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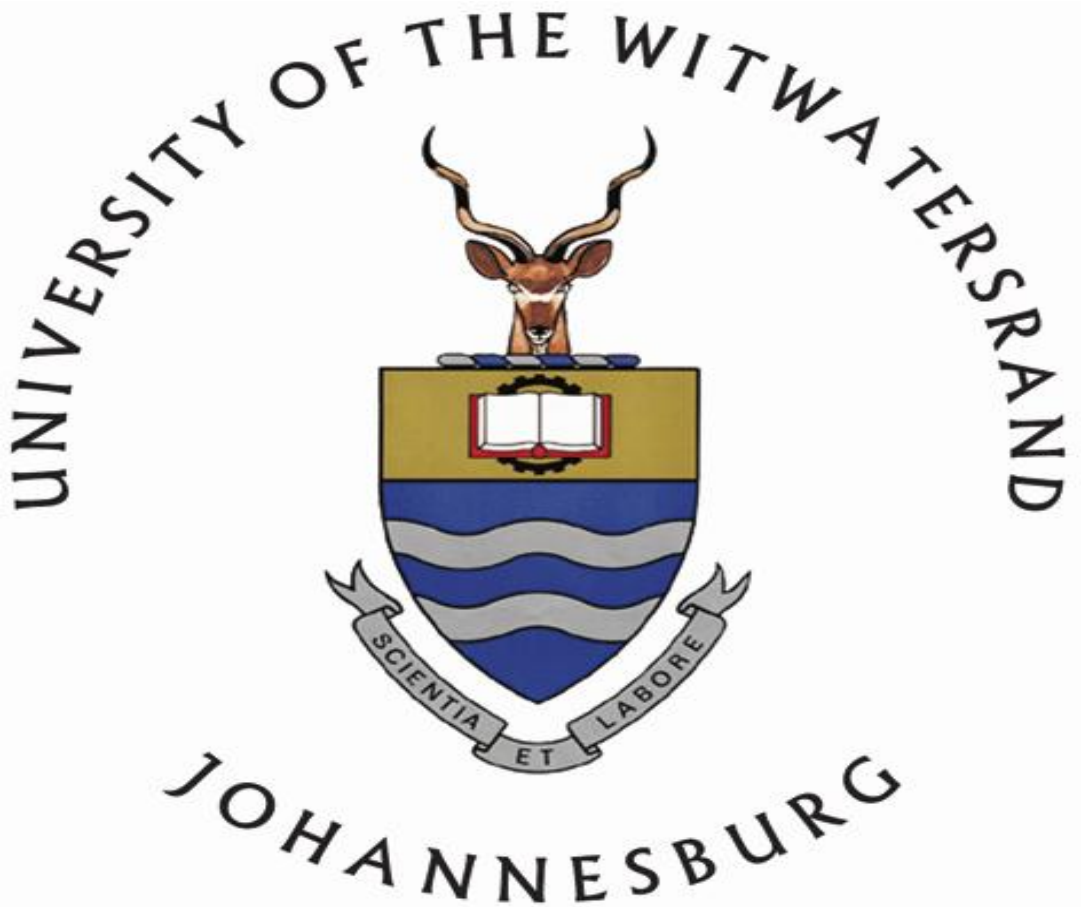
Establishment of a flow cytometric assay in the setting of renal transplant for T and B cell crossmatching

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ESTABLISHMENT OF A FLOW CYTOMETRIC ASSAY IN THE SETTING OF RENAL TRANSPLANT FOR T AND B CELL CROSSMATCHING



Narisha Ramparsad

A research report submitted to the Faculty of Health Sciences, University of Witwatersrand, Johannesburg, in partial fulfillment of the requirements for the degree

Of

Master Medicine in the branch of Haematology

Declaration

I, Narisha Ramparsad declare that this research report is my own work. It is being submitted for the degree of Master of Medicine (Haematology) at the University of the Witwatersrand, Johannesburg. This work has not been submitted before for any degree or examination at this or any other university.

This 3rd day of April 2012



Narisha Ramparsad

Ethics Approval

Approval for this research was granted by the University of the Witwatersrand Human Research Ethics Committee (Protocol Number: M080218).

Acknowledgements

1. Professor Wendy Stevens (Supervisor, Department of Molecular Medicine and Haematology)
2. Dr Elizabeth Mayne and Dr Melinda Suchard (Department of Molecular Medicine and Haematology)
3. Professor Russell Britz (Department of Surgery, Renal Transplant Unit)
4. Mr. Derrick Nelson and Dr Robert Crookes (South African National Blood Transfusion Service
5. Dr G Paget, Dr T Gerntholtz, Dr M Mashabane, Dr V Rekviashvilli, Dr B Bloy, Dr S Wadee (Adult Nephrology)
6. Dr D Hahn (Paediatric Nephrology)
7. Patricia Wyllie (Donald Gordon Medical Centre)
8. Dr Raymond Fernando and Dr Henry Stephens (Anthony Nolan Trust in Hampstead, United Kingdom)
9. Flow cytometry unit (Charlotte Maxeke Johannesburg Academic Hospital)
10. Patients of the Renal Transplant Units

Dedication

To my husband, son and mother

&

In memory of my father,

Krushna Ramparsad

Presentations

Poster presentations

1. Establishment of Flow cytometric crossmatching in renal transplantation, PathTrack Congress, 2008, Cape Town

2. Flow Cytometric Crossmatching in Renal Transplantation:

   The Johannesburg experience, PathTech Congress, 2009, Durban

Abstract

Donor specific crossmatching is performed prior to renal transplantation in order to determine the presence of pre-existing antibodies against donor HLA antigens which can result in hyperacute rejection. Flow cytometric crossmatching is reported in the literature to be a more sensitive and objective method of testing than the complement dependent cytotoxicity (CDC) method that is currently used in the Gauteng Province.

A prospective analysis of the flow cytomeric crossmatch (FCXM) assay using the Luminex technology as the reference method was conducted. Forty-three samples were analysed. The T cell crossmatch (using a cutoff value of 2) revealed a sensitivity of 66.7%, a specificity of 83.8%, a positive predictive value (PPV) of 40% and negative predictive value (NPV) of 93.9%. The B cell crossmatch (using a cutoff value of 5) gave a sensitivity of 100%, specificity of 92.7%, and a PPV and NPV of 40 and100%, respectively.

In addition, a retrospective analysis of clinical data for all patients transplanted during the period January 2008 to May 2009 was performed. Of a total of 50 patients assessed post transplant, none of the patients showed signs of hyperacute rejection, while twelve percent (12%) of patients revealed signs and symptoms suggestive of acute rejection.

The validation of the flow cytometric crossmatch analysis was complex as there is no gold standard reference method. The assay was validated based on the clinical relevance of its high negative predictive value and the absence of hyperacute rejections in the clinical follow up. The rate of acute rejection found in this study is similar to that reported in literature.

Key Words

Donor specific crossmatching

Flow cytometric crossmatching

Complement dependent cytotoxic assay

Luminex technology

Sensitivity, specificity, negative predictive value, positive predictive value

Acute and hyperacute rejection

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List of Abbreviations

HLA:	Human Leucocyte Antigen
MHC:	Major Histocompatibility Complex
MIC –A:	Major Histocompatibility Class I related Chain A
UNOS:	United Network of Organ Sharing
EFI:	European Federation of Immunogenetics
ASHI:	American Society of Histocompatibility and Immunogenetics
CDC:	Complement Dependent Cytotoxicity
AHG:	Anti- Human Globulin
FCXM:	Flow cytometric cross-match
SANBS:	South African National Blood Services
PRA:	Panel Reactive Antibodies
ELISA:	Enzyme Linked Immuno-Sorbent Assay
MFI/s:	Mean Flourescence Intensity/Intensities
DSA:	Donor Specific Antibodies
ELISPOT:	Enzyme Linked Immunosorbent Spot Assay
PBMCs:	Peripheral Blood Mononuclear Cells
CKD:	Chronic Kidney Disease
GFR:	Glomerular Filtration Rate
ESRD:	End Stage Renal Disease
HIVAN:	HIV associated nephropathy
HAART:	Highly Active Anti- Retroviral Therapy
AMAR:	Antibody Mediated Acute Rejection
Tx:	Transplant

ACD:	Acid Citrate Dextrose
SOP/s:	Standard Operating Procedure/s
WBCs:	White Blood Cells
ALG:	Anti- Lymphocyte Globulin
PBS:	Phosphate Buffered Saline
BC:	Beckman Coulter
BD:	Becton Dickinson
EQA:	External Quality Assurance
NPV:	Negative Predictive Value
PPV:	Positive Predictive Value
CMJAH:	Charlotte Maxeke Johannesburg Academic Hospital
CHB:	Chris Hani Baragwanath
HJH:	Helen Joseph Hospital
MMF:	Mycofenalate Mofetil
CYA:	Cyclosporin
ATN:	Acute Tubular Necrosis

1.0 Introduction

The field of transplantation encompasses two broad areas: the transplanting of solid organs and the transplanting of tissues (Britz, 2007). Solid organ transplantation includes kidney, heart, small bowel, lung and liver, while tissue transplantation includes corneas, bone marrow, skin, stem cells etc. (Britz, 2007). Donations of the allografts may be obtained from cadavers, related living donors or non- related living donors.

