of Medical Microbiology, Faculty of Medicine, at the University of Natal, under the supervision of Professor A. W. Sturm. This study was given ethical approval before commencement.



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LIST OF ABBREVIATIONS

IgG	immunoglobulin G
IgA	immunoglobulin A
IgM	immunoglobulin M
IgE	immunoglobulin E
ELISA	enzyme- linked- immunosorbent assay
STD	sexually transmitted disease
Ab	antibody
SIgA	secretory immunoglobulin A
M cells	microfold cells
MALT	mucousa- associated- lymphoid tissue
GALT	gut- associated- lymphoid tissue
APC	antigen presenting cell
FAE	follicle- associated epithelium
SC	secretory component
MHC	major histocompatibility complex
NALT	nasal- associated lymphoid tissue
HIV	human immunodeficiency virus
OMVs	outer membrane vesicles
µm	microns
nm	nanometre
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
ATP	adenosine trophosphate

BV	bacterial vaginosis
PBS	phosphate buffered saline
rpm	revs per minute
xg	gravitational force
µl	microlitre
mins	minutes
Ag	antigen
P/N	positive/ negative
µg/ml	microgram per millilitre
NaOH	sodium hydroxide
3M	three molar
°C	celcius
mg/ml	milligram per millilitre
γ- globulin	gamma globulin
STIs	sexually transmitted infections
Fig.	figure
Da	Dalton
OMP	outer membrane protein
LPS	lipopolysaccharide
pH	hydrogen- ion concentration
bp	base pair
pNNP	para- nitrophenyl phosphate
AP	alkaline phosphatase
Pos	positive
Neg	negative

OD	optical density
e.g.	example
ANC	antenatal clinic

CHAPTER ONE

1.0 INTRODUCTION

It has long been known that the presence of antibody (Ab) in local secretions correlates better with protection against pathogenic organisms than serum Ab. Tomasi and Hanson discovered in 1968 that the major Ab in external secretions was immunoglobulin A (IgA) in a unique secretory configuration (secretory IgA; sIgA). With this came the belief that sIgA was responsible for immune protection of mucosal surfaces (Ernst *et al.*, 1987). While it is still true that IgA remains the best marker of local mucosal responses, other humoral and cellular mechanisms must also be considered in the assessment of mucosal resistance. Very little is known about the immunological mechanisms functioning at the vaginal mucosa, and much of what is known comes from animal studies rather than human data (Fidel and Sobel, 1996).

Establishing precise correlates for mucosal protection against genital tract pathogens by the humoral immune system requires quantitative information about specific antibody concentrations in genital tract secretions. The most convenient and widely used method for collection of such secretions from the female genital tract is cervical-vaginal lavage (Bélec *et al.*, 1995). However, this sampling technique results in a considerable and non-standardised dilution of the secretions and this may render antibody concentrations too low and non-comparable to be detected by available assays. It should also be noted that the amount of secretion varies between women and over time which would cause the concentration of the immunoglobulins to vary due to changing dilution factors.

Furthermore, lavage samples cannot provide quantitative information about the concentrations of antibodies or immunoglobulin isotypes present on mucosal surfaces at specific locations in the genital tract (Quesnel *e. al.*, 1997). A study aimed at collecting secretions from specific sites in the vagina was carried out by Quesnel *et al.* (1997) using Sno-strips (Akorn Inc., Abita Springs LA). The method utilizes absorbent filter strips that are placed at a specific site to collect secretions, however, the Sno-strips are pliable and difficult to handle, while mucus adheres without being absorbed.

Therefore, we applied a method of specimen collection using commercially available tampons that can be self-administered and are easy to use. With this method the amount of material collected cannot be standardized and therefore the dilution factor varies. The introduction of the immunoglobulin/ total protein ratio overcomes this problem.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Mucosa-associated lymphoid tissue (MALT)

Mucous membranes have a specialized immune system which is evident from the extensive studies on the gastrointestinal-associated lymphoid tissue (GALT) (Ernst *et al.*, 1987). All mucosal surfaces are protected by similar systems termed “mucosa-associated lymphoid tissue” (MALT). MALT arose from the realisation that not only did mucosal surfaces share organisational similarities in their lymphoid elements, but also functional ones (Bienenstock *et al.*, 1978). MALT is characterised by the predominance of local IgA production and by the finding that activated lymphocytes derived from one mucosal surface can recirculate and localise selectively to other mucosal surfaces.

2.2 Vaginal Mucosa

The wall of the vagina consists of three layers: the mucosa, the muscular coat and the adventitial connective tissue. The mucosa consists of a surface epithelium and an underlying lamina propria. The vaginal epithelium is made up of stratified squamous epithelium (Fig 1a) 150 to 200 μm in thickness, consisting of about 45 layers of cells during the follicular phase of the cycle and about 30 in the luteal phase (Bloom and Fawcett, 1986). The cytoplasm in the superficial cells is filled with glycogen, especially at midcycle. In pregnancy, the vaginal mucosa becomes thicker, the vaginal muscle

hypertrophies and there is an alteration in the composition of the surrounding connective tissue (Llewellyn- Jones, 1999).

Superficial cells are continually shed from the surface of the vaginal epithelium throughout the cycle, but this desquamation is greater late in the luteal phase and during menstruation. During pregnancy there is an increased desquamation of the superficial vaginal mucosal cells with increased vaginal discharge. These changes are initiated by oestrogen and occur early in pregnancy (Llewellyn-Jones, 1999).

The amount of glycogen in the epithelium is controlled by oestrogen, the pH of the vaginal fluid is lowest at midcycle (Bloom and Fawcett, 1986, Kerr, 1999). Glycogen from the exfoliated cells is a rich substrate for Lactobacilli, which break it down to lactic acid, lowering the pH of the vagina (Mims *et al.*, 1999). During pregnancy there is an increase in oestrogen production, which reduces the vaginal pH substantially.

The vagina is devoid of glands and goblet cells and much of the lubricating fluid is contributed by secretions from mucin secreting columnar epithelial cells of the cervix (Coombs *et al.*, 2003). It is generally agreed, however, that there is true vaginal fluid that increases in abundance during sexual stimulation. Therefore fluid collected from the vagina is best referred to as cervico-vaginal secretions. The intercellular spaces of the epithelium are accessible to mononuclear leukocytes. Lymphocytes normally breach the basal lamina, open the desmosomes and invade the enlarged intercellular spaces (Bloom and Fawcett, 1986). Accumulation of lymphocytes is numerous, and sometimes lymph nodules are present (Bloom and Fawcett, 1986). A well developed system of lymph vessels is present in the wall of the vagina (Bailey, 1978). It has been shown that the vaginal

epithelium includes a population of Langerhans cells, which appears as scattered clear cells occupying expanded intercellular channels in its basal and intermediate layers.

The lamina propria is a moderately dense connective tissue (Fig 1b). Immediately under the epithelium there is a dense network of fine elastic fibres. From there, fine fibres run downward to the muscular layer. The deeper layers of the lamina propria contain a dense plexus of small veins (Fig 1i).

The muscular coat is made up predominantly of longitudinal (Fig 1f) and obliquely arranged bundles of muscle fibres (Fig 1h) and the interstitial connective tissue is rich in elastic fibers (Bailey, 1982).

The adventitial coat (Fig 1g) is a thin layer of dense connective tissue, which merges into the loose connective tissue joining the vagina to the surrounding structures. In this connective tissue, an extensive venous plexus, nerve bundles and small groups of nerve cells can be found (Bloom and Fawcett, 1986). Vaginal tissues receive blood supply from uterine arteries and sometimes branches of the internal iliac artery. Blood returns to the venous system through veins that empty into the internal iliac veins. Lymphatic drainage is via the external and internal iliac lymph nodes and superficial inguinal lymph nodes (Bloom and Fawcett, 1986).

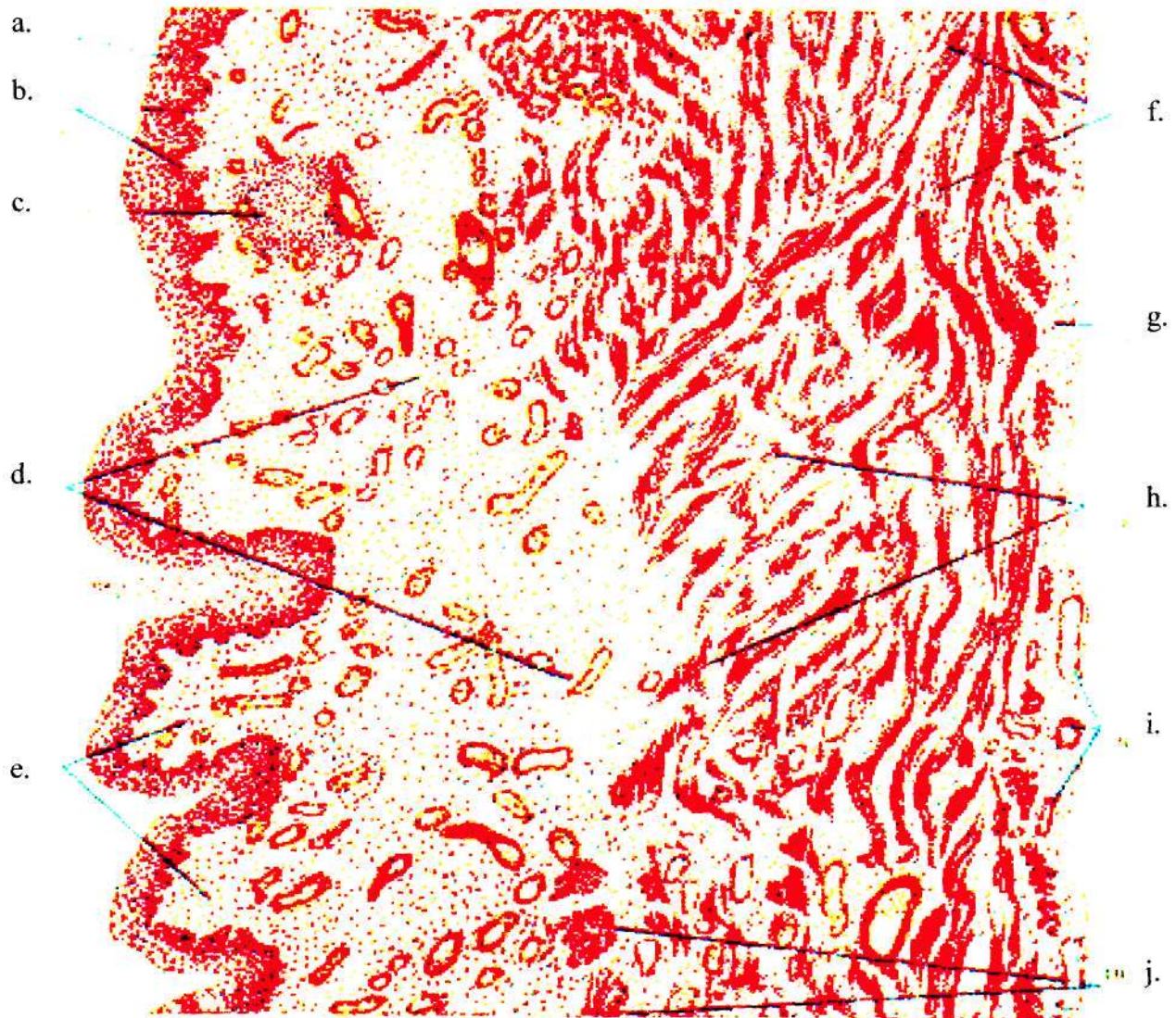


Figure 1. Haemotoxylin- eosin stain (30 X) of the human vagina, longitudinal Section (Di Fiore, 1974).

- a- stratified squamous epithelium
- b- papillae in the superficial layer of the lamina propria
- c- lymphocyte nodule
- d- lamina propria
- e- folds of the mucosa
- f- longitudinal bundles of smooth muscle fibers
- g- adventitia
- h- oblique bundles of smooth muscle fibers
- i- veins and artery
- j- transverse bundles of smooth muscle fibers

2.3 Host Defense

2.3.1 Non-specific Mechanisms

2.3.1.1 Mucous Membranes

Mucous membranes are the surfaces that line the gastrointestinal, the respiratory, and the urogenital tract. Mucous membranes comprise one or more layers of living cells and are constantly moist as a result of the secretion and absorption of chemical substances (Salyers and Whitt, 1994). This provides a warm wet environment, which is readily colonized by normal vaginal flora. Colonisation is allowed when this involves useful, non-pathogenic organisms that do not interfere with the primary function of the mucosal membrane. Protection against colonisation with harmful, pathogenic organisms is provided by a number of defences.

Goblet cells (which produce mucus), M (microfold) cells (which aid in the presentation of antigens to underlying cells of the immune system) and ciliated cells (which propel fragments of mucus away from the area) perform a protective function. In addition the secreting or absorbing cells perform the basic physiological functions of the membrane.

2.3.1.2 Mucosal Barrier

2.3.1.2.1 Mucous Coat

The vaginal mucous membrane is covered by a layer of mucus (or mucin), which provides an important protective function. Mucus is a thick, sticky substance composed of polysaccharides and proteins, secreted by goblet cells. Mucus acts as a lubricant that prevents any particulate matter from damaging the delicate cell layers that form the

mucous membrane. It also functions as a physical barrier that traps bacteria before they can reach the membrane itself. The adhesive property of mucous is due in part to the fact that it contains carbohydrate structures similar to the ones on mucosal cells. Bacteria that can adhere to mucosal cell surface carbohydrates will also adhere to mucus and will thus be prevented from attaching to mucosal cells (Salyers and Whitt, 1994).

Mucus contains an antibody called secretory immunoglobulin A (sIgA). One part of sIgA binds to antigens on the bacterial surface and another part (the Fc portion) binds to mucin components, thus contributing to the ability of mucin to trap bacteria (Kato and Owen, 1994). Blobs of mucus containing trapped bacteria are expelled by either peristalsis or ciliated cells that propel them away from the site.

The mucin layer is often but not always depicted as a homogenous layer of constant thickness. Streams of mucus emerging from the goblet cells create layers of mucus separated by areas of lower viscosity (Salyers and Whitt, 1994). The continued expulsion of mucus blobs creates areas of uneven thickness. Bacteria may be able to take advantage of the non-homogenous nature of the mucin layer by moving along planes of low viscosity to reach mucosal cells (Salyers and Whitt, 1994).

2.3.1.2.2 Mucosal Shedding

Bacteria that manage to colonize the surface of the mucous membranes are prevented from passing through this membrane by tight junctions between the mucosal cells. Few, if any, bacteria are capable of disrupting tight junctions and crossing the membrane by moving between cells (Salyers and Whitt, 1994).

Bacteria that succeed in reaching and adhering to mucosal cells also have to cope with the rapid turnover of mucosal cells. Mucosal cells are among the most rapidly dividing populations of cells in the body and are constantly being produced and released from the membrane. Bacteria that manage to attach themselves to mucosal cells would be expelled from the membrane as the cells are released and must thus be able to divide and reattach rapidly enough to remain at the site.

2.3.1.3 Innate Humoral Factors

In addition to acting as a physical barrier, mucus also contains substances that either kill bacteria or inhibit their growth. Innate humoral factors are products that are secreted onto mucosal surfaces by exocrine glands and are able to carry out protective functions independently of the presence of specific antibodies.

These factors include lysozyme and lactoferrin (Kato and Owen, 1994). Lysozyme is an enzyme that degrades bacterial peptidoglycan. It is effective mainly against gram-positive bacteria because the peptidoglycan of gram-negative is protected by their outer membrane (Tramont and Hoover, 2000).

Lactoferrin is a protein that binds iron with a high affinity. Iron is essential for bacterial growth. Most bacteria are unable to compete with lactoferrin for free iron. Thus, lactoferrin reduces the amount of iron available for bacterial growth (Weinberg, 2001). However, effective pathogens such as *Neisseria gonorrhoeae* produce substances that can compete

As lymphoid aggregates similar to Peyer's patches are found in the female genital mucosa, it can be assumed that similar processes occur at this site. This suggests the existence of a relatively independent compartmentalised immune response in the female genitalia.