In the context of the South African transplant program, organ transplantation occurs in Bloemfontein, Cape Town, Durban, Johannesburg and Tshwane. Within Johannesburg, renal transplants are performed at the Charlotte Maxeke Johannesburg Academic Hospital, the Donald Gordon Medical Centre and by the Netcare group which includes Garden City Clinic, Milpark Hospital and Olivedale Clinic. Renal transplantation comprises the bulk of solid organ transplantation in South Africa. According to the Organ Donor Foundation, the most recent statistics for organ transplantation in South Africa revealed that the majority of transplants occur in Cape Town (42%) and Johannesburg (39%) (http://www.odf.org.za). In 2009, of the 376 solid organs transplanted, 77.1% (290 of 376) were renal transplants (http://www.odf.org.za). Between 2004 and 2009 more than 200 renal transplantswere performed annually (http://www.odf.org.za). See figures 1, 2 and 3 below.

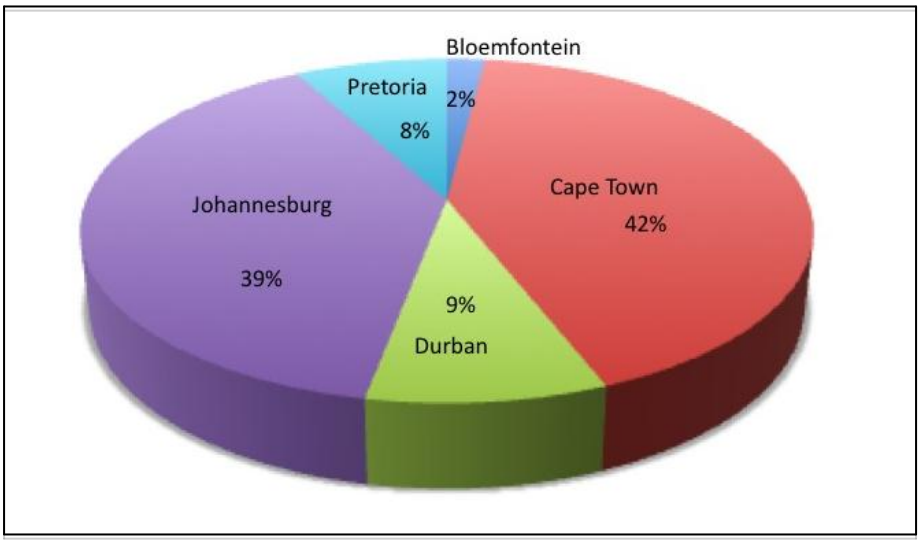


Figure 1: Pie Graph depicting the percentages of organ transplantation as performed in various regions in South Africa during 2009 (http://www.odf.org.za)

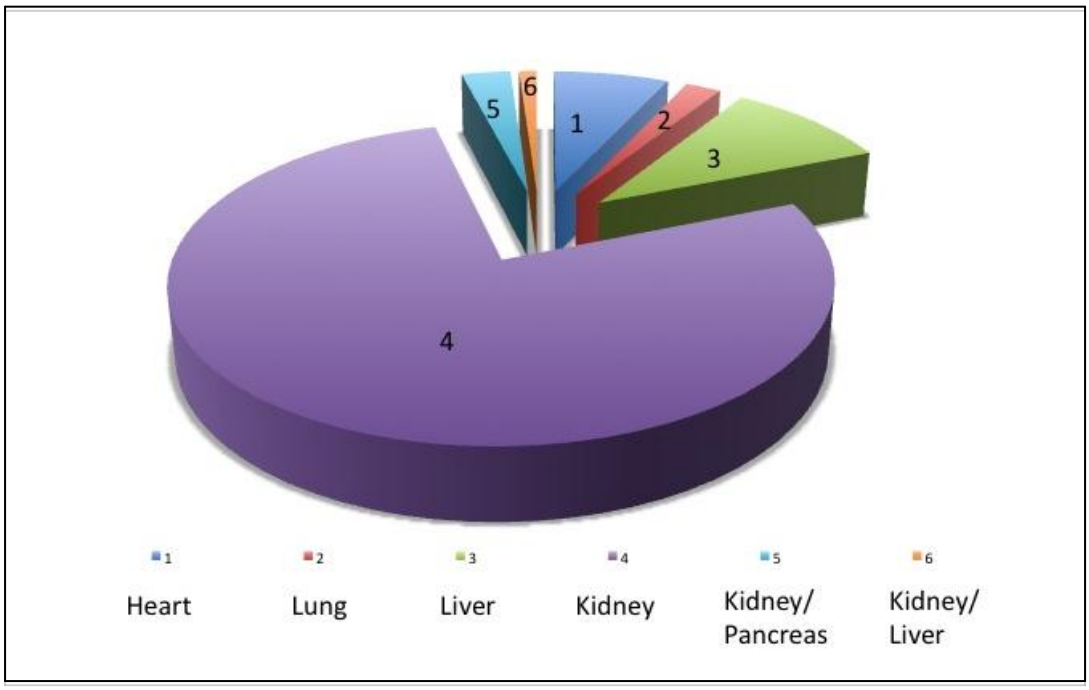


Figure 2: Pie Graph depicting percentages of solid organ transplantation in 2009. Heart (6.9%), Lung (2.1%), Liver (9.8%), Kidney (77.1%), Kidney/Pancreas (2.9%), Kidney/Liver (1.1%) (http://www.odf.org.za)

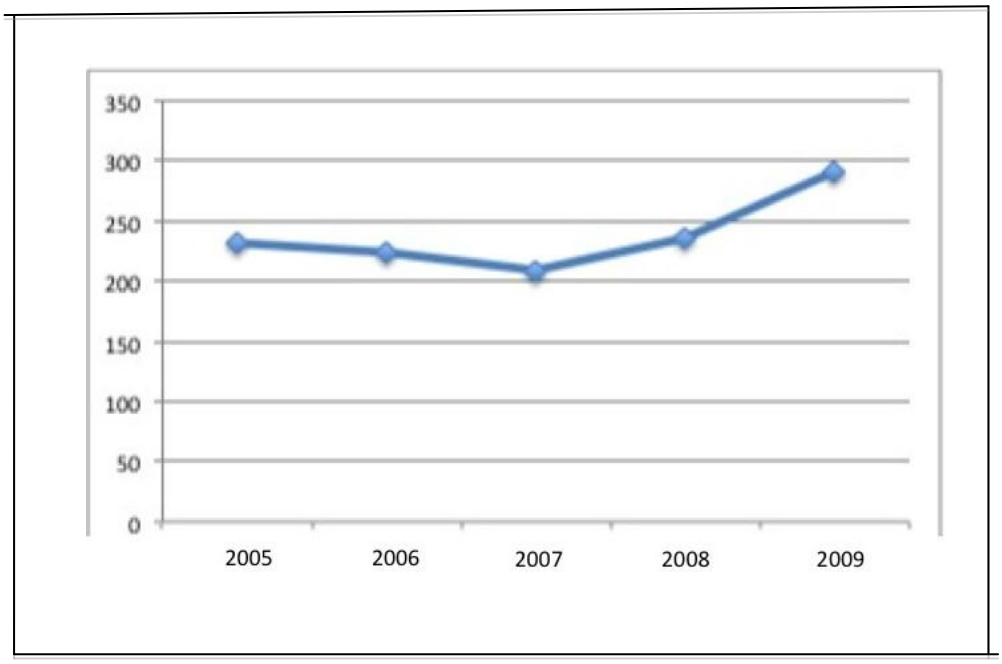


Figure 3: Line Graph of number kidneys transplanted from 2005 to 2009 in South Africa (http://www.odf.org.za)

To facilitate atransplant program of this nature, a thorough understanding of the immune system is required, as well as a sophisticated, dedicated laboratory program.