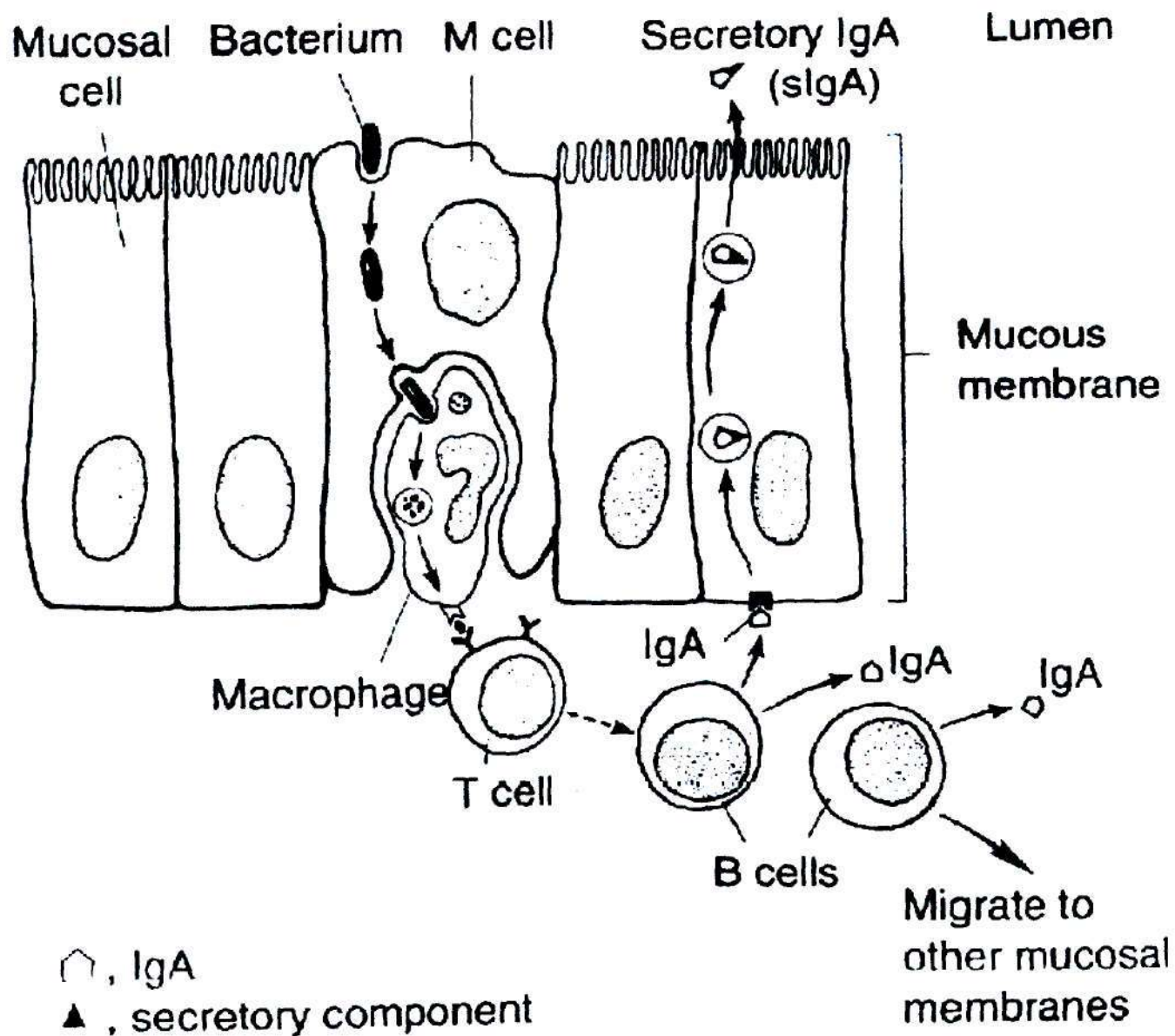


Figure 2: Interaction between M cells, macrophages as APCs, T-helper cells and B- cells leading to the production of secretory IgA at the site of infection.

2.4.2 Efferent Arm of MALT

2.4.2.1 Humoral Immunity

The role of IgG and IgM in the protection of mucosal surfaces is incompletely understood. They are not transported nearly as efficiently as IgA into the mucosal secretions, and they are not as resistant to proteolysis.

2.4.2.1.1 IgA

IgA, discovered by Heremans (Heremans *et al.*, 1959), was shown to exist in the unique form of secretory IgA as the predominant immunoglobulin in external respiratory and gastrointestinal tract secretions (Chordirker and Tomasi, 1963; Tomasi *et al.*, 1965). The predominance of IgA in mucosal secretions is thought to be the result of the collection of IgA-selective regulatory T cells (Croitoru and Bienenstock, 1994). IgA-committed cells form part of a recirculating pool that may be stimulated locally to produce antibody or may have emigrated from a distant site where they were previously primed by antigen.

Once IgA is secreted by mucosal plasma cells in the lamina propria, it is selectively transported across the mucosal epithelium to function in the external secretions. The selective secretion of IgA is promoted by certain structural features of this immunoglobulin that allow it to bind to a specific transport receptor termed the secretory component (SC) in the basal membrane of the epithelial cell (Ernst *et al.*, 1987). This then results in endocytosis and transcytosis to the apical membrane (Brandtzaeg *et al.*, 1988) (Fig 3).

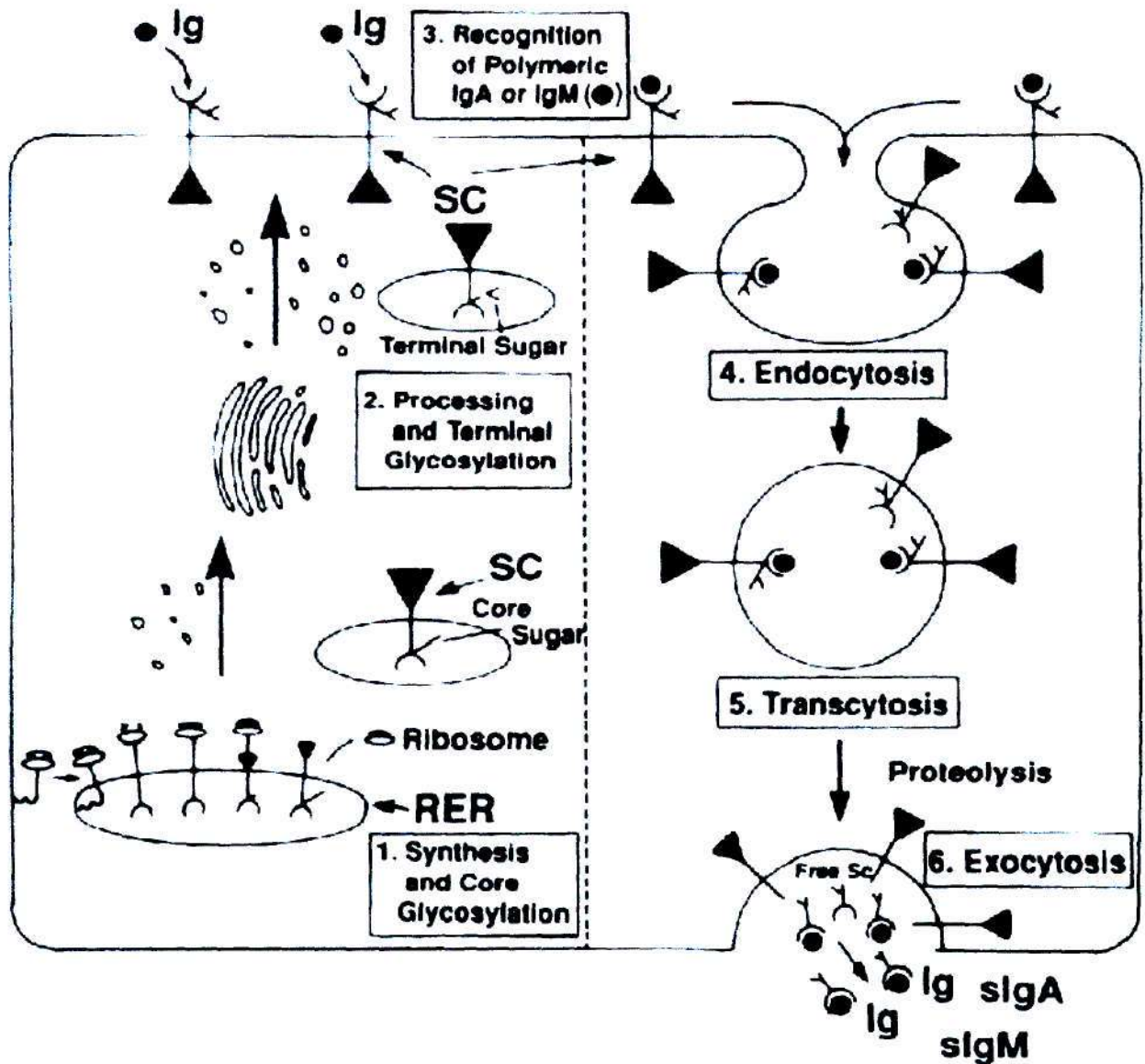
The mechanism of transport of IgA is one of the best examples of the non-degradative transcellular transport of proteins through cells. Secretory component, which mediates the

transport of IgA, is a protein expressed as an integral membrane protein on the basolateral membrane of mucosal epithelial cells. This receptor is apparently continuously endocytized and transported across the epithelial cell and secreted at the apical membrane into the mucosal secretions. The transport of IgA occurs following its synthesis and secretion by plasma cells in the lamina propria. IgA binds with high affinity to SC on the epithelial cell and is transported to the mucosal secretion bound to its receptor in the form of a complex (SC-IgA). Mucosal secretions therefore contain a mixture of secretory IgA and free SC (Ernst *et al.*, 1987).

Secretory IgA appears to have unique properties that may explain its predominance at the mucosal surface. Both the dimeric nature and the addition of SC confer on secretory IgA considerable resistance to proteolysis. IgA is more resistant to proteolytic degradation than IgG or monomeric IgA (Shuster, 1971; Underdown and Dorrington, 1974). IgA does not activate complement via the classical pathway and, therefore, tends not to induce potentially damaging inflammatory reactions (Colten and Bienenstock, 1974) but functions to neutralise toxins and viruses (Ogra *et al.*, 1968; Holmgren *et al.*, 1975) and to prevent bacterial adherence to epithelial cells (Williams and Gibbons, 1972). IgA antibodies may exert their microbial function primarily by binding to antigenic epitopes on microorganisms. This binding restricts their mobility or prevents their binding to mucosal epithelium

It has also been claimed that sIgA may perform antibody-dependent cell-mediated cytotoxicity and promotes phagocytosis via Fc α receptors on various cell types (Brandtzaeg *et al.*, 1987).

BASAL – LATERAL OR SINUSOIDAL SURFACE



LUMINAL OR BILE CANALICULAR SURFACE

Figure 3. Model for transport of IgA and IgM across the mucosal cell.(Tomasi, 1994).

- 1-synthesis and core glycosylation of SC.
- 2-processing and terminal glycosylation in Golgi complex.
- 3-expression of receptor and complexing with polymeric IgA or IgM.
- 4-endocytosis of ligand- receptor complexes and unoccupied receptor.
- 5-transcytosis from basolateral to luminal surface.
- 6-exocytosis of sIgA and IgM and free SC.

2.4.2.1.2 IgM

IgM is a pentamer with a single J chain and makes up 10% of immunoglobulins in sera (Nairn, 2001). In the primary immune response IgM is the major immunoglobulin involved. IgM has five units linked by disulphide bonds giving it ten antigen binding sites and is therefore far more effective in agglutination (Nairn, 2001). Due to its five Fc portions, IgM is also more effective at activating complement (Nairn, 2001).

IgM in the external secretions also is associated with SC, which results from its transport to the secretions by SC (Braendtzaeg, 1985). Secretory IgM has been shown to follow the same intracellular route through secretory epithelia as sIgA. However the concentration of secretory IgM is substantially lower than that of secretory IgA because of the lower proportion of IgM-producing cells in mucosal tissue.

2.4.2.1.3 IgG

IgG is a monomeric immunoglobulin and makes up 75% of serum immunoglobulins (Nairn, 2001). Because of its small size (150 000Da), IgG is the only immunoglobulin that crosses the placenta (Nairn, 2001). IgG protects the newborn for about 6-9 months. There are 4 major subclasses of IgG, IgG1; IgG2; IgG3 and IgG4 (Rassanti and Kipps, 1997). IgG is best at opsonizing bacteria but is less effective at activating complement than IgM. Intra- and extravascular spaces are evenly distributed with IgG and IgG is the major antibody involved in the secondary immune response (Rassanti and Kipps, 1997).

In most species, the concentration of IgG in mucosal secretions is approximately the same as or somewhat greater than that of IgM. IgG is thought to enter the mucosal secretions non-specifically via paracellular transport or fluid phase endocytosis.

2.4.2.2 Cell Mediated Immunity in the Female Genital Tract

Technological advances in the ability to identify cells in mucosal tissue have revealed that the vaginal mucosa of mice contains large numbers of, dendritic-like Langerhans cells, macrophages, and T-cells. Vaginal Langerhans cells are major histocompatibility complex (MHC) class II dendritic-like cells capable of antigen presentation in the vaginal submucosa (Fidel and Sobel, 1996). Intraepithelial lymphocytes in the submucosa are capable of migrating towards the epithelial surface or into the uterine lumen in response to chemotactic factors (Fidel and Sobel, 1996). Thus, the presence of T-cells and antigen-presenting cells suggests that the vaginal mucosa may be an immunocompetent tissue capable of a cell mediated immune response (Fidel and Sobel, 1996).

This, together with the fact that the vaginal epithelium is permeable to proteins with different molecular weights, suggests that antigens present in the vaginal lumen could gain access to Langerhans cells, which could in turn stimulate resident T lymphocytes (Fidel and Sobel, 1996). This hypothesis is supported by studies performed in animal models of genital *Chlamydia trachomatis* infections, in which vaginal lymphocytes from infected animals were found to proliferate and produce cytokines in response to chlamydial antigens (Igiertseme and Rank, 1991; Cain and Rank, 1995). Epithelial cells were originally

thought to be immunologically inert. However, epithelial cells can be induced to express MHC class II antigens and thus play a role in antigen presentation (Fidel and Sobel, 1996).

T lymphocytes found in the vaginal mucosa have been described as phenotypically distinct from those in the periphery (Fidel and Sobel, 1996). It has been shown that although T-cells expressing the CD4 or CD8 receptor have been identified in the murine vaginal mucosa, lymphocytes isolated from the vaginal tissue of naïve mice and analysed by flow cytometry contained few CD8 cells, resulting in a high CD4/ CD8 cell ratio, and that CD4 cells did not recognise some epitope-distinct anti-CD4 antibodies normally recognised by peripheral T-cells (Fidel *et al.*, 1996). These observations suggest a different distribution or migration of lymphocytes within the vaginal mucosa and a possible atypical expression of the CD4 receptor on vaginal CD4 cells compared with T-cells in the periphery or at other mucosal sites (Fidel and Sobel, 1996).

2.5 Site Specific Variations In Immunoglobulin Output

Waldman *et al.* (1971) found that secretory IgA was the predominant (~45%) immunoglobulin class in normal cervicovaginal secretions, the remainder consisting mainly of monomeric IgA (~20%) and IgG (~30%). Others have reported predominance of IgG also in normal vaginal fluid, mainly existing as intact molecules (Hocini *et al.*, 1995). Several studies have shown that both IgA and IgG are present in cervico- vaginal fluids and that IgG appears to be the predominant immunoglobulin, but the reported ratios of IgG to IgA vary from approximately 2:1 to 10:1 (Chordirker and Tomasi, 1963; Masson *et al.*, 1969; Schumacher, 1973, 1980; Tjokronegoro and Sirisinha, 1975; Coughlan and Skinner,

1977; Jalanti and Isliker, 1977; Usala *et al.*, 1989). A recent study by Bard *et al.* (2002) compared the rates of immunoglobulins in genital fluids and found a higher IgG prevalence (80%) in cervico- vaginal and endocervical secretions than IgA (12%).

This fits with a detailed study showing considerable variations, with IgA dominating in mucous from the endocervix and less so ectocervix, and IgG dominating in vaginal fluid (Quesnel *et al.*, 1997). Measurements of immunoglobulins in human uterine fluid also indicate that IgG is the predominant immunoglobulin present (Schumacher *et al.*, 1979). Nevertheless the uterus may be the primary source of IgA in cervico- vaginal fluids, since the concentration of IgA in vaginal fluid from hysterectomized women was only about 10% of normal (Jalanti and Isliker, 1977).

When interpreting these observations one has to keep in mind that specimen collection methods can be responsible for differences in observations, in particular when absolute quantities are inferred.

2.6 Influences on Mucosal Immune Reactivity

2.6.1 Resident Microflora

Resident microflora are known to co-exist within the intestinal tract and maintain a stable environment by precluding attachment of enteropathogens. The flora can eliminate foreign pathogens by producing antimicrobial substances (colicins, short chain fatty acids), (Iglewski and Gerhardt, 1978; Byrne and Dankert, 1979) and by stimulating the growth of mucosal epithelium (Thompson and Trexler, 1971).

The vaginal tract is normally colonized by a complex resident microflora that probably plays an important protective role. It consists of a complex mixture of gram-positive and gram-negative bacteria. The vagina is predominantly colonised by species from the genus *Lactobacillus*, a genus of lactic acid producing gram-positive bacteria. It has been suggested that lactobacilli help to prevent pathogens from colonizing the vagina by keeping the pH low (around 4.5) by the production of hydrogen peroxide (Klebanoff *et al.*, 1991), and by forming a biofilm that prevents pathogens from gaining access to the mucosal surface of the vagina or cervix, but other members of the microflora probably also contribute to protection of the site.

2.6.2 Endocrine Regulation

The secretion of IgG and IgA antibodies into vaginal fluids was shown to be reduced in the presence of high levels of estrogen (Schumacher, 1980). In vitro studies similarly showed that antigen presentation of ovalbumin to cloned lymph node cells by cells isolated from the vaginal mucosa (presumably Langerhans cells) was inhibited by estradiol (Wira *et al.*, 1994). The addition of progesterone reversed the inhibitory effect (Wira and Rossoll, 1995).

In a study by Wira *et al.* (1994) it was found that the uterine response was specific for estradiol, that progesterone blocks estradiol-stimulated increases in uterine IgA and IgG. However, progesterone either alone or with estradiol decreases cervico-vaginal levels of IgA and IgG. It was also found that when estradiol was given to ovariectomised rats, SC levels increased sharply, in parallel with IgA in uterine secretions (Sullivan *et al.*, 1983). In

contrast, SC levels in cervico- vaginal secretions were reduced markedly in response to estradiol treatment (Wira and Sullivan, 1985).

2.7 Sexually Transmitted Infections (STIs)

Sexually transmitted agents have long been recognised to be responsible for many acute genital infections, but only recently has a wider role been established for these agents in the epidemiology of maternal and infant morbidity and mortality. Although men can have long term complications from STIs, they are often more symptomatic early in the infection cycle and thus may seek care before the infection becomes deeply established. Women, on the other hand, are more susceptible to infection and may be asymptomatic in the early stages of infection. Thus, they may be less likely to seek health care and more likely to transmit disease to other sexual partners.

In addition to causing widespread infections, STIs have a major impact on reproductive health; infertility has been linked to some genital infections (e.g. *Chlamydia* and *Mycoplasma* species) and maternal-fetal transmission of genital organisms remains a common cause of fetal and infant morbidity and mortality (Moodley and Sturm, 2000). The most common non- ulcerative STIs in sub-Saharan Africa are discussed below.

2.7.1 Gonorrhoea

Gonorrhoea is an infectious disease primarily involving the genitourinary tract. Members of the genus *Neisseria* are gram-negative rods or cocci, represented by more than fifteen species (Knapps, 1988). Gonorrhoea is caused by the small gram-negative diplococcus *Neisseria gonorrhoeae*, an extracellular parasite whose only natural host is humans. Transmission is most commonly by sexual contact, and infection usually occurs in the more sexually active age groups. Severe infection involving the eyes can develop in the newborn that becomes infected during passage through the birth canal.

The acute disease is manifested by a urethritis, usually purulent in nature, in the male and may or may not be associated with symptoms of urethritis or vaginal discharge in the female. Approximately 30- 50 % of females colonised with *N. gonorrhoea* are asymptomatic (Youmans *et al.*, 1985; Sturm *et al.*, 1998). In woman the complications due to infections are more prevalent as patients are often unaware of their infection. Ascending infection involving the fallopian tubes and ovaries may result in pelvic inflammatory disease, which produces a chronic, debilitating infection that can cause partial or complete obliteration of the patency of the fallopian tubes.

As in most mucosal infections, the function of local antibody is thought to be prevention of attachment and subsequent infection of the host mucosa (Mestecky and McGhee, 1987). Urethral exudates from men with gonococcal infection have demonstrated that all three antibody classes (IgA, IgG and IgM) are produced in response to infection (Kearns *et al.*, 1973; McMillan *et al.*, 1979a). In women, vaginal washings after infection with gonococci have been shown to contain both IgG and IgA antibodies against pili, lipopolysaccharide (LPS), and the outer membrane proteins (OMPs) implicated in attachment (Lammel *et al.*,

1985; Ison *et al.*, 1986). Although IgA and IgG specific for gonococci have been demonstrated, production of specific IgM has not been well documented (O'Reilly *et al.*, 1976; McMillan *et al.*, 1979b; Ison *et al.*, 1986). Despite the formation of these antibodies, little is known about their functional activity. Rapid re- infection (Moodley *et al.*, 2002) indicates that its protective effect is limited.

2.7.2 Chlamydiae

Long classified as large viruses due to their size (200-400 nm in diameter) and their intracellular parasitism (Schacter and Dawson, 1979; Ward, 1983), chlamydia organisms are now considered as a special type of bacteria. Recent studies have shown chlamydia to have both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) as well as a cell wall similar in structure and chemical content to that of bacteria. Chlamydiae are intracellular parasites in that they are incapable of synthesising adenosine triphosphate (ATP) and are dependent on the host cell for energy and as a source of metabolism (Moulder, 1984; Cook and Honeybourne, 1994). Multiplication occurs by binary fission and in contrast to viruses, chlamydiae are susceptible to antibiotics.

Chlamydia trachomatis is a major cause of genital tract infections in humans. Although it is well established that chlamydiae are associated with infectious urethritis in males, culture for *Chlamydia trachomatis* from females with mucopurulent cervicitis has shown a carriage rate of approximately 20 percent (Youmans *et al.*, 1985). This finding suggests that the cervix is the primary site of infection in the female.

The antibody response to *C. trachomatis* has been well documented (Rank *et al.*, 1979; Monnickendam and Pearce, 1983; Schachter *et al.*, 1983; Monnickendam, 1988). Although humoral antibodies are generated in *C. trachomatis* infection (IgM, IgG and IgA), their role in subsequent protection is partial or transitory, at best (Katz *et al.*, 1987; Rank *et al.*, 1988).

2.7.3 Bacterial Vaginosis (BV)

Bacterial vaginosis, previously known as nonspecific vaginitis or Gardnerella vaginitis, is the most common cause of vaginal discharge. It may be the cause of up to one half of cases of vaginitis (Eschenbach *et al.*, 1988) in all women and the cause of from 10 to 30 percent of cases in pregnant women (Colli *et al.*, 1996). The local prevalence of BV among pregnant women was found to be 21% (Sturm *et al.*, 2002).

This clinical syndrome is now recognised as a polymicrobial event involving a loss of the normal lactobacilli and an overgrowth of anaerobes. The pathogen of this condition is still obscure. While commonly found in increased numbers in women with bacterial vaginosis, *Gardnerella vaginalis* is not invariably present. *G. vaginalis* has been reported in 16 to 42 percent of women with no signs or symptoms of vaginitis (Hill, 1993) and *G. vaginalis* is now accepted as just one of the BV associated organisms.

BV is associated with an increased risk of several pathologic conditions, including adverse outcome of pregnancy (Hillier *et al.*, 1990), pelvic inflammatory disease (Eschenbach *et*

al., 1988), postpartum endometritis (Watts *et al.*, 1990), urinary tract infections (Sturm, 1989; Hughes and Hillier 1990) and vaginal cuff cellulitis (Soper *et.al.*, 1990).

Bacterial vaginosis is diagnosed by the presence of three of the following clinical and microscopic findings viz. homogenous vaginal discharge (colour and amount may vary), presence of clue cells (greater than 20%), amine (fishy) odour when potassium hydroxide solution is added to vaginal secretions, vaginal pH greater than 4.5 and the absence of the normal vaginal lactobacilli (Majeroni, 1998).

Some controversy remains over the sexual transmission of bacterial vaginosis. While it occurs more commonly in women with more than one sexual partner, bacterial vaginosis can also occur in women who are not yet sexually active (Bump and Buesching, 1988). Treatment of male partners has not resulted in improved cure rates or a reduced rate of recurrence (Moi *et al.*, 1989).

2.7.4 Trichomoniasis

Trichomonas vaginalis is the cause of one of the most common sexually transmitted diseases, trichomoniasis (Draper *et al.*, 1993). *T. vaginalis* is a pathogenic protozoan which resides only in the lower genitourinary tract of human beings, its only natural host. Worldwide, the World Health Organisation estimates that 180 million persons are infected annually.

Trichomoniasis is transmitted sexually and both males and females may be infected. Persons infected with *T. vaginalis* may not show any signs of infection, thus one of the difficulties encountered in any study of *T. vaginalis* is asymptomatic carriage (Hammil, 1989). The appearance of symptoms may be influenced by local immune factors and inoculum size. Vaginal discharge is found in more than half of symptomatic cases.

T. vaginalis activates the alternative complement pathway (Shaio *et al.*, 1991) and attracts polymorphonuclear neutrophils (Shaio *et al.*, 1992) which can kill the protozoans (Rein *et al.*, 1980). On the other hand *T. vaginalis* moves away from the products of polymorphonuclear neutrophil oxidative metabolism (Styrt *et al.*, 1991). Monocytes and macrophages can also kill trichomonads in vitro, but their role in natural infection is uncertain (Mantovanic *et al.*, 1991). Local and systemic humoral responses and delayed hypersensitivity are seen in human infection (Alderete *et al.*, 1991) but they have insufficient sensitivity and specificity to permit serologic diagnosis. Whether these immune responses confer any protection is currently uncertain.

2.8 Immunization and Immune Regulation

After MALT B and T cells at a particular site have been stimulated by an antigen, most of them remain in the area and continue to produce IgA locally, but some migrate to other mucosal surfaces and produce IgA there. Thus, exposure of one mucous membrane to a bacterial pathogen results in production of sIgA at other mucosal surfaces and in breast milk (Ernst *et al.*, 1987).

Although the urogenital tract apparently lacks classical organized MALT structures, the squamous epithelium of the vagina and ectocervix contains MHC class II-positive putative

antigen presenting cells (APCs) of the Langerhans type (Bjercke *et al.*, 1983; Wira and Rossoll, 1995). Furthermore, there is experimental evidence in rodents to suggest that uterine MHC class II-positive stromal and epithelial cells may perform antigen presentation (Wira and Rossoll, 1995). Such cells are also present in the human endometrium, and candidate APCs occur in the cervical and vaginal stroma as well (White *et al.*, 1997). This may explain why topical antigen application together with mucosal adjuvant induces a prominent vaginal IgG response in mice (Menge *et al.*, 1993).

Substantial dissemination of primed immune cells from GALT to exocrine effector sites beyond the gut is the rationale for many desired oral vaccines (Mestecky *et al.*, 1994; Staats *et al.*, 1994). Oral vaccines work by stimulating the MALT of the gastrointestinal tract directly. The secondary migration of MALT T and B cells to other mucosal surfaces spreads immune protection to these other areas (Fig 4).

A preferential link between nasal-associated-lymphoid tissue (NALT) and the female genital tract in terms of homing determinants for primed B cells could explain why particularly high levels of specific IgA and IgG antibodies were found in cervico- vaginal secretions of mice after nasal immunization with transgenic live attenuated *Mycobacterium bovis* as an immunogen vector (Langermann *et al.*, 1994), recombinant adenovirus vector expressing herpes simplex glycoprotein B (Gallichan and Rosenthal, 1996), an HIV peptide vaccine candidate (Staats *et al.*, 1996), or group B streptococci (Hordnes *et al.*, 1997). Similar results have been obtained in Rhesus monkeys (Russell *et al.*, 1996). A more recent study compared intranasal and intravaginal routes of immunisation in mice with a protein antigen coupled to cholera toxin B subunit. Mice immunised intranasally developed substantially stronger vaginal IgA and IgG and serum IgA and IgG antibodies

than those immunised intravaginally (Russell, 2002). In addition, the nasal route is generally found to be efficient for stimulation of systemic immunity and serum-derived antibodies appear to be of considerable importance in female genital secretions (Brandtzaeg, 1997). Based on the promising results obtained in mice, similar studies on the immunogenicity of intranasally applied outer membrane vesicles (OMVs) prepared from group B meningococci have been initiated in humans (Haneberg *et al.*, 1997). A study by Johannsson *et al.*, (2001) involved vaccinating human volunteers nasally or vaginally with the model mucosal antigen cholera toxin B subunit. The study concluded that a combination of nasal and vaginal vaccination might be the best vaccination strategy for inducing protective antibody responses in both cervical and vaginal secretions.

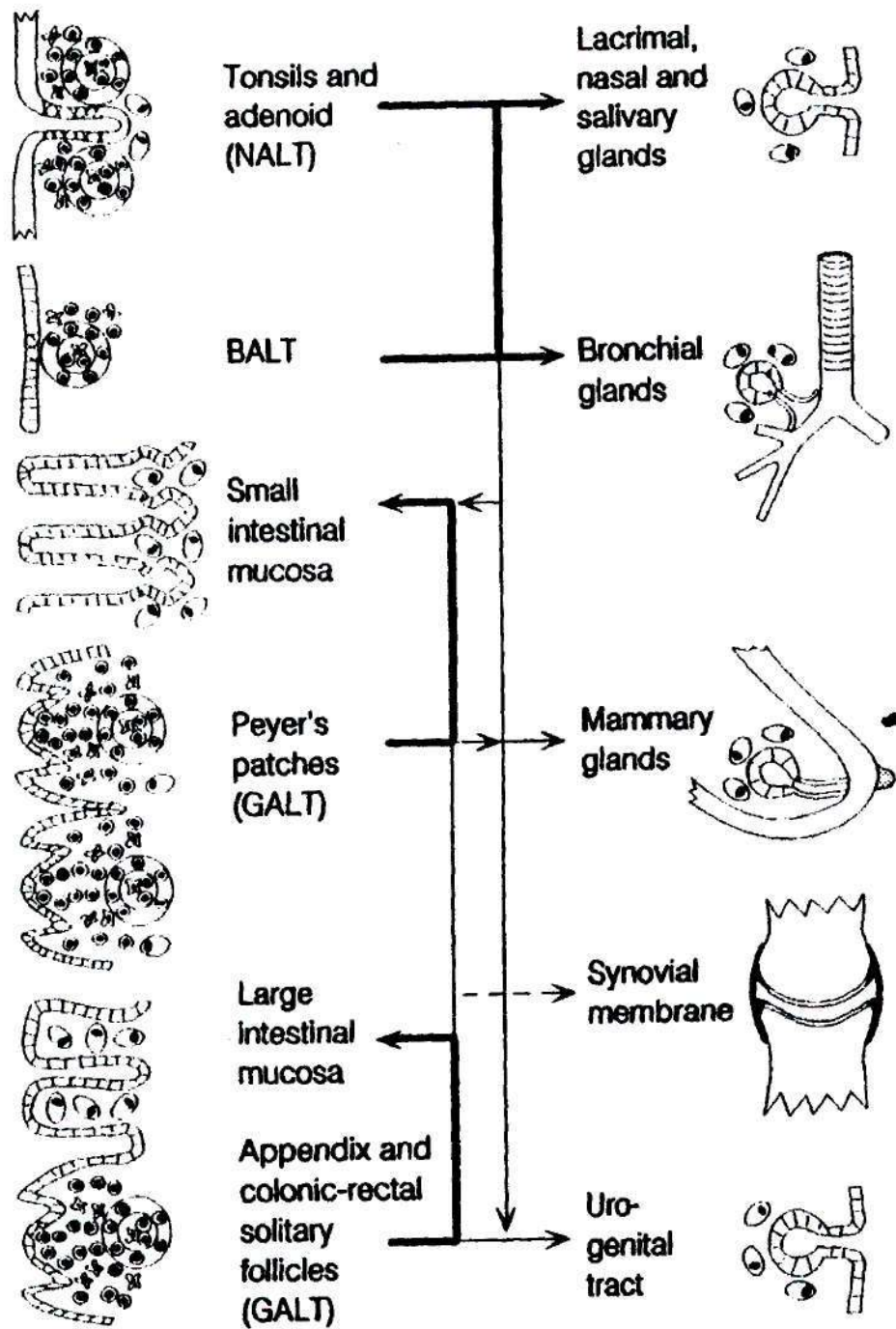


Figure 4. Model for homing pathways of primed lymphoid cells from inductive sites to effector sites (Brandtzaeg, 1997).