1.1 Overview of the Immune System

The immune system represents the body's defence system against pathogens and molecules regarded as foreign. The immune response has two major divisions i.e., the innate system and adaptive immune system.

1.1.1 Innate and Adaptive Immunity

The innate immune system represents the first line, non specific, temporary response against foreign peptides (Mendelow, Ramsay & Chetty, 2006) . This may include physical barriers like

the skin or respiratory epithelium and pattern recognition of pathogen characteristics such as carbohydrate moieties or bacterial membranes that can trigger complement resulting in cell lysis (Mendelow,et al., 2006). Effector cells of the innate immune system includes cells capable of phagoctyosis such as monocytes, neutrophils and macrophages; as well as cells that are able to stimulate an inflammatory response includingbasophils, mast cells, eosinophils and natural killer cells (Mackay & Rosen, 2000).

The adaptive immune response is a specific, slower, tailor-made response to a certain type of foreign peptide that has not been destroyed by the innate immune system. This response is long-lived and a second encounter with the same antigen results in a more effective, faster response (Hoffman, Benz & Shattil, 2009). The adaptive immune system is dependent on lymphocytes (B and T) and antigen presenting cells (these are cells that present the antigens to lymphocytes) (Mackay, et al., 2000). Humoral and cell mediated immunity form part of the adaptive immune response.

1.1.2 Humoral and Cell Mediated Immunity

Humoral immunity is mediated by B lymphocytes which produce antibodies (Schwartz, 2003). Each B cell has a unique B cell receptor which can be secreted as an antibody when it encounters an antigen or bind to antigen and function as an antigen presenting cell to T cells (Parkin & Cohen, 2001).

The T cell receptor is comprised of alpha and beta regions (Jiang &Chess, 2006). T helper (CD4 positive) and T cytotoxic (CD8 positive) cells form the major components of the T cell population. T helper cells are involved in promoting B cell function (antibody secretion) while

cytoxic T cells drives the Cell Mediated Immunity (CMI) which culminates in cell lysis (Parkin et al., 2001).

1.2 Human Leucocyte Antigen (HLA) and Transplantation

The HLA system, also known as the Major Histocompatibility Complex (MHC) is the primary regulator of graft rejection or tolerance helping to distinguish self from non-self (Abdul, 2007). The HLA is encoded on chromosome 6 and can be separated into 3 different areas (Choo, 2007), (Robinson, Waller, & Fail, 2009), that include genes encoding for Class I, II and III antigens.

Class I antigens (HLA - A, HLA - B, HLA -C) are expressed on all nucleated cells, while Class II antigens (HLA - DR, HLA – DQ,HLA - DP) are found on professional antigen presenting cells (dendritic cells, B cells and macrophages/monocytes) only (Abdul, 2007),(Smith, 2002). The Class III region encodes genes for complement and tumour necrosis factors(Choo, 2007). While renal glomeruli express MHC class II molecules, the expression of MHC class II on renal tubular epithelium is variable (Daar, Fuggle & Fabre, 1984).

1.2.1 Structural differences between Class I and Class II MHC molecules

Class I molecules: The MHC Class I molecule is comprised of α chains and beta 2 – microglobulin which represent the light chain of the class I molecule. The α chain is comprised of 5 regions – this includes 2 peptide binding domains (α1 and α2), one immunoglobulin like domain (α3), a transmembrane region as well as a cytoplasmic tail (Mackay, et al., 2000). Endogenous peptides (e.g. viral proteins) are predominantly processed by forming a complex with Class I molecules which results in activation of CD8 T lymphocytes

(cytotoxic T lymphocytes) (Abdul, 2007),(Smith, 2002). The peptide binding groove for Class I molecules process shorter peptides than the Class II molecule (Refer to Figure 4).

Class II molecules: The class II molecule is comprised of α and β chains. Each of these chains is comprised of 4 regions, which include peptide binding domains (α1 andβ1), an immunoglobulin-like domain (α2 and β2), a transmembrane region and the cytoplasmic tail (Mackay, et al., 2000). Exogenous peptides (e.g. bacterial proteins) are predominantly processed by forming a complex with Class II molecules and result in activation of CD4 T lymphocytes (Abdul, 2007),(Smith, 2002).

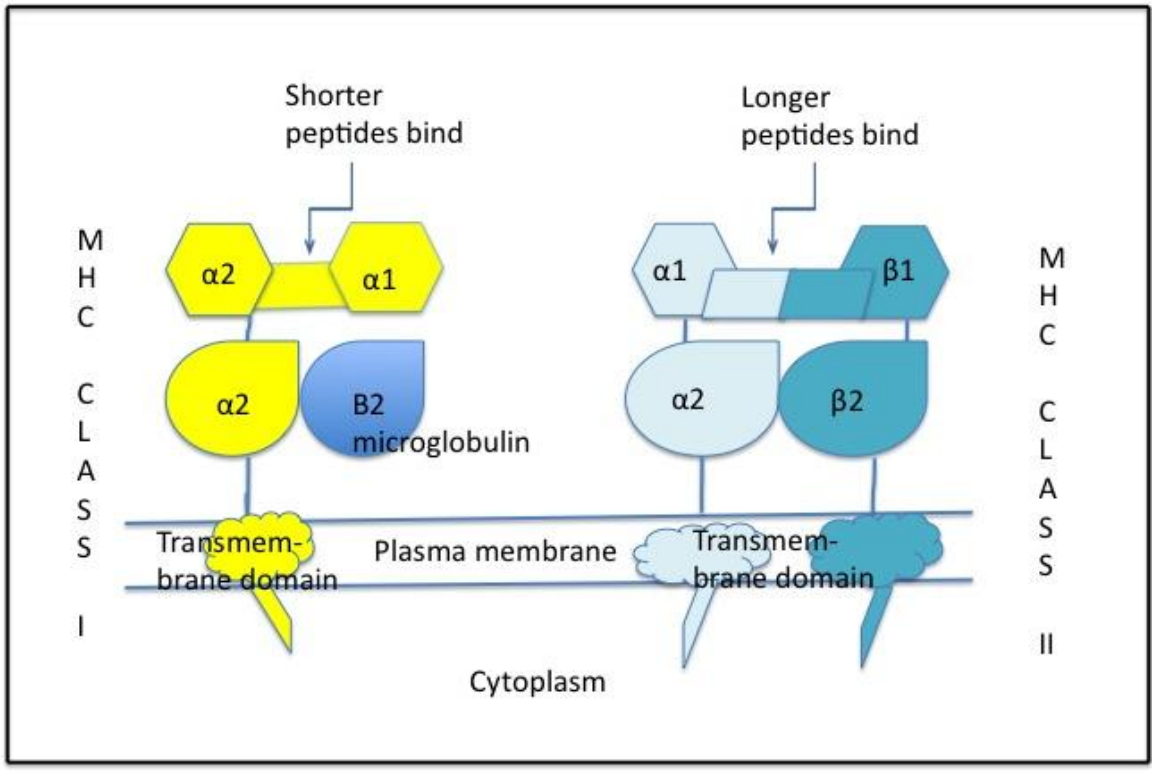


Figure 4: Diagram illustrating differences between MHC Class I and MHC Class II molecules (Adapted from Mackay, et al., 2000)

1.2.2 Major Histocompatibility Class I related chain A (MIC-A)

Major histocompatibility complex (MHC) class I related molecules are structurally similar to MHC Class I molecules, but do not necessarily bind peptides or associate with B2 microglobulin (Quiroga, Salio & Koo, 2006). MIC molecules are encoded on chromosome 6 where seven different MIC loci (MIC-A to MIC-G) are located. Of the 7 MIC loci, only MIC-A and MIC-B loci are able to transcribe proteins (Collins, 2004),(Morales-Buenrostro & Alberu, 2008).

1.2.3 Role of MIC-A in renal transplantation

Endothelial cells, gastric epithelium and fibroblasts have been reported to express MIC-A molecules on cell surfaces during times of stress (Sumitran-Holgersson, Wilczek & Holgersson, 2002). Anti MIC-A antibodies to endothelial cells in renal tissue may develop post transplantation (Zachary& Lefell, 2008). The presence of antibodies to MIC-A has been identified in the sera of patients post- transplant and are associated with a greater risk of graft failure (Panigrahi, Gupta & Siddiqui, 2007), (Terasakia, Ozawab & Castro, 2007). Screening for the presence of these antibodies would represent another avenue of investigation in patients who have features of graft failure not explained by classic HLA incompatibility. Currently, in South Africa, there are no screening tests available for determination of anti MIC-A antibodies. As MIC-A antigen expression has not been described on peripheral blood lymphocytes, routine crossmatch assays are unable to identify anti-MIC-A antibodies (Zou, Stastny & Susal, 2007).