There is growing interest in development of vaccine strategies for induction or enhancement of local mucosal immune protection against sexually transmitted diseases (STDs) including HIV (Miller *et al.*, 1992). Evaluation of anti-STD vaccines requires quantitative information about specific antibody concentrations in female genital secretions. The most convenient and widely used method for collection of these secretions is cervical-vaginal lavage (Bélec *et al.*, 1995b), but lavage inevitably involves unpredictable dilution of secretions and this may render antibody concentrations too low to be detected by available assays.

This study aims to assess humoral immunity using a site specific method for collection of vaginal secretions with minimal dilution. Such information will be important for establishing precise correlates of mucosal protection against specific STDs and for evaluating the potential protective abilities for candidate vaccines. In addition, antibody responses in both pregnant and non-pregnant females will be assessed and compared to better understand their differences in immune responses if any. The benefits would be dual: not only could the infection rate and subsequent morbidity and mortality in newborns be decreased but it may be possible to reduce the rampant spread of STIs through immunisation programs in the ensuing future.

CHAPTER THREE

3.0 MATERIALS AND METHODS

AIMS

- To assess humoral immunity in the vaginal mucosa in pregnant and non-pregnant females using cervico- vaginal secretions from tampon samples.
- To determine the difference, if any, between the systemic humoral response (using blood samples) and the results from cervico- vaginal secretions.

OBJECTIVES

- To extract protein from tampon samples.
- To determine total protein concentration using BIORAD.
- To quantitate immunoglobulins using ELISA.

3.1 Study Participants

Ethical approval was obtained from the University Research Ethics Committee, Ref H123/00. Patients were recruited once consent was given and specimens were collected between February and September 2001. Subjects were divided into four groups i.e. non-pregnant healthy women, pregnant healthy women, pregnant women with an STD and non-pregnant women with an STD. Those without an STD were recruited from the third year medical students at the Nelson R. Mandela School of Medicine. The pregnant women with and without an STD were recruited from the Kwamsane Antenatal Clinic. Those non-pregnant women with an STD were recruited from the Prince Cyril Zulu Communicable Disease clinic in Warwick Avenue, Durban.

3.1.1 Inclusion criteria

For the two STD groups, all non-pregnant and pregnant women presenting with an STD were recruited. Thereafter, a routine STD workup determined if these women qualified for the study. Women recruited with an STD had to be positive for at least one of the following pathogens: *Chlamydia trachomatis*, *Neisseria gonorrhoea*, *Trichomonas vaginalis*, or bacterial vaginosis. Pregnant women were recruited regardless of gestational age.

3.1.2 Exclusion Criteria

All non-pregnant women in the study were not on any form of contraceptives. Women who were menstruating were not included in the study.

3.1.3 Sample Size

For each group there were 45 women recruited. However after STI testing, some women had to be regrouped which changed the sample size for each group. The sample size for each group was as follows:

- i non-pregnant, non- STD: 22
- ii non-pregnant, with STD: 60
- iii pregnant, non-STD: 22
- iv pregnant, with STD: 65

3.2 Collection of Secretions

The collection of secretions was done using commercially available tampons (Kotex). The tampons were self-administered, and remained in the vagina for 15 minutes after the patient had inserted the tampon. Once the tampon had been removed it was placed in 10 ml of phosphate buffered saline (PBS) (Appendix One), pH 7.4. Specimens were transported on ice and were stored at 4°C. The storage period did not exceed 24 hrs.

The vaginal secretions were squeezed from the tampon using autoclaved wooden tongue depressors. The PBS containing the secretions was aliquoted into 2 ml eppendorf tubes and stored at -20°C. The vaginal fluid was used in further tests as follows:

- i Four mls was aliquoted for use in the BD Probetech method. This method is used to detect *Chlamydia trachomatis* and *Neisseria gonnorrhoea* and uses Strand Displacement Amplification (SDA) technology for direct, qualitative detection. The assay is based on the simultaneous amplification and detection of target DNA using amplification primers and a fluorescent labeled detector probe. The method followed was as for manufacturers instructions.
- ii One ml was aliquoted for an in- house PCR use to detect *Trichomonas vaginalis*. Primers were specific for a family of repeats that yielded a 413 bp product that was detected by gel electrophoresis,
- iii One ml for protein extraction (method below),
- iv a drop of fluid for a slide for BV detection. A routine Gram stain was performed for BV detection and diagnosis was based on Nugent's criteria (Nugent *et. al.*, 1991).

Nugent's scoring system is as follows:

0-3 : negative

4-6 : intermediate

7-10 : positive

3.3 Extraction of protein from vaginal fluid

Proteins were extracted using the method described by Quesnel *et.al* (1997). The thawed specimen was vortexed following which 50 μ l of specimen was used for extraction. Before protein extraction, 300 μ l of PBS containing protease inhibitors (0.2 mM 4-(2-aminoethyl)-benzenesulfonylfluoride {Sigma, Germany}, 10 μ M leupeptin {Sigma, Germany}, 1 μ g/ml aprotinin {Sigma, Germany} and 3.25 μ M bestatin {Sigma, Germany}) was added in each 2 ml eppendorf tube. Protein was allowed to precipitate for 30 min on ice (vortexed 3 or 4 times). Tubes were then centrifuged at 16000 X g (9000 rpm) for 10 min at 4°C. To avoid protein reabsorption, supernatants were removed immediately after the spin.

3.4 Total Protein Determination

A 96 well microtitre plate (Costar, Corning Incorporated, New York) was used for this assay. The Bio-Rad Protein Assay was used in which the standard was γ -globulin (Bio-Rad, Germany) in two- fold serial dilutions to yield a standard curve. Ten μ l of each standard and specimen solution was pipetted in duplicate into separate microtitre plate wells. The kit contained a dye reagent concentrate (Bio-Rad, Germany), which was added to each well (200 μ l). The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The plates were incubated at room temperature for at least five minutes. Absorbance was read at 595nm on the Anthos 2000 plate reader. For each specimen the absorbance reading was plotted on the standard curve (Fig 7) and the

concentration of protein was then extrapolated from the curve. The average concentration of the two samples was taken as a final result.

3.5 Enzyme- Linked- Immunosorbent Assay (ELISA)

The enzyme- linked- immunosorbent assay (ELISA) combines the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an enzyme.

The technique used was the two- antibody ‘sandwich’ ELISA. This ELISA is used to determine the antigen concentration in unknown samples provided a purified antigen standard is available. The change in enzyme activity as a result of the enzyme- Ab- Ag reaction is proportional to the concentration of the antigen and can be measured spectrophotometrically or with the naked eye. The ELISA applied was a double antibody sandwich method which was used for the detection and measurement of immunoglobulins in sera as well as in vaginal secretions. (Fig 5).

3.5.1 Optimisation

Sterile tissue culture grade 96 well microtitre plates (Costar, Corning Incorporated, New York) were used. To determine the optimal concentrations for the primary antibody and secondary antibody, titration dilution curves were done and those yielding the highest positive/ negative (P/N) ratio were chosen.

Plates were coated with a standard preparation of primary antibody concentrations starting with a 1: 125 dilution and thereafter a two- fold serial dilution was done for each primary antibody. For each dilution three rows were incubated with diluted serum as a positive (serum dilutions given below) and three rows were incubated with PBS as a negative. This allowed for the negatives and positives to be done in triplicate. Only one variable was changed at a time, in this case it was the primary antibody. The secondary antibody was added at a set concentration for each primary antibody concentration. For each assay, the primary antibody was diluted across the plate while one at a time the secondary antibody was changed starting from a 1:125 dilution. The average absorbance of the three positive rows was divided by the average absorbance of the three negative rows to give a P/N ratio (Figure 6).

Primary antibody was diluted 1: 500 for goat anti- human IgG and IgA and 1:125 for goat anti-human IgM. For the secondary antibodies, goat anti- human IgA (Jackson ImmunoResearch, USA) was diluted 1:1000; goat anti-human IgG (Zymed, Germany) diluted 1:500 and mouse anti-human IgM (Zymed, Germany) diluted 1:250. All secondary antibodies were conjugated to alkaline phosphatase (AP).

3.5.2 Construction of a Standard Curve

Purified human IgG, IgA and IgM were used as standards (Biogenesis, UK). Each standard was serially diluted two- fold from a 10 µg/ml stock solution with a final dilution of 1.25 µg/ml. Each standard was used to construct a standard curve from which immunoglobulin

concentration could be extrapolated (Fig 8). Standards were run with every plate to prevent inter-assay variability in results. Plate 2 shows how each plate was set up.

3.5.3 Detection of Immunoglobulins

3.5.3.1 Cervico- vaginal Secretions

Plates were coated with 100µl of primary antibody, a goat antibody to human IgA or IgM or IgG (Zymed, Germany), and left overnight at 4⁰C in a humid container. All primary antibodies were diluted in a sodium carbonate coating buffer (Appendix One). The plates were then washed with PBS containing 0.05% Tween 20 (Sigma, Germany) to remove excess antibody and blotted on absorbent paper. To prevent non-specific binding, fetal calf serum (Sigma, Germany) was applied as a blocking agent to all wells for ten minutes at room temperature. The excess blocking agent was washed off and plates were blotted dry. Next 50µl of the specimen was added and incubated in a humid a container for 30 mins at 37⁰C. Serial double dilutions of purified human IgA or IgG or IgM were added as standards (Biogenesis. England, UK). The excess specimen was washed off and the plates blotted dry. Thereafter 50µl of secondary antibody conjugated with alkaline phosphatase was added and incubated in a humid container for 30 mins at 37⁰C . All secondary antibodies were diluted in PBS. Plates were allowed to develop in the dark by addition of 50µl of pNPP (para- nitrophenyl phosphate) (Zymed, Germany) substrate. A 100µl of a 3M sodium hydroxide (NaOH) solution was added to stop the reaction and optical densities were read at 405 nm with the Anthos 2000 plate reader. Immunoglobulin concentrations were determined by reference to the standard curves. Each plate had a negative control using PBS as a blank. All specimens were assayed in duplicate.

3.5.3.2 Serum Specimens

All serum specimens had to be diluted to obtain a ~3-5 µg/ml range. Sera has a very large concentration of immunoglobulins and it is estimated that (Rassenti L. Z and Kipps T. J, 1997):

IgG has 8 - 16 mg/ml

IgM has 1.4 - 4 mg/ml

IgA has 0.5 - 2 mg/ml

Therefore sera were diluted 1: 3000 for IgG, and 1: 250 for both IgM and IgA.

Determinations of immunoglobulin concentrations were performed as for vaginal fluid.

3.6 Statistical Methods

A statistician used medians and ranges to summarise the data. The Wilcoxon Rank sum test was used to compare groups. A nonparametric measure was used because the assumption of normality was violated and could not be corrected by a transformation.

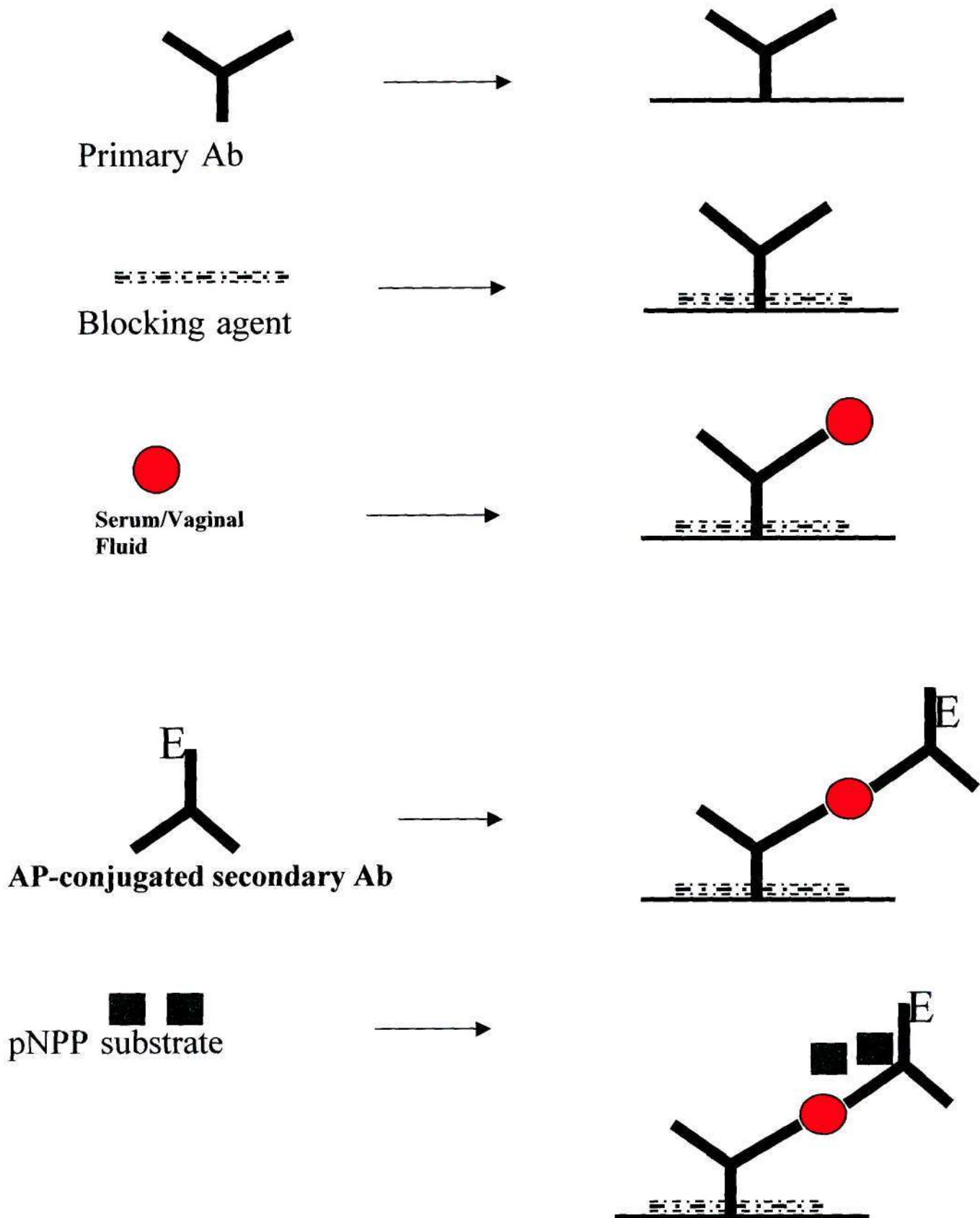


Figure 5. Diagram showing the method used in the double sandwich ELISA

	Coating	1/125	1/250	1/500	1/1000	1/2000
	Ab conc.					
pos	A	1.579	1.324	0.773	0.323	0.110
	B	1.50	1.312	0.754	0.299	0.086
	C	1.596	1.483	0.796	0.286	0.080
neg	D	0.227	0.176	0.133	0.113	0.045
	E	0.221	0.173	0.131	0.112	0.050
	F	0.226	0.163	0.131	0.128	0.052
	P/N	7.07	8.08	5.91	2.62	1.88

Figure 6. Calculation of P/N ratio for immunoglobulins e.g. IgA to determine coating antibody concentration.