1.3. Mandatory Laboratory Testing In Renal Transplantation

Globally, the number of patients requiring renal transplantation is escalating while the donations available are unable to meet the demand (http://www.bts.org.uk/transplantation/standards-and-guidelines/),(Thomas, 2007). Effective screening programmes and allocation of available

donations, which is largely dependent on accurate laboratory testing, are mandatory to ensure successful outcomes.

Histocompatibility testing involves Human Leucocyte Antigen (HLA) matching and donor specific crossmatching (Kasiske, Cangro & Hariharan, 2001),(Winsett, Martin & Reed, 2002), (http://www.bts.org.uk/transplantation/standards-and-guidelines/), (Thomas, 2007).

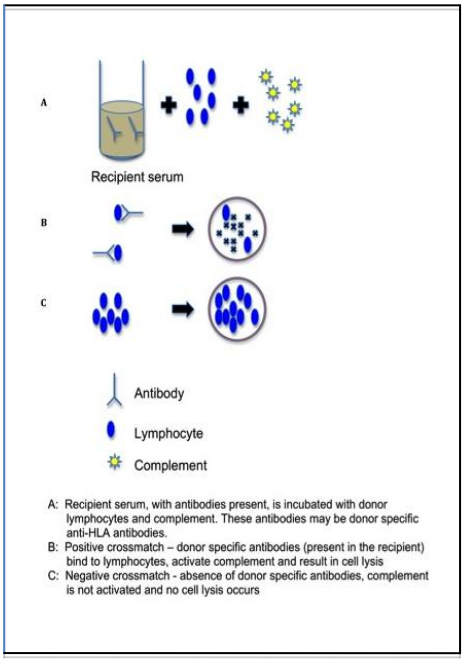
In an attempt to standardize laboratory testing, various organizations have developed guidelines which describe the minimum testing that is required for an accurate histocompatibility assessment. Collectively, the United Network of Organ Sharing (UNOS), the European Federation of Immunogenetics (EFI), the American Society of Histocompatibility and Immunogenetics (ASHI) / College of American Pathologists, consider the following mandatory testing in renal transplantation (Marrari & Duquesnoy, 1994), (European Federation of Immunogenetics, 2006):

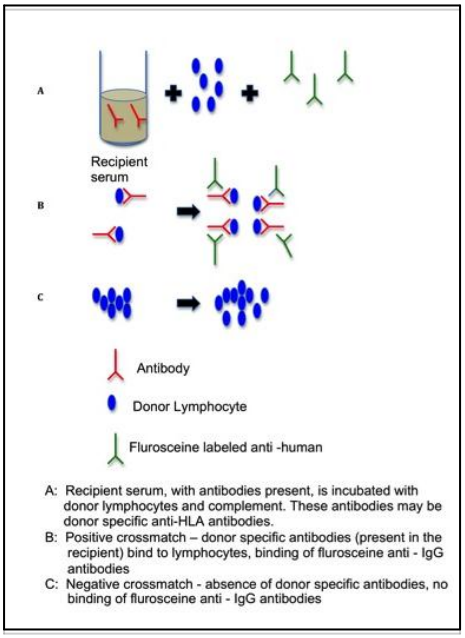
1) Donors and recipients must be typed for HLA – A and HLA - B class I antigens, as well as HLA - DR class II antigen
2) Anti - HLA antibody screening must be performed on the recipient. Patients must be screened after potentially sensitizing events. A sensitizing event occurs when donor specific antibodies develop post- exposure to non- self antigen e.g. blood transfusion, pregnancy, infections, previous allografts, vaccinations or development of an autoimmune disease (Karpinski, Rush &Jeffery, 2001), (Lefell, Donnenberg &Rose 2008).

3) A crossmatch between the donor and the prospective recipient must be performed to detect the presence of donor - specific antibodies in the recipient's serum.

1.4 Summary of Current Technologies

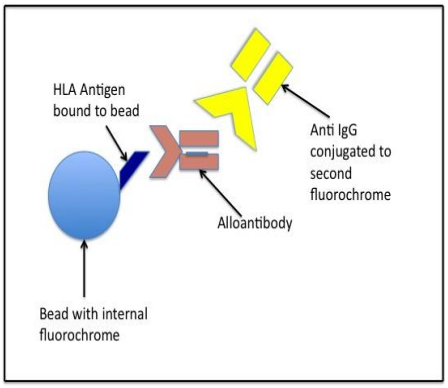
Table 1: Summary of the techniques used for crossmatching, HLA typing as well as antibody detection.

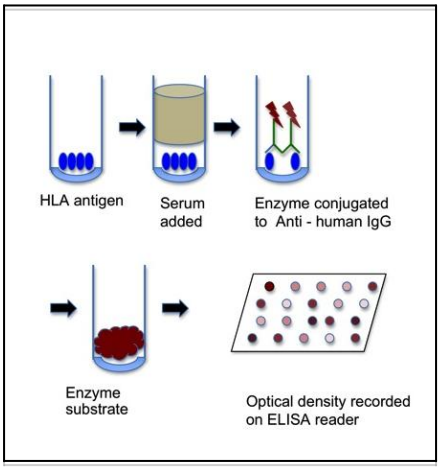
Technology	Advantages	Disadvantages	Diagram
Complement Dependent Cytoxicity: Donor lymphocytes are separated using commercial bead separation kits (One Lambda, California, USA) (Book, Agarwal & Milgrom, 2005). In the case of anti HLA antibody screening, commercially available panels are also used. Sera or, anti-sera in the case of HLA typing, from the patients are incubated with these lymphocytes in individual wells on the tray (together with complement and permeable dye). The percentage of cells which demonstrate lysis and hence uptake of the dye is recorded. Cytotoxicity (and hence degree of positivity of the crossmatch) is determined by the addition of dyes such as acridine orange and ethidium bromide (an intracellular/ permeabilisation fluorescent dye combination) (Book, et al., 2005). With respect to anti-HLA antibody detection, the percentage of cells which demonstrate lysis and hence uptake of the dye, post incubation with the recipient's sera (due to the presence of anti-HLA antibodies) is documented as the percentage of panel reactive antibodies (PRA).	Large numbers of tests can be crossmatched using pre-plated samples.	*Poor sensitivity * Limited by viabililty of sample * May not detect non complement antibodies *Subjective assessment operator dependent *In the setting of anti-HLA antibody detection, low titre antibodies my not be detected due to poor complement fixation (Tinckam, 2009). Commercially available panels e.g. DynaChip Antibody Analysis (Invitrogen Corporation, Carlsbad, CA), are not representative of local population HLA.	 Applications: Crossmatching, HLA typing & HLA antibody screening. Figure 5: Illustration of CDC technology (adapted from Mulley RW & Kanellis J, 2011)
AHG enhanced crossmatch	Improved sensitivity over conventional CDC crossmatching.		

Flow Cytometry: Incubation of patient lymphocytes or antigen coated beads with donor serum. Anti human IgG (immunoglobulin) monoclonal antibody labeled with fluorescein is added. Changes in fluorescence intensity compared to negative control used to determine positive (i.e. presence of donor specific antibodies bound to anti IgG) and negative (Absence of donor specific antibodies) results.	*Improved sensitivity compared to the CDC crossmatch and ELISA assessment for PRA. *Less likely to be affected by poor lymphocyte viability *Less subjective *Can detect non complement antibodies	*Low volume testing	 Figure 6: Ilustration of flow cytometry technology (adapted from Mulley RW & Kanellis J, 2011 Applications: Crossmatching, HLA antibody detection

Solid Phase assays

A solid phase assay is the interaction between any molecule and its receptor which is bound to a solid surface. Molecules used for radiolabelling are not part of the initial reaction and are only passed through the solid medium once the initial interaction between antigen and receptor has occurred. (Ishii, Nakayama & Katayama, 1997). The solid phase assays currently being used for anti-HLA antibody identification include coated bead assays e.g. Luminex technology and ELISA based assays (Tait, Hudson & Cantwell, 2008), (Zachary, Lucas & Detrick, 2009).