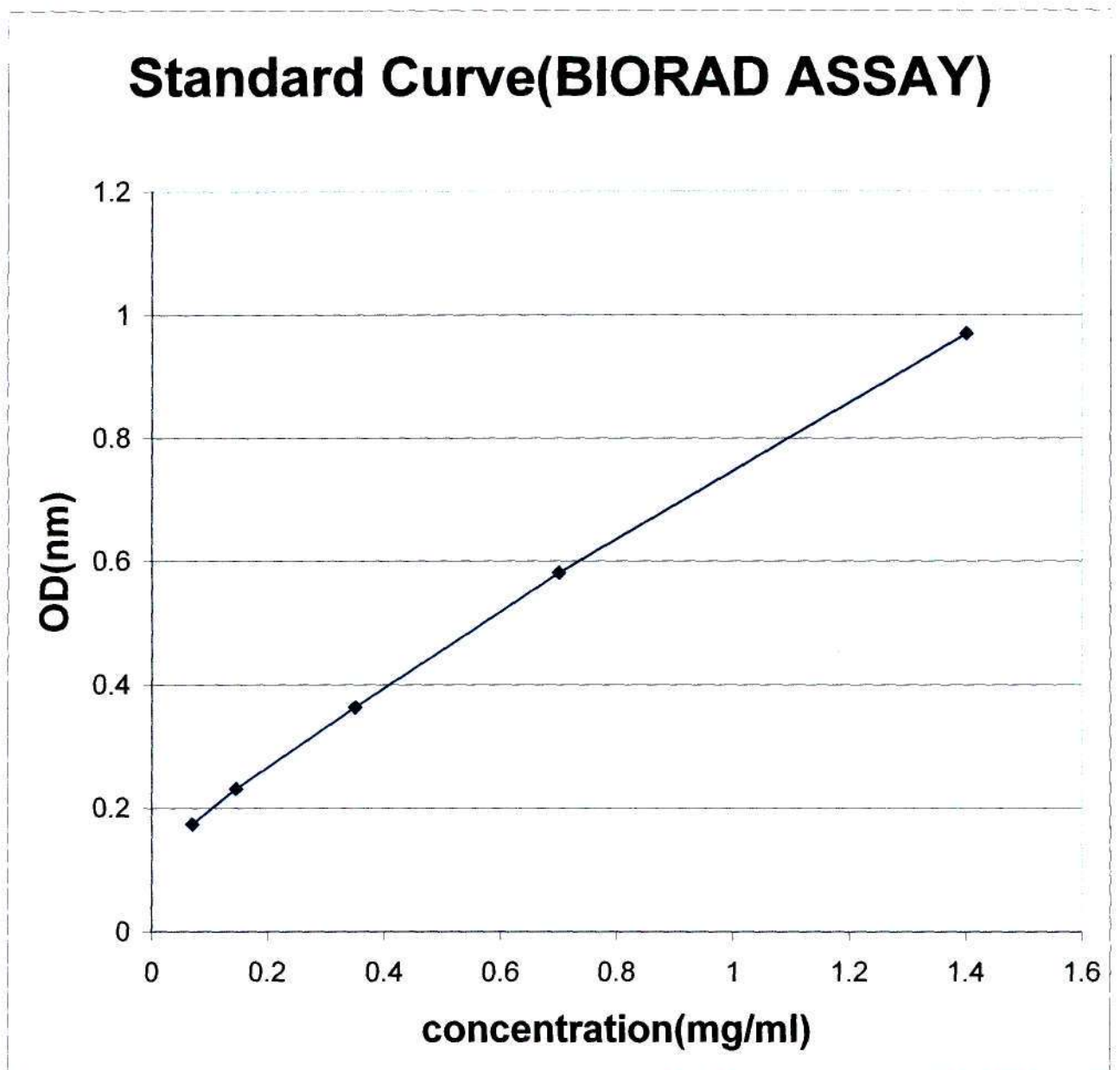


Figure 7. Standard curve plotted using γ -globulin as standard. Optical density read at 595nm.

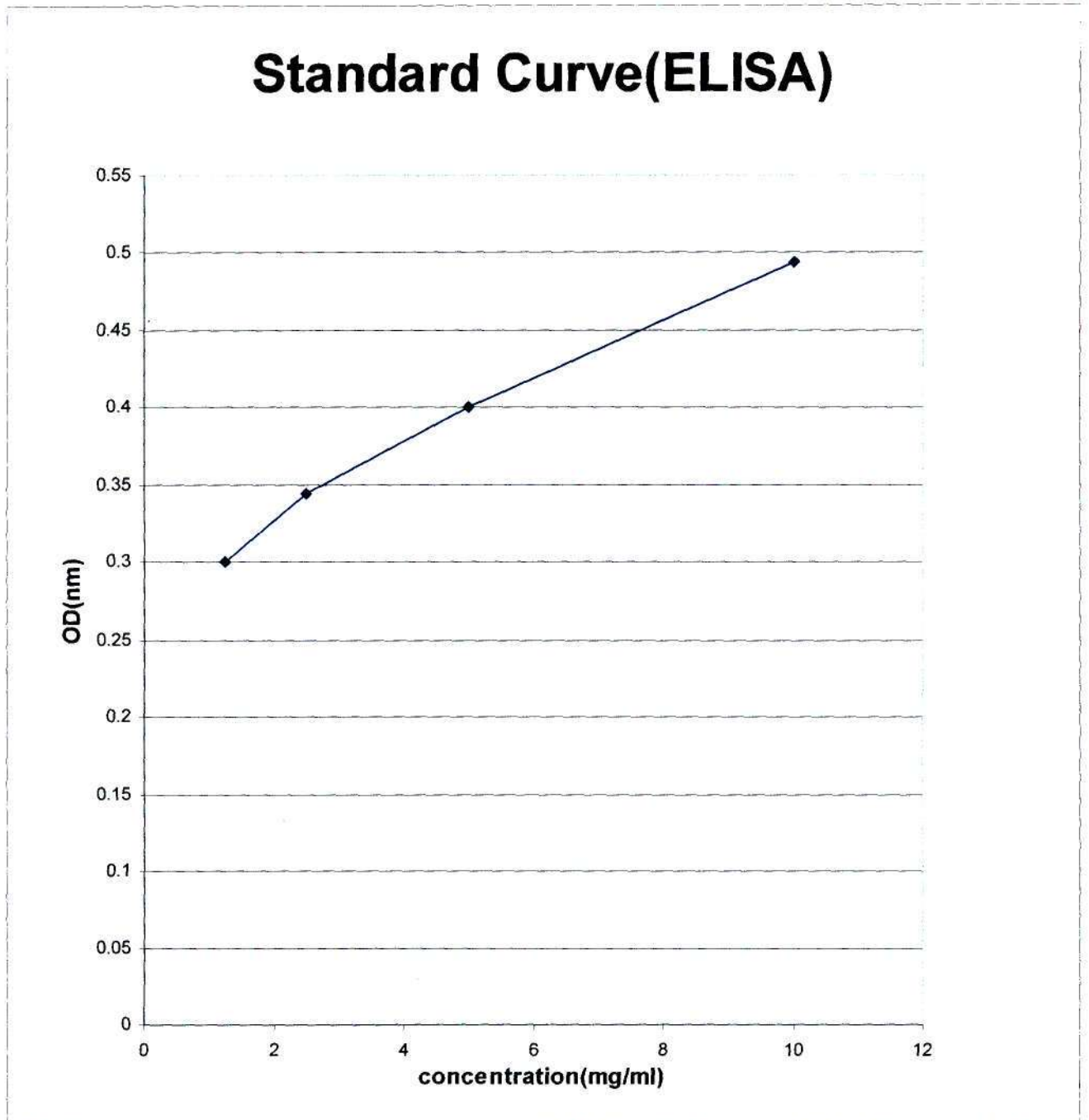


Figure 8. Standard curve plotted for ELISAs e.g. IgA. Optical density read at 405nm.

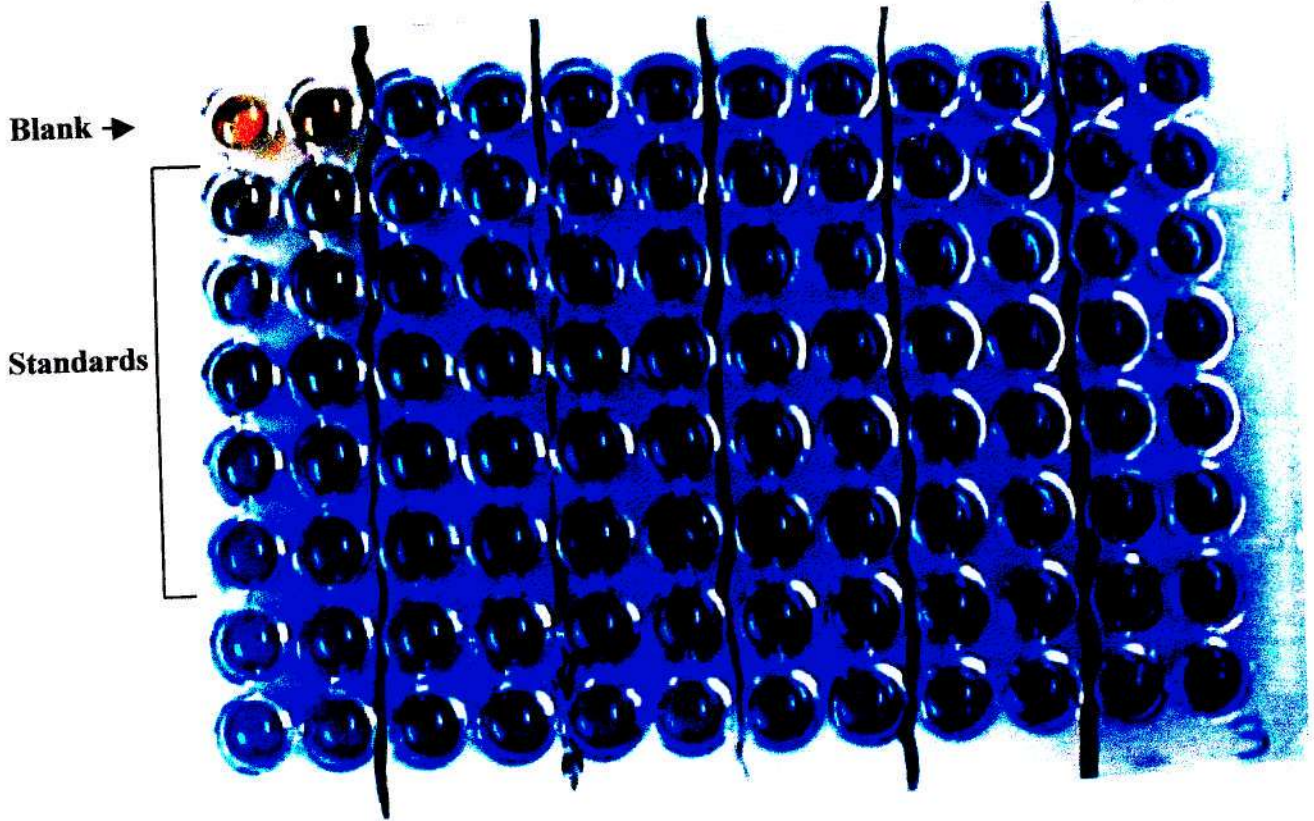


Plate 1 Protein determination assay

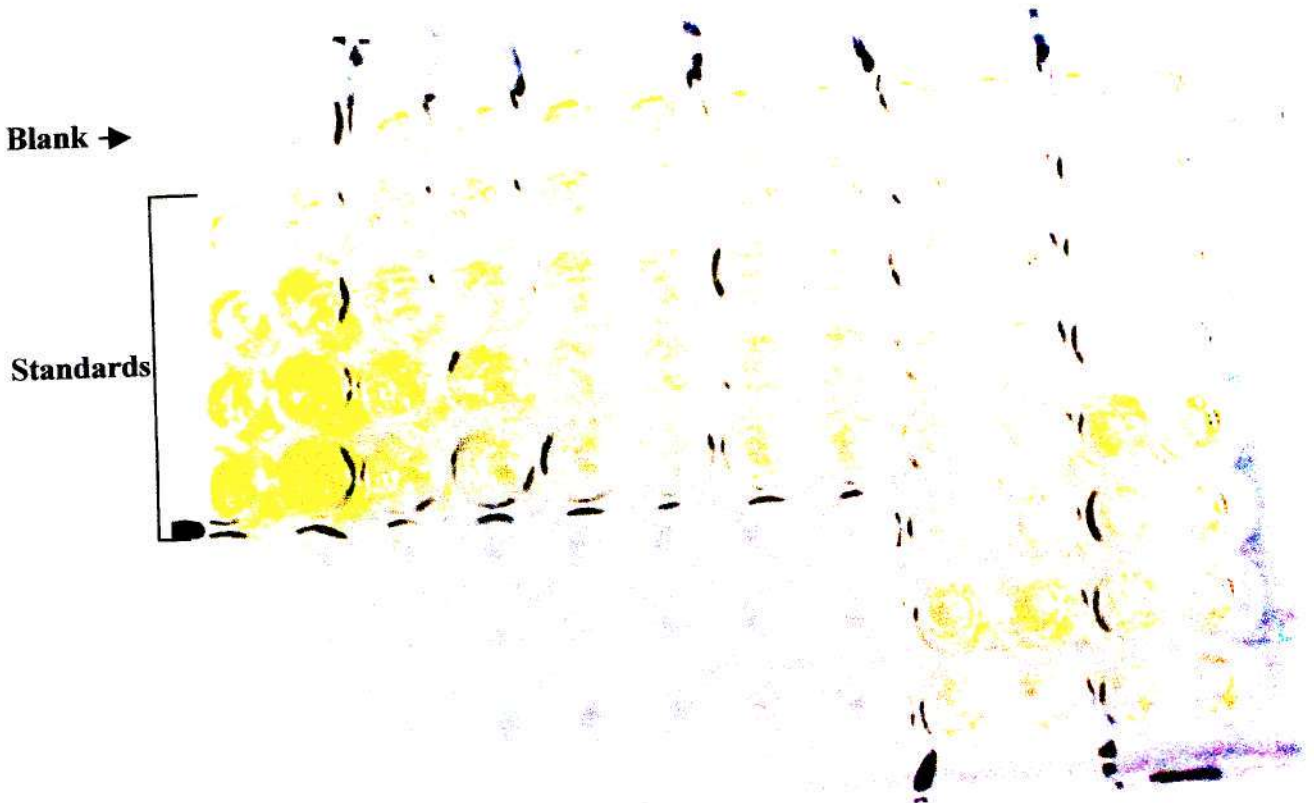


Plate 2 ELISA plate

CHAPTER FOUR

4.0 RESULTS

4.1 Analysis of Immunoglobulins

For all immunoglobulins, values were expressed as a ratio of the average quantitative ELISA result for each immunoglobulin over the patient's average total protein content. This ensured a standardised approach to exclude variable dilution factors of cervico- vaginal secretions. Results presented in the tables in this chapter are the median ratios for each group.