Luminex technology: Luminex technology is an example of a solid phase assay where the target molecules are soluble HLA antigens. (Tait, et al., 2008), (Eng, Bennet & Bardy, 2009) or bound anti-HLA monoclonal antibodies in the case of Luminex crossmatching which are internally conjugated to a fluorochrome. A second fluorochrome conjugated to anti-human IgG is then added. Thus each bead exhibits its own specific fluorescence pattern (of the internal fluorochrome as well as that of the fluorochrome attached to the IgG) (Tait, et al., 2008) (See Figure 7). The average or mean fluorescence intensities (MFIs) of the test samples are compared to negative control beads and negative control sera to determine whether a sample	Increased sensitivity compared to FCXM and ELISA for antibody detection	*Clinical significance of low titre antibodies not clear *The range of epitopes represented on each bead is limited *Reactivity to altered epitopes present on the beads but not present on the patient's HLA molecules may result in false positive results	 Figure 7: Diagram depicting Luminex technology (adapted from Tait et al, 2008) Applications: Virtual crossmatching, Luminex

is positive or negative for a specific antibody (http://www.gen-probe.com/pdfs/documents/product_inserts2/ Three levels of sensitivity are described as follows: 1) Screening - yields positive or negative results for the presence of Class I and Class II Anti- HLA antibodies 2) Antibody identification – 2 alleles of each of the Class I loci (HLA-A, B, C) and Class II loci (HLA-DR, DQ) are attached to the beads 3) Single antibody testing – represents the most sensitive level of testing. Here single antigens are attached to each bead. (Tait et al., 2008)			crossmatching, HLA typing and HLA antibody assessment
ELISA (Enzyme Linked Immuno-Sorbent Assay): Purified HLA antigens are used to coat ELISA plates and can be used as a screening tool for Class I and Class II anti HLA antibodies. Kits are also available to detect specific Class I and Class II antibodies (Gebel, Moussa & Eckels, 2009). Serum is added to the plates. Following incubation and washing, an enzyme conjugated to an anti human imuunoglobulin is added. In the last step the enzyme substrate is added to detect colour changes (Fuggle & Martin, 2008).	High throughput system which allows for rapid antibody testing. When compared to the flow cytometric or Luminex detection of specific anti-HLA antibodies, the ELISA methodology appears to be less sensitive (Worthington, Robson & Sheldon, 2001), (Phelan, Mohankumar & Ramachandran, 2009).	The ELISA method is described as being more sensitive than the CDC method of antibody screening (Kerman, Susskind & Buelow, 1996	 Figure 8: Illustration of ELISA technology (adapted from Fuggle & Martin, 2008) Application: HLA Antibody detection
PCR (Polymerase Chain Reaction): PCR involves the use of specific primer pairs which anneal to DNA sequences of interest (after a process of denaturation). with subsequent nucleotide extension. The DNA sequence of interest is amplified and identified using gel electrophoresis or melting curves (real time PCR). PCR with sequence specific primers: used to identify HLA alleles. This is a rapid system, however typing is low resolution and not suited to large volume testing. PCR with specific oligonucleotide probes are used to detect HLA polymorphisms – high volume testing can be performed with improved resolution. (Howell, Carter & Clark, 2010)			

1.5 Principles of Crossmatching

The process of crossmatching was first described by Terasaki and colleagues about 40 years ago (Bishara, Sherman & Brautbar, 1997). The principle of crossmatching is to determine which patients are sensitized due to the presence of antibodies in their serum that may react with a potential donor's lymphocytes and result in possible humoral mediated graft rejection(Susal & Opelz, 2007). A crossmatch assay is performed prior to transplantation and a number of different methodologies of crossmatching may be employed as reflected in Table 1.

1.6 Current Assays Available for Crossmatching

Most of the available assays are primarily measures of the status of the humoral arm of the adaptive immune system. These tests aim to reflect antibody production postsensitization events e.g. blood transfusion, pregnancy etc. prior to transplantation, as well as to the transplanted allograft in the post transplant period.

Donor specific anti-HLA antibodies present in the serum of potential recipients is known to result in hyperacute and acute rejection of allografts (Takeda, Uchida & Haba, 2000). Acute rejection may also be secondary to T cell mediated injury to the graft (Sis, Mengel & Haas, 2010). Sensitive techniques of crossmatching have evolved over the last 40 years (O'Rourke, Osorio & Freise, 2000). The implementation of these crossmatch tests and the detection of anti-HLA antibodies pre-transplant, resulted in dramatically lower rates of hyperacute and acute rejection (Scornik, 1995),(Takeda, et al., 2000), (Karpinski, et al., 2001). With the introduction of the Complement Dependent Cytotoxic crossmatch (CDC) assay the risk of early graft failure decreased by about 50-60% (Lobashevsky, Senkbeil & Shoaf, 2000) and the rate of hyperacute rejections decreased to 0.2 % (Colvin, 1996). However more sensitive crossmatch methods wereneeded to further decrease this risk. The assays subsequently developed include the Anti-

Human Globulin (AHG) Enhanced CDC assay, flow cytometric crossmatch (FCXM) assay and newer methods including virtual crossmatching.

1.6.1 Complement Dependent Cytotoxicity Assay

A pre-transplant crossmatch is performed to determine the presence of antibodies against donor HLA antigens (European Federation of Immunogenetics, 2006). A positive crossmatch due to the presence of HLA specific antibodies is a contraindication to transplantation and is thought to predict hyperacute/accelerated rejection (http://www.bts.org.uk/transplantation/standards-and-guidelines/), (Rebibou, Bittencourt & Saint-Hillier, 2004).

In Gauteng, at the time of this study, crossmatch practice for renal transplants involved the use of the CDC assay performed by the South African National Blood Services (SANBS). The current grading system used by SANBS for determining positive and negative results is shown below (See Table 2 below).

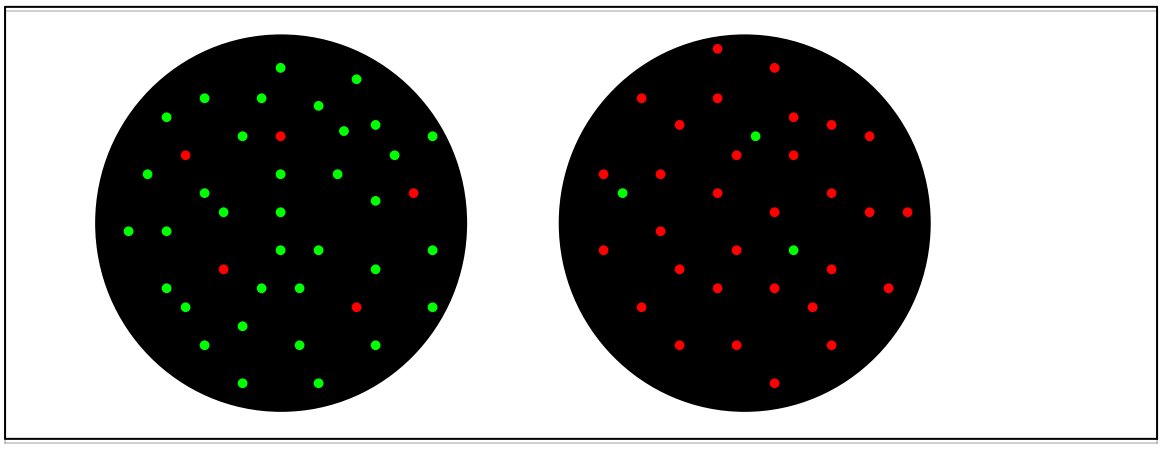


Figure 9: Graphical representation of CDC assay: 1) representation of a negative crossmatch and 2) representation of a positive crossmatch. Viable cells demonstrated in green and lysed cells demonstrated in red.

Table 2: Current grading system being used by SANBS.

Score	Interpretation	Dead Cells	Result
0	Not readable		
1	Negative	0 – 10 %	Negative
2	Doubtful negative	11 – 20%	Positive
4	Weak positive	21 – 50 %	Positive
6	Positive	51 - 80%	Positive
8	Strong positive	81-100 %	Positive

Samples graded ≥ 4 are considered positive. In the literature, a cut off ≥20% cell lysis has been used to determine positive results (Karpinski, et al., 2001).

Using this approach, antibodies to both Class I and Class II antigens can be detected. IgM and IgG antibodies can thus be identified simultaneously (European Federation of Immunogenetics, 2006). Unlike anti-donor HLA IgG antibodies, the clinical implications of the presence of anti-donor IgM antibodies has not been well elucidated (Takakura, Kiuchi & Kasahara, 2001),(Panigrahi, 2005). Some centres use Dithiothreitol,(which cleaves disulphide linkages) (Khodadadi, Adib & Pourazar, 2006), prior to crossmatch, to remove IgM antibodies (Martin, Davies & Robson, 1994). Anti-Human Globulin can be added to the traditional CDC assay. This has been shown to improve the sensitivity of the assay (Karpinski, et al., 2001) by detecting non-complement fixing antibodies. The current CDC crossmatch that is being done at SANBS does not include the use of AHG.

1.6.2 Flow Cytometric Crossmatch (FCXM)

In this study, an alternative approach to the current pre-transplant CDC crossmatch using flow cytometry was validated. The flow cytometric assay was not validated previously in Johannesburg. However, internationally the flow cytometric crossmatch is part of routine compatibility testing (Wen, Wu & Dmitrienko, 2006). It has been determined to be a more sensitive assay than the current CDC method of crossmatching (Fettouh, Cook & Flechner, 2001),(Wen, et al., 2006).

As flow crossmatching is dependent on the specific gating of lymphocytes, as well as the use of specific cut-offs (as determined by the local centre) for determination of positive and negative results, this method of crossmatching lends itself to less subjective interpretation and improved sensitivity over the CDC method (Pelletier, Adams & Hennessey, 1999),(Rebibou, et al., 2004). The assay specifically detects anti IgG antibodies (as anti- human IgG monoclonal antibodies are used in the assay), thus reducing the rate of false positive results arising due to the presence of IgM antibodies(Scornik, et al., 1995).

As the CDC method of crossmatching is reliant on the extent of cell lysis for determining positive and negative results, samples with poor lymphocyte viability are likely to yield a no result or a false positive result. The FCXM is therefore less likely than the CDC method to be limited by sample viability (Scornik, et al., 1995), as non viable cells would not be included in the gating strategy during analysis. Non- complement dependent antibodies are also detected, in contrast to the CDC method(Fettouh, et al., 2001), (Bryan, Wakefield & Reese, et al. 2007).

In the studies described below, CDC crossmatch negative patients who were found on retrospective analysis to be FCXM positive, revealed poorer graft outcome. In a study of 143 individuals who underwent primary renal transplant (all patients were AHG-CDC T cell and CDC B cell crossmatch negative at the time of transplant), retrospective flow cytometry crossmatches revealed that 13% of these patients were subsequently found to have positive T cell crossmatches by FCXM. Thirty three percent (33%) of these T cell crossmatch positive patients experienced early graft loss within 2 weeks. The group of FCXM positive patients in general demonstrated poorer graft function and demonstrated an increased chance of being refractory to conventional treatment(Karpinski, et al., 2001) .

Similar findings were demonstrated in a separate study, where 14 of 170 patients (who initially tested negative by CDC methodology) was found to be FCXM T cell positive (Rebibou, Carvalho Bittencourt & Saint-Hillier,1998). These patients demonstrated significantly increased levels of anti-HLA antibodies than the FCXM negative group (58.3% vs. 24.7%) and poorer graft survival (72.4% vs. 47.3%)(Rebibou, et al.,1998).

In a further study, poor graft survival at one year was predicted by a positive T cell crossmatch (Panigrahi, 2005). A close correlation with apositive T cell crossmatch (pre and post transplant) and early, refractory acute rejection has been described (O'Rourke, et al., 2000), (Takakura, et al., 2001).

1.6.3 Virtual Crossmatching

Virtual crossmatching is performed by comparing the anti-HLA antibody repertoire of the recipient and the HLA-typing of the donor. Hence, a recipient with anti-HLA antibodies directed

against antigens found in a potential donor will not be transplanted (without the performance of conventional crossmatching such as CDC or FCXM) (Deutsch, Kauke & Sadoni, 2010). As an example, if the HLA type of a donor is found to be HLA-A01, HLA-B 05 and HLA-DR 03, while the recipient is found to have anti-HLA antibodies to HLA-B 05 - this would be considered to be crossmatch positive and a contra-indication to transplantation.

1.7 Donor Specific Antibody Testing

Previously, donor specific antibodies were detected using the CDC method only. However, new and sensitive technologies such as the Luminex described above in Table 1, together with ELISA (described in Table 1) have been developed for antibody detection.

1.8 Assessment of cell mediated immunity in transplantation

Assays for assessment of the humoral arm of the immune system are widely reported in the literature. Tests available for assessment of cell mediated immunity are less well characterized.

1.8.1 Cylex ImmuKnow Assay

This assay (from Cylex Incorporated, Columbia, USA) was designed to assess the response of CD4+ T cells in immunosuppressed patients. After isolation of CD4+ T cells from whole blood samples using magnetic beads bound to a monoclonal antibody, the T helper cells are stimulated with phytohaemmaglutin. After washing, cells are lysedusing a lysis reagent and the quantity of ATP released is measured to assess the integrity of the immune response. A luminescence reagent (luciferin/luciferase) is added and thequantity of ATP emitted measured using a luminometer (http://www.cylex.net/pdf/ImmuKnow_Insert-cx.pdf).

In a meta-analysis performed across 10 transplant centres in the United States, ATP levels in post transplant patients (including recipients of kidney, kidney-pancreas, heart, liver and small bowel grafts) could be correlated to various states of stability, infection and acute cellular rejection (Kowalski, Post & Mannon, 2006). The strength of an immune response was stratified based on the level of ATP production as follows: strong (≥ 525 ng/ml of ATP, moderate (226- 524 ng/ml of ATP) and weak (<225 ng/ml of ATP). Patients with an ATP value of approximately 25ng/ml had a twelve fold increased risk of developing an infection compared to those subjects with stronger immune responses, patients with ATP values around 700ng/ml had a 30 fold increased risk of developing cellular rejection. Patients with ATP values of around 280ng/ml represented the most immunologically stable group.

1.8.2 ELISPOT

The ELISPOT (Enzyme Linked Immunosorbent Spot Assay) can be used to assess T cell response to a graft by measuring the production of cytokines e.g. Interferon γ by T cells. Donor cells or peptides of donor cells are combined with T cells from the recipient. As the reaction between the T cell and donor cells proceeds, various cytokines are secreted within a plate. The wells of the plate are coated with monoclonal antibodies so that the cytokine/s of interest can be isolated. With the use of dyes, the cytokine response of the T cells can be evaluated by measuring thespot on the plate using an Elispot reader. Software programs are used to interpret the intensity of the cytokine response(Tinckam, 2009).

In a study of 52 patients, an ELISPOT assay assessing interferon-γ production was performed pre-transplant and up to 10 times post transplant (testing was done weekly up to 8 weeks and between months 3 and 6, post transplant) (Nickel, Presber & Bold, 2004). In this study, the frequency of interferon-γ spots was assessed in relation to episodes of acute rejection and renal function (up to 12 months post-transplant). The frequency of spots ranged from undetectable to

>1000 spots/300 000 peripheral blood mononuclear cells (PBMCs). Patients with >200 spots/300 000 PBMCs pre-transplant were at higher risk of acute rejection within 6 months, independent of PRA status. In addition, patients with high frequency interferonγ ELISPOTs during week 2 and 3 post transplant were also more likely to have poorer renal function at 6 and 12 months post transplant (Nickel et al, 2004).

1.8.3 Soluble CD30

CD30, a member of the tumour necrosis factor family is expressed on T cells which express cytokines associated with a T helper 2 response. Elevated pre-transplant soluble CD30 levels have been associated with poorer 2 year post transplant graft survival, irrespective of the PRA status (Pelzl, Opelz & Wiesel, 2002).

1.9 Background to Chronic Kidney Disease

1.9.1 Definitions – Chronic Kidney Disease, Glomerular Filtration Rate, End stage Renal Disease

Chronic kidneydisease (CKD) may be defined as a functional or structural abnormality in the kidney with or without a decreased glomerular filtration rate (GFR), or a decreased GFR in the absence or presence of structural or functional renal damage persisting for 3 months or longer (Winsett, et al., 2002). GFR refers to the rate at which an ultrafiltrate of plasma is formed at the glomerulus (Kasper, 2005). Various factors may influence the GFR including the hydrostatic pressure inthe glomerular capillaries (which enhances filtration) and in Bowman's space, as well as colloid oncotic pressure in the capillaries (which opposes filtration) (Kasper, 2005).

Equations such as the Modification of Diet in Renal Disease Equation and the Cockroft Gault formula have been developed to estimate GFR (Poggio, Wang & Weinstein, 2006). End stage renal disease (ESRD) refers to the loss of kidney function such that waste can not be cleared from the circulation (Winsett, et al., 2002)

1.9.2 Aetiology of ESRD/CKD in the South African Setting

In South Africa, chronic diseases such as diabetes, cardiovascular disease and HIV associated chronic kidney disease are common aetiologies of ESRD (Gokool, Fabian & Venter, 2010). While outreach programmes and screening protocols are aimed to identify and help prevent end-stage renal disease, the number of patients requiring renal care is increasing in South Africa (Katz, 2006).