The IgM levels in the cervico- vaginal secretions were very low and in many specimens undetectable. These low levels are not thought to be due to a technical problem given the fact that for every IgM ELISA the positive and negative controls ran optimally. As a result of these low levels, the median values for IgM in cervico- vaginal secretions were zero. However, the maximum absolute values (not as a ratio) for IgM in vaginal secretions were looked at in each group instead of the median. This is not a true statistical result but gives an idea of what an IgM response might be in the presence of a more sensitive test.

4.2 Prevalence of STIs and BV

Table I shows the prevalence of sexually transmitted infections diagnosed in the ANC and STD clinic attendees as well as in the control group. The overall prevalence of STIs and BV in

the control group that perceived themselves as not having a genital tract infection was 51.1%. None of them was pregnant. This STI prevalence was significantly higher in the STD clinic attendees. The ANC attendees showed a prevalence of 71% ($p=0.04$).

In the analysis of the immune response parameters, women originally enrolled as controls but with an STI or BV were added to the non-pregnant group with infection. Table II shows the distribution of causes of discharge in the two final groups with infection. When women with BV were excluded, 56 % women had a single infection and 2.4 % a mixed infection. Trichomoniasis was significantly more prevalent in the pregnant as compared to the non-pregnant women ($p=0.0009$) while gonorrhoea was seen with higher frequency in the non-pregnant group ($p=0.01$). Chlamydia infections had a similar prevalence in both groups. BV prevalence was similar in both pregnant and non- pregnant women, however the pregnant women were more likely to have BV associated with another pathogen, in particular *T. vaginalis*.

Table 1: Sexually transmitted pathogens and bacterial vaginosis in STD and ANC clinic attendees and controls.

Number (%) with:				
	<i>N. gonorrhoeae</i>	<i>C. trachomatis</i>	<i>T. vaginalis</i>	BV
Controls (n=45)	5 (11)	1 (2)	0	18 (40)
STD clinic attendees (n=45)	6 (13)	6 (13)	14 (31)	33 (73)
ANC clinic attendees (n=90)	2 (2)	6 (7)	30 (33)	47 (52)

Table II: Causes of vaginal discharge in pregnant and non- pregnant women.

Number (%) with:				
	Total (n=125)	Non- pregnant (n=60)	Pregnant (n=65)	P value
<i>N. gonorrhoeae</i>	13 (10)	11 (18)	2 (3)	0.01
<i>C. trachomatis</i>	13 (10)	7 (12)	6 (9)	
<i>T. vaginalis</i>	44 (35)	14 (23)	30 (46)	0.0009
<i>N. gonorrhoeae</i> + <i>C. trachomatis</i>	1 (1)	1 (2)	0	
<i>N. gonorrhoeae</i> + <i>T. vaginalis</i>	1 (1)	1 (2)	0	
<i>C. trachomatis</i> + <i>T. vaginalis</i>	1 (1)	0	1 (2)	
Bacterial vaginosis (BV)	98 (78)	51 (85)	47 (72)	
BV + any STI pathogen	28 (22)	10 (17)	18 (28)	

was significant in all three groups. The increase in IgM levels was seen in women with BV and in women with multiple STIs. The highest increase in IgM occurred in women with BV only.

Table III: Immunoglobulin ratios in non- pregnant women with a sexually transmitted infection and the control group.

Median (range) of Ig/ protein ratio:				
		IgG	IgA	IgM
Serum	Controls (n=45)	11.8(5.96-18.3)	1.3(0.85-2.24)	0.6(0.17-4.25)
	STIs (n=60)	18.9(0.55-100)	2.3(0-35)	0.6(0.12-10)
Cervico- vaginal secretions	Controls (n=45)	0.015(0.0067-0.117)	0.057(0.027-0.267)	0.003*
	STIs (n=60)	0.009(0.001-0.197)	0.023(0-0.556)	0.086*

* values given are maximum levels of IgM in each group and not median ratios.

Table IVa: Ig/ protein ratios in serum of women with single and multiple STIs and bacterial vaginosis.

Median (range) of Ig/ protein ratio:			
	IgG	IgA	IgM
Control group (n=44)	12.7(5.22-23.6)	1.3(0.19-3.83)	0.5(0.04-4.25)
Women with a single STI (n=22)	12.9(6.67-8130)	1.71(0.31-857.5)	0.6(0.14-5653.8)
Women with multiple STIs (n=48)	17.7(5.6-37.3)	2.5(0-24.1)	0.6(0.13-3.18)
Women with BV (n=55)	13.8(0.55-71.3)	1.9(0.64-5.6)	0.7(0.03-3.16)333

Table IVb: Ig/ protein ratios in cervico- vaginal secretions in women with single and multiple STIs and bacterial vaginosis.

Median (range) of Ig/ protein ratio:			
	IgG	IgA	IgM*
Control Group (n=44)	0.013(0.0065-0.12)	0.046(0.003-0.27)	0.004
Women with a single STI (n=22)	0.012(0.0046-0.06)	0.016(0.005-0.17)	0.013
Women with multiple STIs (n=48)	0.011(0.002-0.2)	0.014(0-0.56)	0.004
Women with BV (n=55)	0.009(0.0014-0.91)	0.02(0.002-2.29)	0.086

* values given are maximum levels of IgM in each group and not median ratios.

4.5 Immunoglobulin ratios in women with *N.gonorrhoeae* and *C.trachomatis* infection versus those with trichomoniasis and BV

To explore whether there is a difference in humoral immune response if the infection originates in the cervix or the vagina, a comparison was made between immunoglobulin ratios in women with *N. gonorrhoeae* or *C. trachomatis* infection and those with trichomoniasis and BV. When compared to healthy women, serum immunoglobulins showed no change in the presence of cervical pathogens. However, serum IgG ($p=0.04$) and IgA ($p<0.0001$) were significantly increased in the presence of vaginal pathogens (Table Va). Similarly, in cervical secretions, cervical pathogens do not alter immunoglobulin ratios. IgG ($p=0.0002$) and IgA ($p<0.0001$), in the presence of a vaginal pathogen, is significantly reduced when compared to healthy women. IgM shows an increase but this was not significant.

When cervical pathogens were compared with vaginal pathogens, serum immunoglobulins showed no change. However, in cervico- vaginal secretions, IgG ($p=0.008$) and IgA ($p=0.005$) are significantly reduced in the presence of vaginal pathogens. IgM shows a higher increase in the presence of a vaginal pathogen but this was not significant.

Table Va: Immunoglobulin ratios (serum) in women with *N. gonorrhoeae* and/ or *C. trachomatis* infection versus those with *T. vaginalis* and/ or BV.

Median (range) of Ig/protein ratio			
	IgG	IgA	IgM
Control group (n=44)	12.7(5.2-23.6)	1.32(0.19-3.83)	0.48(0.04-4.25)
Women with <i>N. gonorrhoeae</i> / <i>C. trachomatis</i> (n=8)	11.4(6.67-8130)	1.55(0.31-857)	0.7(0.14-5653.8)
Women with <i>T. vaginalis</i> / BV (n=88)	14.4(0.55-71.32)	1.97(0.43-5.61)	0.62(0.03-3.18)

Table Vb: Immunoglobulin ratios (cervico- vaginal secretions) in women with *N. gonorrhoeae* and/ or *C. trachomatis* infection versus those with *T. vaginalis* and/ or BV.

Median (range) of Ig/protein ratios:			
	IgG	IgA	IgM
Control group (n=44)	0.013(0.006-0.12)	0.05(0.003-0.27)	0.0041*
Women with <i>N. gonorrhoeae</i> / <i>C. trachomatis</i> (n=8)	0.04(0.01-0.1)	0.09(0.01-0.56)	0*
Women with <i>T. vaginalis</i> / BV (n=88)	0.01(0-0.91)	0.02(0-2.29)	0.09*

* values given are maximum levels of IgM in each group and not median ratios,

4.6 Immunoglobulin ratios in pregnancy

In the absence of one of the causes of abnormal genital discharge, serum immunoglobulins during pregnancy showed no change but the cervico- vaginal secretions showed a significant decrease in IgA levels in the pregnant non-STD women (Table VIa; $p= 0.003$). The trend observed in IgM was similar, with lower values in the vaginal secretions of pregnant women (Table VI).

A change in immunoglobulins in pregnant women with an STD was seen when the non-pregnant women with an STD were compared to the pregnant women with an STD. In serum the IgG and IgA were significantly increased in the non-pregnant STD group (Table VIb; $p < 0.001$ and $p= 0.02$ respectively). The vaginal secretions showed the opposite with a significantly increased IgG in pregnant women with an STD (Table VI; $p= 0.04$). The increased IgG was still, however lower than the control group.

Differences among pregnant women were determined by looking at pregnant women with an STD compared with pregnant women without an STD. IgA, in serum, was increased in pregnant women with an STD (Table VI; $p= 0.009$; Fig 10). In vaginal secretions, the opposite was seen. IgA was significantly decreased in pregnant women with an STD (Table VIc; $p= 0.001$; Fig 13) but IgM showed a trend in the opposite direction (Fig 14). Pregnant women with an STD have much higher IgM levels than pregnant women without an STD (Table VIc).

Table VIa: Immunoglobulin ratios in pregnant and non- pregnant women without STIs.

Median (range) of Ig/ protein ratio:				
		IgG	IgA	IgM
Serum	Control (n=45)	11.8(5.96-18.3)	1.3(0.9-2.2)	0.6(0.17-4.3)
	Pregnant (n=65)	13(5.2-23.6)	1.2(0.19-3.8)	0.5(0.04-2.8)
Cervico-vaginal secretions	Control (n=45)	0.015(0.007-0.12)	0.057(0.03-0.27)	0.003*
	Pregnant (n=65)	0.012(0.007-0.04)	0.037(0.003-0.11)	0.004*

* values given are maximum levels of IgM in each group and not median ratios.

Table VIb: Immunoglobulin ratios in pregnant and non- pregnant women with STIs.

Median (range) of Ig/ protein ratio:				
		IgG	IgA	IgM
Serum	STIs (n=60)	18.9((0.55-100)	2.3(0-35)	0.6(0.12-10)
	Pregnant STIs (n=65)	13.1(5.6-25.8)	2.0(0.31-24.1)	0.6(0.03-3.2)
Cervico-vaginal secretions	STIs (n=45)	0.009(0.001-0.2)	0.023(0-0.56)	33.6*
	Pregnant STIs (n=65)	0.012(0.004-0.3)	0.016(0.001-1)	21.2*

* values given are maximum levels of IgM in each group and not median ratios.

Table VIc: Immunoglobulin ratios in pregnant women with and without STIs.

Median (range) Ig/ protein ratio:				
		IgG	IgA	IgM
Serum	Pregnant (n=22)	13.0(5.2-23.6)	1.2(0.19-3.8)	0.5(0.04-2.8)
	Pregnant STIs (n=65)	13.1(5.6-25.8)	2.0(0.31-24.1)	0.6(0.03-3.2)
Cervico-vaginal secretions	Pregnant (n=22)	0.012(0.007-0.04)	0.037(0.003- 0.011)	3.7*
	Pregnant STIs (n=65)	0.012(0.004-0.3)	0.016(0.001-1)	21.2*

* values given are maximum levels of IgM in each group and not median ratios.

4.7 Extreme Values in Serum and Vaginal Secretions

Extreme values were observed for two subjects. In one subject's serum sample, all immunoglobulin levels were extremely high. This subject was positive for only one STI pathogen, *N. gonorrhoea*, and was not pregnant.

The other subject had extreme values for IgG and IgA in her vaginal secretions. This subject was positive only for BV and was pregnant.

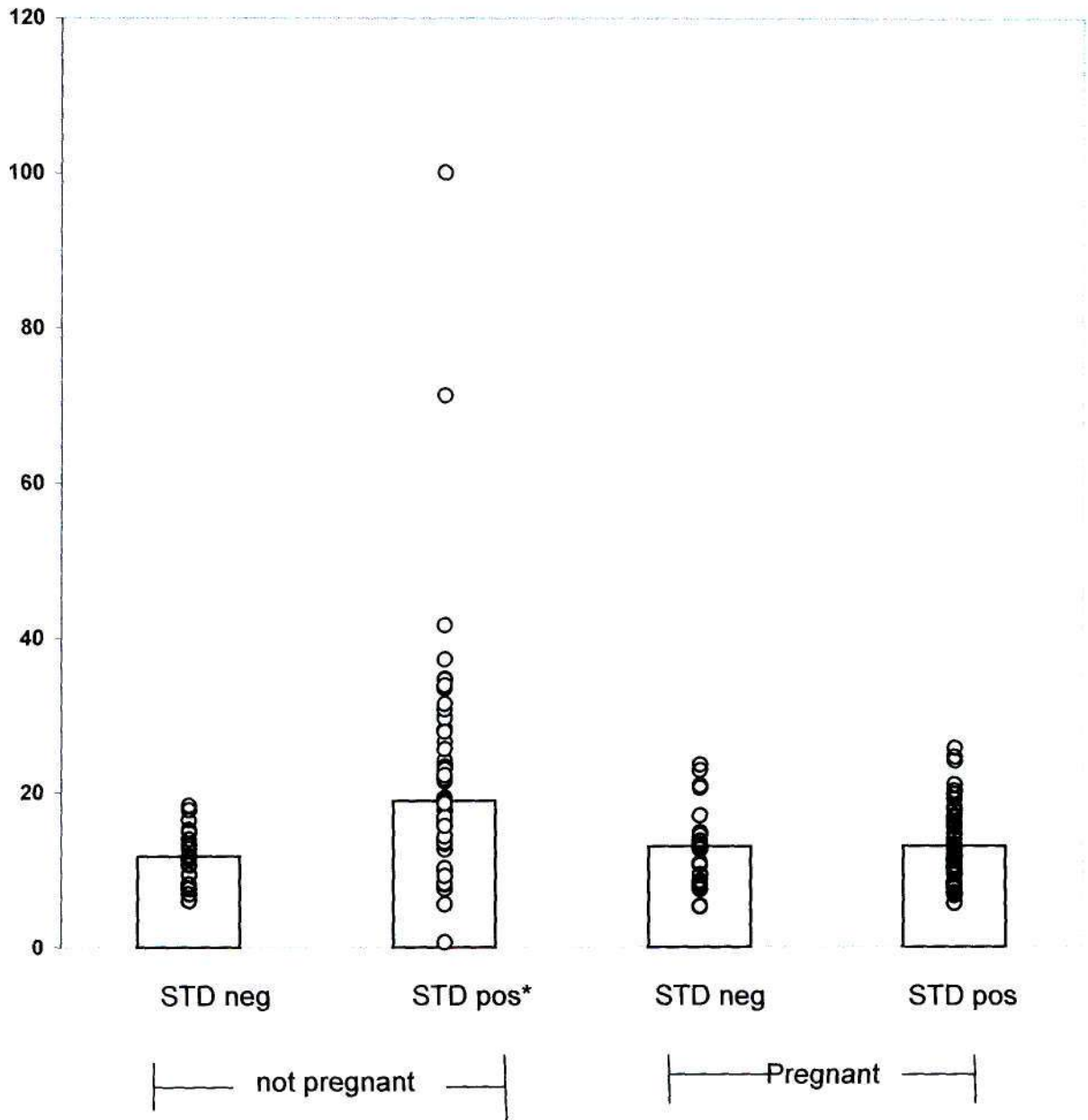


Figure 9. Serum ratios for IgG for each group.

* extreme values plotted at 100 ; actual value 8130.