An example of such an outreach program was the South African Chronic Disease Outreach Primary Prevention Program which was implemented in primary health care clinics in Soweto and was conducted in 2 phases spanning 2001-2003 for the first phase and 2004-2005 for the second phase (Katz, 2006). The aim of this program was to implement algorithms into primary health care facilities to identify patients with chronic illnesses (diabetes, hypertension, obesity and kidney disease). This outreach program was adapted from a similar program run in Australia (Hoy, Zhiqiang & Philip, 2003). Phase I of the study involved data collection and monthly feedback sessions with the primary health care workers. Phase II of the study differed from Phase I in that staff performing the study received training in Australia as well as locally before implementation of the program. Feedback sessions occurred quarterly with training of appropriate health care workers based on the results of the data analysis. Eight hundred and seventy one patients were screened during Phase I of the study, of which ~60% patients were

obese, ~35% had evidence of chronic kidney disease and between 37%-49% of patients had signs of diabetes mellitus with poor glycaemic control. With the implementation of more aggressive therapy in the second phase of the program, improvements in blood pressure and glucose control were noted (during phase I,average blood glucose levels were >12mmol/l at presentation and subsequently improved to < 12mmol/l at 6 months compared to <8mmol/l in Phase II). The difficulties experienced in this resource poor setting included poor staffing, high patient numbers, loss of patients to follow up and lack of information technology infrastructure.

Treatment options for CKD are generally limited by resources. Renal dialysis is a treatment modality used for patients with ESRD, however renal transplantation remains the most effective way of restoring near normal functioning (http://www.bts.org.uk/transplantation/standards-and-guidelines/), (Thomas, 2007).

In 1994, in South Africa, glomerulonephritis (52.1%) and hypertension (45.6%) were documented to be the commonest aetiologies of ESRD according to the South African Dialysis and Transplant Registry(Naicker, 2003). These statistics were, however, not entirely representative of the aetiology of chronic renal failure as they include only those patients who were eligible for transplant (Naicker, 2003), (Rayner B 2003). It is thought that the disease burden associated with Diabetes Mellitus will increase dramatically in the future as ~300 million people are expected to be affected by it in 2025 (Naicker, 2003).

In recent years HIV- associated nephropathy (HIVAN) has been well described and is now reported to be the third commonest cause of end- stage kidney disease in black people aged between 20 and 64 years in the United States of America (Winston, Deray & Hawkins, 2008).

HIVAN is characterized by proteinuria, haematuria, hypertension and progression to renal failure (Bihl, 2006), (Roefs A, van der Ende M, Ijzermans J, 2009). Other causes of ESRD in the setting of HIV include drug- related nephrotoxicity and the development of chronic illnesses such as diabetes and hypertension in patients with longer lifespans (in the post HAART era)(Roefs A, et al 2009).

Previously, the presence of HIV infection was considered a contra-indication to renal transplantation as concerns regarding the effect of cumulative profound immunosuppression (i.e the effect of the HIV infection itself and the use of immunosuppressive drugs), the use of limited allografts in patients with limited lifespans and drug interactions, were expressed (Stock & Roland, 2007). Successful transplantation in patients with higher CD4 counts has been reported internationally (Thomas, 2007). HIV positive patients have demonstrated similar 5 year graft and patient survival rates when compared to HIV negative patients who also received kidney allografts from the same donor (Qiu, Terasaki & Waki, 2006).

In South Africa, treatment of end-stage renal disease in the setting of HIV infection includes the use of HAART and other drugs (e.g. angiotensin converting enzymes, possibly corticosteroids), the prevention of co-infection with Hepatitis B and C as well as other opportunistic infections and malignancies and dialysis (haemodialysis or peritoneal dialysis) (Bihl, 2006), (South African Renal Transplant Society, South African Transplant Society, South African HIV Clinicians Society Guidelines, 2008). Patients may be considered for transplantation if they demonstrate good compliance with HAART and are stable, have CD4 counts > 200/μl, undetectable viral loads and the absence of an AIDS- defining condition (South African Renal Transplant Society, South African Transplant Society, South African HIV Clinicians Society Guidelines, 2008).

In a local study, the concept of transplanting HIV positive individuals with renal allografts from HIV positive donors was deemed acceptable by both patients and health care workers. At the time of this study, 4 HIV infected kidneys were successfully transplanted into HIV positive individuals and ongoing monitoring of these patients continues (Gokool, et al., 2010).

1.10 Factors affecting outcome in Renal Transplantation

The primary aim of renal transplantation is the maintenance of a functioning graft for as long as possible. To ensure successful outcome, various factors need to be considered at the time of transplantation spanning the pre-, peri and post - transplant periods.

1.10.1 Pre-transplant factors

These include patient factors such as a co-morbidity, prior transplant history and Panel Reactive Antibody (PRA) status. Other pre-transplant factors include those that are organ- related e.g. baseline functioning of the transplanted kidney, donor organ retrieval during surgery and cold ischaemia time, amongst others. Cold ischaemia time refers to the period of time the kidneys remain in cold preservation after retrieval from the donor and prior to being transplanted into the recipient (Salahudeen & May, 2008)). An increased cold ischaemic time is reported to be associated with poor graft survival (Thomas J 2007), (http://www.bts.org.uk/transplantation/standards-and-guidelines/).

1.10.2 Peri-transplant factors

Intra-operative factors that may affect outcome include surgical technique, anaesthetic or other complications associated with general surgery.

1.10.3 Post transplant factors

Post operative outcomes vary depending on the type of maintenance therapy used (e.g. immunosuppressive agents) or the availability of post transplant monitoring (e.g.antibody titres).

1.11 Immunosuppressive Therapy

Immunosuppressive therapy mediates its effects by decreasing both lymphocyte numbers as well as blocking various signal transduction pathways. Effects of such therapy includes inhibition of rejection, immunodeficiency and non- immune toxicity (see Table 3), (Halloran, 2004). The aim of immunosuppressive therapy is to maintain a balance between inhibition of rejection and preventing the side effects associated with drug toxicity.

Table 3: Immunosuppressive drugs and their mechanisms of action

Mechanism of Action	Immunosurpressive Agent
a) Inhibitors of cytokine production by inhibiting gene transcription in various cells e.g. lymphocytes, macrophages	Corticosteroids
b) Inhibitors of nucleotide synthesis 1) Antimetabolites 2) Purine synthesis inhibitors 3) Pyrimidine synthesis inhibitors:	 Aziathioprine Mycophenolate Mofetil Enteric coated MMF Leflunomide
c) Immunophilin binding drugs 1) Calcineurin inhibitors 2) Target of rapamycin inhibitors	 Cyclosporine Tacrolimus Sirolimus Everolimus
d) Protein drugs 1) Depleting antibodies (against B or T cells or both) 2) Non depleting and fusion proteins 3) Intravenous Immunoglobulin	 Polyclonal antibody Muromonab CD3- (OKT3) Alemtuzumab (Humanized anti CD52 monoclonal antibody) Rituximab Anti CD20(B cell) depleting antibody Monoclonal anti CD25 antibody: Daclizumab, Basixilimab

(Adapted from Halloran, (2004), and Pascual, Theruvath & Kawai, et al (2002))

1.12 Possible Complications of Renal Transplantation

A myriad of complications may arise post transplantation and include those due to the surgical procedure (e.g. bleeding, haematoma formation, urinary leaks etc), infections, vascular complications, urinary tract obstruction and rejection of the allograft (Winsett, et al., 2002). The focus of the discussion will largely be on factors related to graft rejection.

1.12.1 Rejection

Allograft rejection occurs as a result of either an antibody mediated mechanisms or due to activity of the cellular mediated immune system or a combination. There are several types of rejection. These include hyperacute, acute or chronic rejection which are defined as follows: (Colvin, 2007)

1) **Hyperacute** rejection occurs within minutes to hours after reperfusion of the transplanted kidney and results in a non- functioning graft. This usually occurs due to the presence of pre-formed donor specific antibodies present at the time of transplantation (hyperacute humoral rejection).
2) **Acute** rejection refers to a very quick deterioration of graft function and may occur any number of days after transplant. Cases of acute rejection, years after transplantation, have been reported in patients managed with reduced intensity immunosuppression.
3) **Chronic** rejection is a process of gradual loss of graft function and may occur months or years after transplantation.