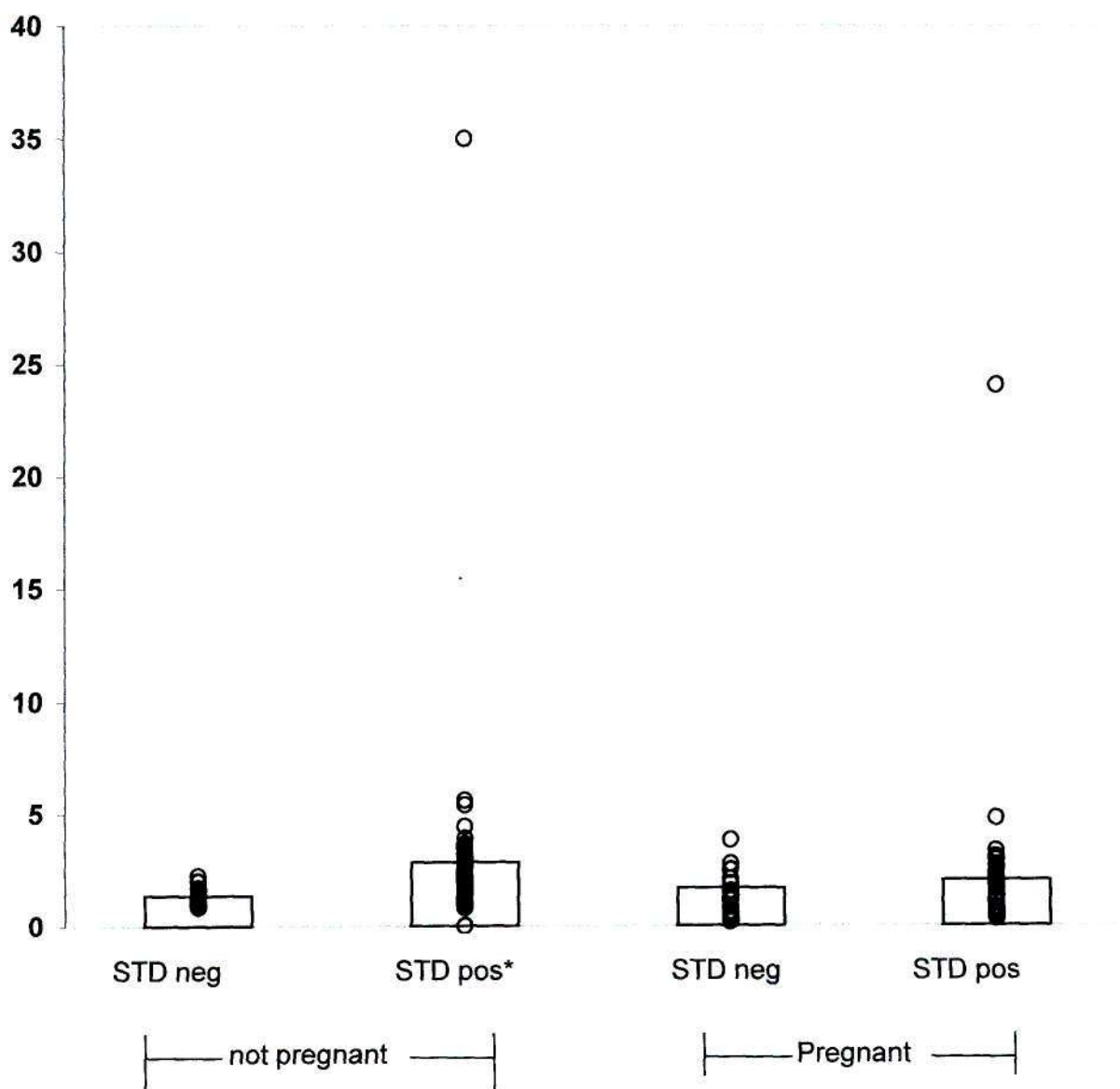


Figure 10. Serum ratios for IgA for each group.

* extreme values plotted at 35 ; actual value 857.

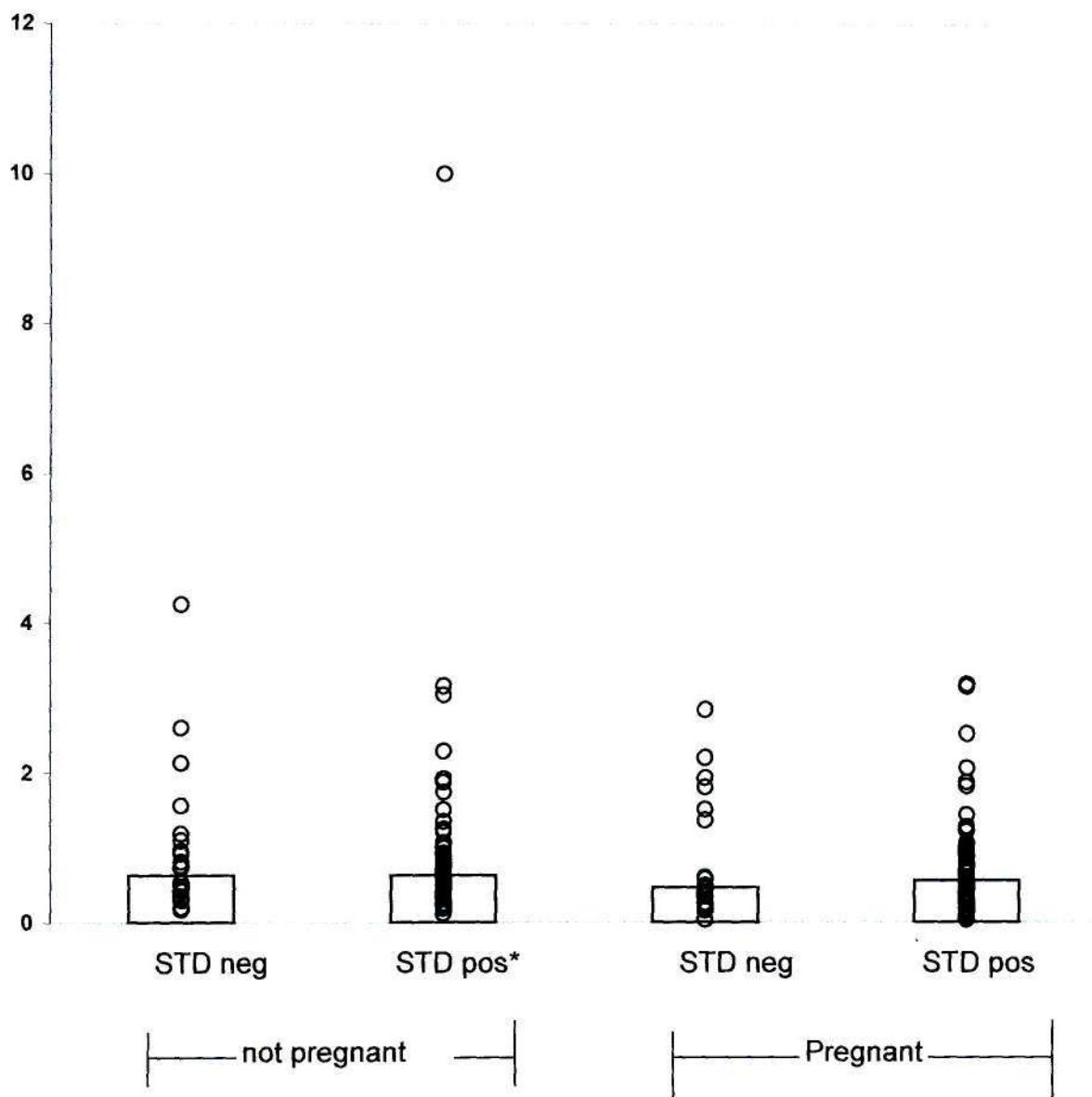


Figure 11. Serum ratios for IgM for each group.

* extreme value plotted at 10 ; actual value 5654.

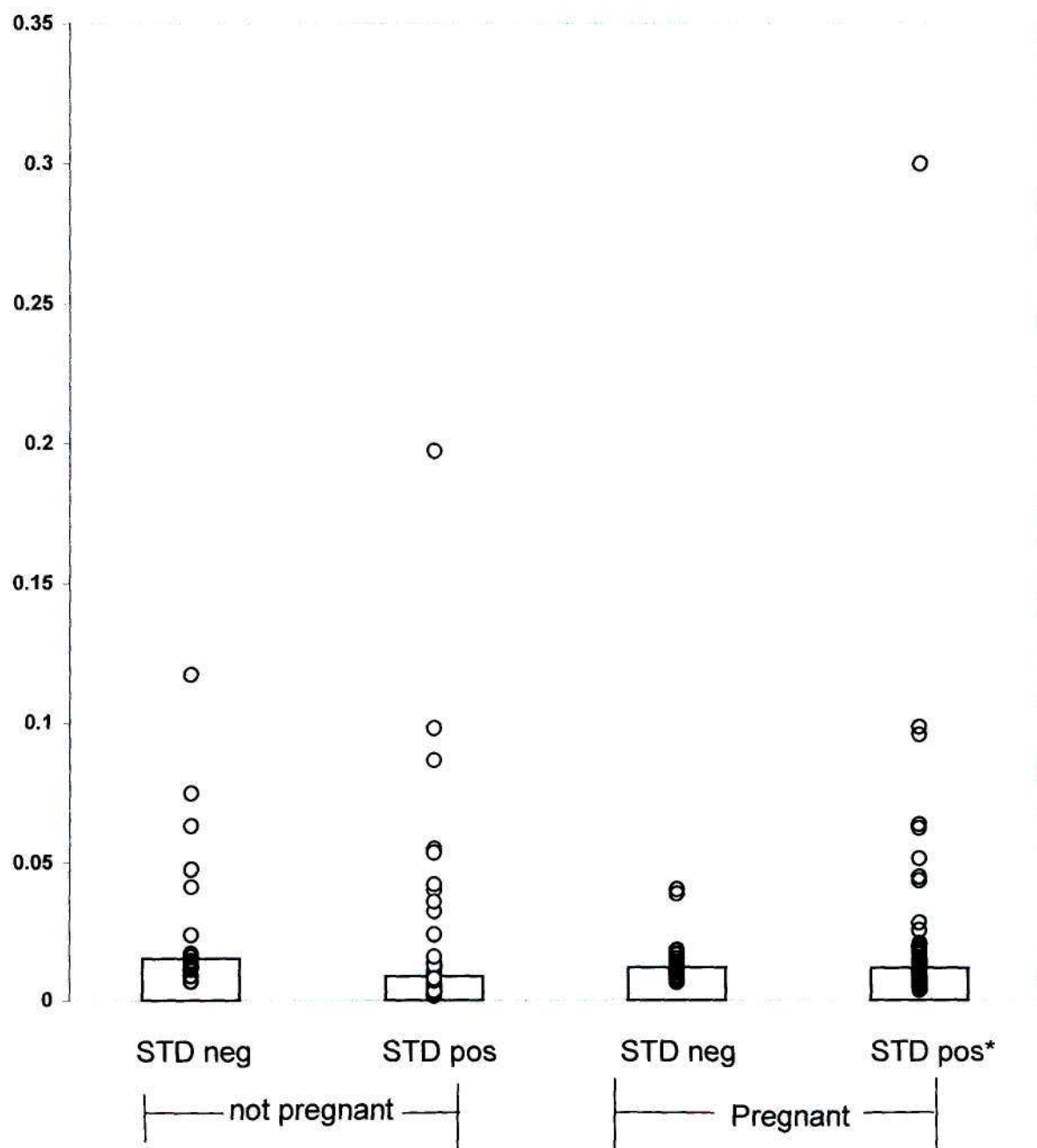


Figure 12. IgG ratios in cervico- vaginal secretions for each group.

* extreme values plotted at 0.3; actual value 0.9.

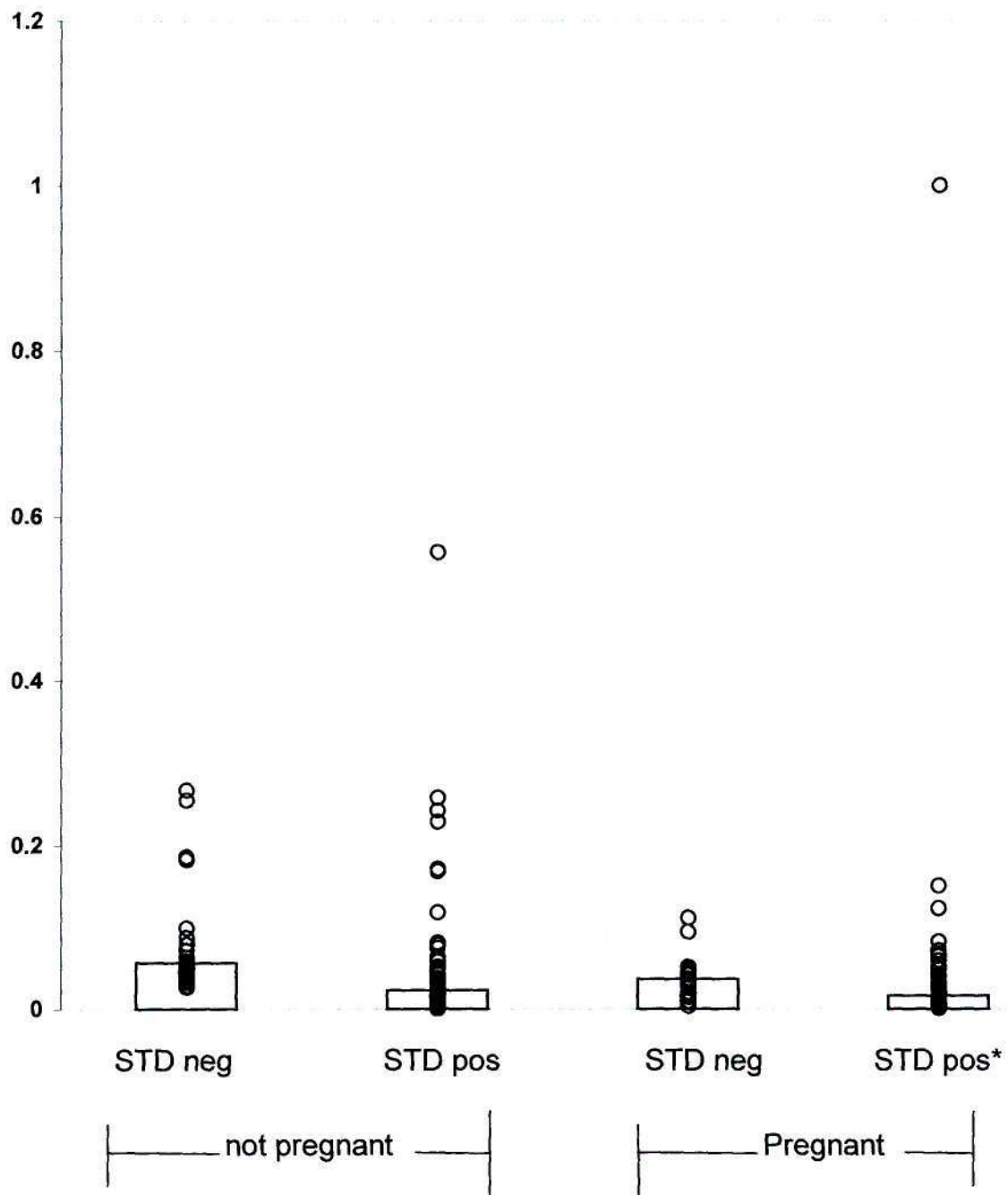


Figure 13. IgA ratios in cervico- vaginal secretions for each group.

* extreme values plotted at 1.0 : actual value 2.3.

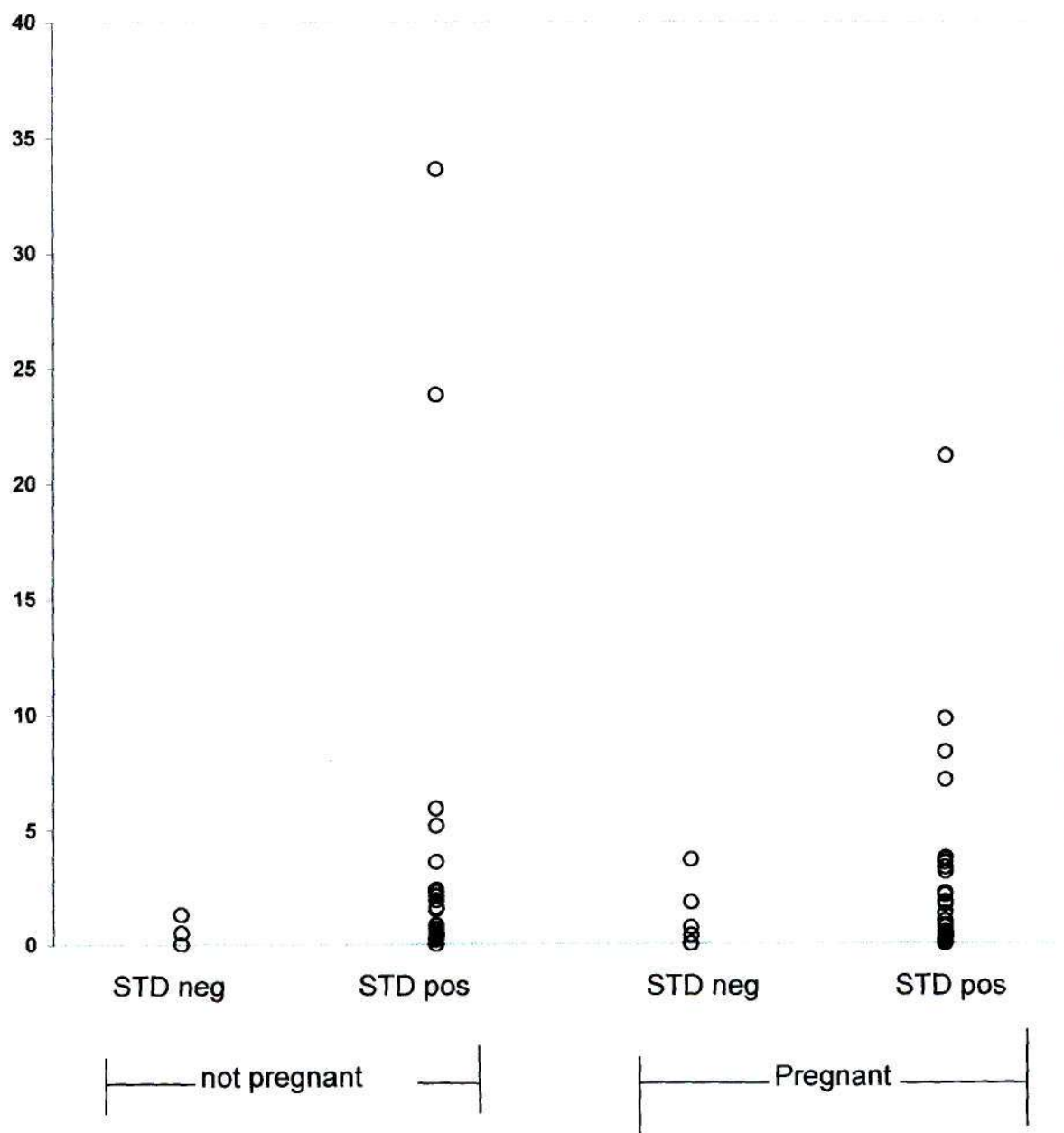


Figure 14 IgM values(ug/ml) in cervico-vaginal secretions for all groups.

CHAPTER FIVE

5.0 DISCUSSION

All women recruited for the study were in the age group ranging from 20-45. Pregnancy testing was not performed for the control group, however there were no births in this group during the study period thereby ruling out early gestation. The majority of these women were familiar with the use of a tampon and those who were not were given assistance. For this study it was not necessary to obtain vaginal secretions from a specific site in the vagina but rather to collect all secretions present in the vagina. Previous studies have described different methods for the collection of vaginal secretions e.g. cervical- vaginal lavage (Bélec *et al.*, 1995), Sno-strips and wicks (Quesnel *et al.*, 1997), and the use of a plastic transfer pipette (Kutteh *et al.*, 1996). However, lavage involves considerable dilution, the Sno-strips are difficult to handle due to their pliability, wicks can be contaminated by blood staining and the pipette method is only feasible during days of the cycle near ovulation. All secretions obtained via tampon were free from blood staining, easy to use and subject to a total protein determination to standardise results.

Specimens were collected from women at any time during their cycle except when they were menstruating. Wide variations seen in the immunoglobulin values in the vaginal secretions among women in the same group can be attributed to this fact. It has been observed that oestrogen and progesterone have an effect on vaginal immunity (Schumacher, 1980). Therefore variations in the oestrogen and progesterone levels during

the menstrual cycle have an impact on immunoglobulin levels. A recent study also reported large individual variations in immunoglobulin levels (Bard *et al.*, 2002).

There are no obvious explanations for the two subjects with extreme values in their serum and cervico- vaginal secretions. It could be that their infections were very severe and caused a pronounced immune response. Alternatively, they could have been co-infected with some other infection other than those tested for.

When comparing the IgA and IgG levels in control groups it is found that IgA is predominant in cervico- vaginal secretions and IgG is predominant in serum. There have been variable reports about the presence of various immunoglobulin subclasses in human female genital secretions. Waldman *et. al.* (1971) found IgA to be predominant while Hocini *et al.* (1995) reported a predominance of IgG in normal vaginal fluid. Such discrepancies probably reflect both differences in the applied sampling method and individual variables such as age and phase of menstrual cycle.

All groups, when compared to the control group, have significantly lower IgA levels in cervico- vaginal secretions. For IgA, both pregnancy and the presence of an STI, lower IgA levels. In pregnant women IgA levels are further lowered with the presence of a STI.

In serum IgA behaves similarly to IgG and both pregnant and non-pregnant women with an STI have significantly higher Ab levels. This increase is due solely to the presence of an STI.

Women with an STI show significantly decreased IgG in cervico- vaginal secretions with this effect being greater in non- pregnant women. In the absence of an STI there is no significant difference between non-pregnant and pregnant women. However, pregnancy does cause a slightly lower IgG level. It appears the STI is the significant cause for the decrease and that pregnancy has some effect but not significantly so. The opposite response is seen in serum. IgG is increased in the presence of an STI. This is true only for the non-pregnant women with an STI. Pregnancy, regardless of an STI, has no effect on IgG in serum.

IgM behaves differently to IgG and IgA in cervico- vaginal secretions. Although IgM in the cervico- vaginal secretions is mostly too low to be detectable, the highest absolute values are in those women with an STI (pregnant and non- pregnant). There appears to be an effect of pregnancy. Detectable IgM levels shows lower values in pregnant women without an STI, however, in the presence of an STI both pregnant and non-pregnant women have much higher values. This increase is greater in non-pregnant women. Serum IgM levels shows no change regardless of pregnancy or the presence of an STI.

In serum, immunoglobulin levels are unaltered in women regardless of pregnancy if they are free of an STI. In keeping with other studies it is shown that pregnancy has no effect on serum immunoglobulins. A study by Xusheng Lü *et al.* (1999) showed that in rhesus macaques, ovarian hormones that drive the menstrual cycle do not affect systemic immunoglobulin or antibody levels.

However, in the presence of an STI, IgA and IgG are increased. It was to be expected that serum immunoglobulin levels would be raised in non-pregnant women with an STI. This is indicative of an immune response. However, it is interesting to note that serum IgM levels were unchanged. This could be attributed to the nature of the STI in those women infected. If the infection was not recent then it follows that secondary immunity has developed bringing about the increased IgG values.

The opposite of the systemic response is seen to take place in the cervico- vaginal secretions. IgA is decreased in all groups when compared to the control group and IgG is decreased in the STI groups. This is contrary to what has been reported in previous studies where colonisation with a bacteria has resulted in an increase in IgG and IgA with either IgG or IgA predominating. *Chlamydia trachomatis* has been reported to act particularly stimulatory on the appearance of sIgA in cervicovaginal secretions (Heisterberg *et al.* 1987).

It is quite clear that pregnancy does have an effect on immunoglobulin levels. This is especially so for IgA in the cervico- vaginal secretions. One possible explanation for the decreased IgA and slightly decreased IgG is the effect of the reproductive hormones. Oestrous-cycle-dependent changes have been documented in the immune cell population of the rat uterus and vagina (Kaushic *et al.*, 1998). It has been reported that the secretion of IgG and IgA into vaginal fluids was reduced in the presence of high levels of oestrogen (Schumacher, 1980; Wira *et al.*, 1994). The decrease in IgA in vaginal secretions during pregnancy could be caused by a shift in immunity away from the vagina towards the uterus. Oestrogen has a stimulatory effect on immunoglobulins in the uterus (Wira *et al.*,

1994) and the opposite effect in the vagina (Wira *et al.*, 1994). Therefore, the decrease in IgA can be explained by the hormonal effect of pregnancy.

However, a recent study looking at the number and distribution of immune cells in the cervico- vaginal mucosa of rhesus macaques, showed that steroid hormones do not influence genital mucosal immunity by changing the number or distribution of immune cells in the lower reproductive tract (Ma *et al.*, 2001). This would explain why there is no significant effect of pregnancy on IgG and IgM in vaginal secretions. However it does not explain the exception in the significantly decreased IgA levels during pregnancy. Whether pregnancy influences IgA solely, and how, is unclear.

An interesting observation is the trend of higher IgM levels in the STI groups in cervico- vaginal secretions. It seems that IgM offers protection against invading pathogens at the site of infection. One hypothesis could be that IgM offers a primary immune response with antibodies arising from the vaginal mucosa itself. Thereafter secondary immunity is more a systemic response and hence the decreased IgG and IgA in the cervico- vaginal secretions but elevated levels in the serum. This primary immune response might explain why IgM levels are so low in the cervico- vaginal secretions since a primary immune response does not last long, a few days at most. If this is so, then IgM is being cleared and is therefore not easily detectable.

There has been much debate about the derivation of vaginal antibodies. It is believed that through functional compartmentalization of its afferent and efferent limbs, MALT is to a significant extent independent of the systemic immune apparatus. In humans the fallopian tubes, cervix and the vagina have been reported to contain sub-epithelial immunoglobulin-

producing immunocytes (Kutteh *et al.*, 1988). This study shows that while there is an increase in immunoglobulins in serum, the opposite is seen in vaginal secretions. This supports the theory of local antibody production. Here, an increase in IgG and IgA in serum does not cause any increase in the cervico- vaginal secretions significant or otherwise. The higher IgM levels in the cervico- vaginal secretions of women with an STI would therefore be locally produced since there is no change seen in serum IgM.

Bacterial vaginosis is the most common cause of vaginal discharge, although its classification as a true STI pathogen is unclear. It was necessary to determine the role, if any, of BV on immunoglobulin levels. The above results show that BV does play a very significant role in serum and vaginal immunoglobulin levels.

It is shown that BV has an interesting effect on IgG and IgM. In serum IgG was significantly increased only in the presence of BV and multiple infections. Single infections without BV did not alter IgG levels but did alter IgA levels. IgM levels remained unchanged regardless of the presence of an infection.

In cervico- vaginal secretions, IgG was significantly decreased, again, only in the presence of BV and multiple infections. The highest increase in IgM was in the presence of BV.

BV has a definite effect on IgG. Why this is so is unclear but it would be interesting to explore the pathophysiology in relation to this immunoglobulin. It has been reported that the presence of sialidase, a hydrolytic enzyme produced by BV- associated bacteria, has an effect on anti- *Gardnerella vaginalis* IgA (Cauci *et al.*, 2002). Sialidase cleaves the terminal sialic acid from glycoproteins, including immunoglobulins and especially IgA.

Thus, sialidases may potentially hamper the local mucosal immune response. This could explain the decreased IgA and IgG levels found in cervico- vaginal fluid in the presence of an STI, especially BV. Why this effect cannot be seen for IgM is unclear. It could be that IgM is structurally different to IgA and IgG. IgM is bigger than the other two immunoglobulins and this could offer some protection against hydrolytic activity.

Although BV is seen to have a significant effect on all three immunoglobulins, other single infections also show a similar pattern. It is hard to determine whether the stage of infection in women with other single infections affects the significance of the results. Especially when considering an STI like syphilis which has various stages of infection. However, whether BV alone has an effect on immunoglobulin levels is unclear given that sample size would be too small to make a definite conclusion. It is clear though that all the STI pathogens follow a similar trend and each pathogen has an impact on immunoglobulin levels.

The analysis of cervical and vaginal pathogens yielded interesting results. It appeared, from the numbers per group, that the tampon specimen picked up mainly vaginal pathogens. When compared to healthy women, vaginal pathogens, unlike cervical pathogens, elicited an immune response in serum. It is interesting that only the vaginal pathogens alter serum immunoglobulins. Vaginal pathogens lowers IgG and IgA levels much more than cervical pathogens in cervico-vaginal secretions. This effect further supports the theory of compartmentalisation. The higher IgM levels in the presence of vaginal pathogens helps prove this too.

Overall the results do not suggest that pregnancy has some effect on immunoglobulin levels except for IgA in cervico vaginal secretions. Changes in immunoglobulin levels are seen regardless of pregnancy and are as a result of the presence of an STI. The only exception is seen in the significantly decreased vaginal IgA levels in pregnant women without an STI. Why the presence of an STI brings about a decrease in vaginal IgG and IgA is unclear. A primary IgM response is seen but thereafter what happens is unknown.

The contrasting results in this study compared to other studies, especially with regard to cervico- vaginal immunoglobulin results, can be attributed to the type of pathogens studied and their affect on each other. Other studies have mainly looked at non- STI pathogens, predominantly streptococcus. The effect of the human immunodeficiency virus (HIV) has not been accounted for. Women in this study were not divided into HIV groups so the effect of HIV is unknown. Whether the contrasting results can be attributed to the effects of HIV needs to be determined. How HIV alters the immune response, if at all, and especially its effect on STI pathogens is a possible avenue for further study. These inter-relationships would provide a clearer picture of vaginal immunity especially considering the high prevalence of HIV in our local setting.

CHAPTER SIX

6.0 Conclusion

The results presented above suggest a distinct immune response in the vaginal mucosa compared to a systemic response. STIs induce a normal systemic Ab response but a reversed IgG and IgA response at the mucosal level. While pregnancy does not alter systemic responses it does have an effect on mucosal immunity, predominantly IgA.

The effect of BV also warrants further attention. It is clear that BV has a significant effect on mucosal immunity and considering its association with poor pregnancy outcome, further understanding of BV interactions at the mucosal level would be beneficial. Systemic and mucosal IgG and IgA responses to both STIs and BV showed opposite trends. This indicates a compartmentalised effect and might explain why organisms that cause STIs are such successful pathogens.

The mucosal immune system should be most efficient in providing protection against pathogens and generating longer-lasting protection through using attenuated pathogens for vaccine purposes. This is the ultimate aim but it is necessary to first understand the effect of different pathogens on the mucosal immune system. Further studies assessing mucosal immunity in relation to pathogens and especially looking at the effect of HIV would improve the understanding of mucosal immunity.

CHAPTER SEVEN

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CHAPTER EIGHT

8.0 Appendix One

1. Coating Buffer, pH 9.6

- Dissolve 2.93 g Sodium Hydrogen Carbonate (NaHCO_3 ; Merck, South Africa) in 500 ml distilled water.
- Add 1.59 g Sodium Carbonate Anhydrous (Na_2CO_3 ; Merck, South Africa) and stir until dissolved.
- Add 0.20 g Sodium Azide (NaN_3 ; Sigma, Germany) and stir until dissolved.
- Check pH and adjust with 5M Hydrochloric Acid (HCL; Sigma, Germany) if necessary.
- Make up to one litre.
- Expiry date is two weeks after preparation. Store at 4 – 8 °C.

2. Phosphate Buffered Saline (PBS)

- Dissolve 1 PBS tablet in 100 ml of distilled water.
- Autoclave for 30 mins and store at 4 – 8 °C.