Both cellular mediated and humoral mediated mechanisms have been recognized to play roles in both acute and chronic rejection(Takemoto, Port & Claas, 2004)

Parameters used for complete assessment of rejection include clinical, biochemical and histological findings. One of the current histological classification systems currently being

utilized globally for the confirmation of rejection is the Banff criteria. Biopsies of the renal graft are assessed for rejection (Solez, Colvin & Racusen, 2008). (See Appendix 1 for the latest update for Banff diagnostic categories)(Sis, et al. 2010).

1.12.2 Antibody mediated acute rejection

Antibody mediated acute rejection (AMAR) occurs due to the presence of donor specific antibodies and has also been described in cases of transplants across ABO incompatible blood groups (Moll & Pascual, 2005). With activation of complement via the classical pathway (antibody- mediated), complement protein C4 is activated and its product C4d is deposited in the peritubular capillaries of the renal graft (Feucht, 2003).

1.12.3 Clinical significance of C4d

In the early 1990s, the presence of C4d on renal allografts post transplant was recognized as an independent prognostic marker of graft outcome (Feucht, Schneeberger & Hillebrand, 1993). Subsequently it was incorporated into the Banff criteria for assessment of antibody mediated rejection. Current guidelines suggest staining for C4d should form part of routine testing in patients undergoing renal biopsies (whether protocol driven or following clinical suspicion of rejection) post transplant. The presence of C4d, in particular diffuse C4d staining, on renal grafts has been associated with a poor graft outcome (Worthington, McEwen & McWilliam, 2007), (Wang, Wang & Chen, 2009).

Currently the Banff classification (2007) describes the following criteria for the diagnosis of AMAR (Sis, et al., 2010) :

1) The presence of circulating donor -specific antibodies
2) C4d+ staining of renal grafts
3) +/- morphologic evidence of acute rejection (See Appendices 1 and 2)

Various techniques are used to identify C4d including immunohistochemical staining on paraffin embedded or frozen tissue (Seemayer, Gaspert & Nickeleit, 2006). Newer flow techniques for assessment of C4d include the C4dFlowPRA where panel reactive IgG antibodies and complement fixing antibodies are detected simultaneously. Patients' sera are incubated with Flow PRA beads as well as serum from a normal healthy volunteer (with normal C4 levels and no obvious PRA positivity of IgG or C4d) as a source of complement. Following incubation with anti- IgG and anti- C4d antibodies, multicolor flow cytometry is used to determine results. A result was considered positive if reactivity was noted in > 10% of the panel used (Bartel, Wahrmann & Exner, 2008)

2.0 Study Objectives

The primary objectives of this study were to:

1) To establish and validate the flow cytometric crossmatch assay using Luminex technology as the reference method
2) To compare the performance of the flow cytometric crossmatch to existing technologies viz CDC crossmatch
3) Perform clinical follow up of transplanted patients to ascertain the rates of hyperacute and acute rejection in patients who had tested negative by the flow cytometric crossmatch

3.0 Methods

3.1 Stakeholders meeting

A meeting of all stakeholders was held that included nephrologists, transplant co - ordinators and members of the South African National Blood Services (SANBS) to confirm the need for the assay development. All participants were made aware ofthe proposal to establish a flow cytometric assay for crossmatching of T and B cells. Ethics approval for the study was obtained from the University of Witwatersrand Human Research Ethics Committee with the following Protocol number M080218 (SeeAppendix 3). See figure 10 below for a complete description of current testing practice at time of study initiation.

Flow Diagram of Local Laboratory Testing for Cadaveric Transplantation

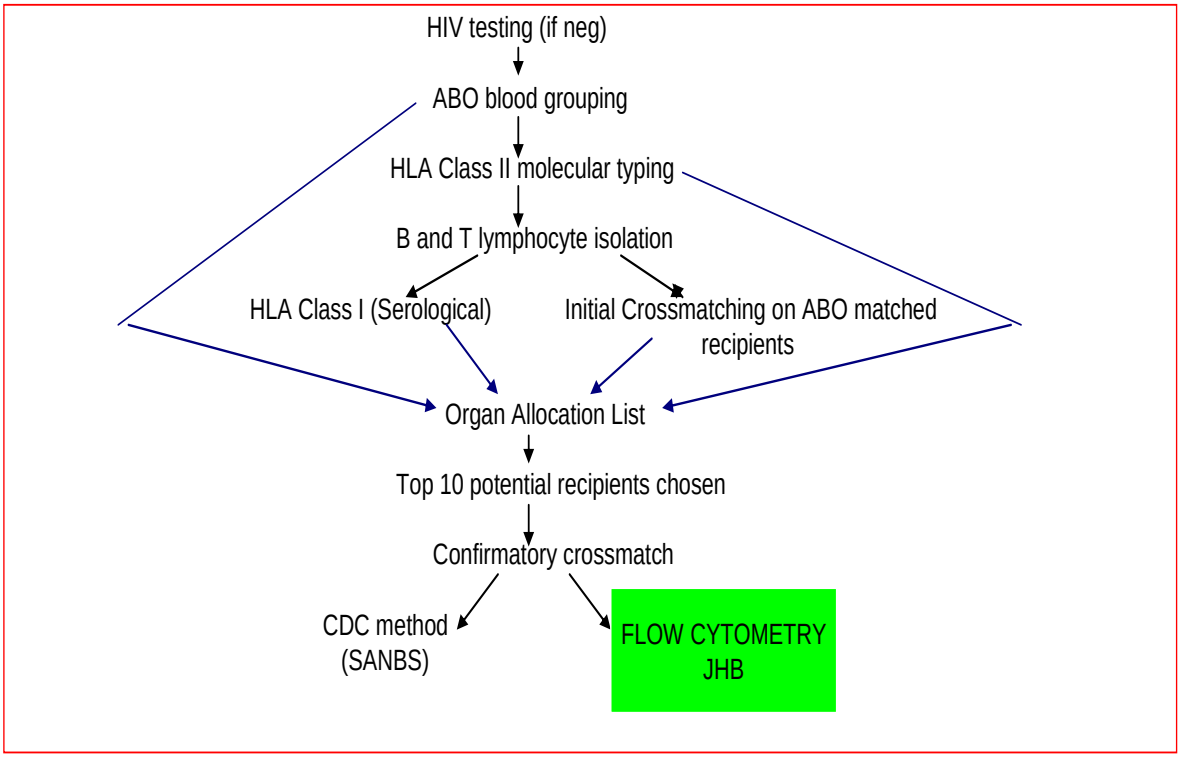


Figure 10: Flow Diagram of laboratory testing for Cadaveric Transplantation at the time of the study. The guidelines for testing is similar to that reported in literature (Kalble, Lucan & Nicita, 2004)

The process of laboratory testing for cadaveric donors at the time of the study included laboratory testing for HIV and other viral markers (such as Hepatitis A, B and C, EBV and CMV). If all viral markers were found to be negative, testing for blood grouping was done. The donor and recipients must be ABO blood group compatible as per local protocol. HLA typing of the donor was determined using a standard complement dependent cytotoxic assay for Class I HLA antigens and PCR analysis for Class HLA II antigens. As per protocol, initial CDC crossmatching was performed on pre-plated sera of ABO recipients. The top 10 patients on the transplant/organ allocation list (see below) who were found to be CDC crossmatch negative were called in for fresh sera to be drawn. The fresh sera were used for the confirmatory CDC as well as flow cytometric crossmatches (performed by the NHLS/ Charlotte Maxeke Johannesburg Academic Hospital laboratory- highlighted in green.)

3.1.1 Transplant List

A points system has been developed with various parameters contributing to the weighting and placement of candidates on the transplant list. Table 4 below illustrates the point's allocation system that was used at the time of the study to determine which patients ranked highest on the transplant list for crossmatching at the time of a cadaver donor. The point allocation system was originally based on the UNOS (United Network for Organ Sharing) point system (Neylan, Sayegh & Coffman, 1999) with some modifications to account for local differences e.g. only very closely HLA matched recipients (5 or 6 HLA match) are weighted highly to prevent racial bias. Factors that contribute include age, previous transplants, waiting time for a transplant, HLA matching, PRA status and previous transplants (See Table 4 below).

Table 4: Diagram of the current point allocation system used for ranking of patients on the transplant list

CURRENT POINT ALLOCATION

Age <18 & Tx No 1& 2		Waiting Time	HLA		HLA –DR		PRA		Previous Tx		Age to Accept >60 yr kidney	Age of Expanded Criteria Recipient
	Points			Points		Points		Points		Points	25	55
0 - 11	2	1 yr =	6 Match	10	0 Match	0	> 79	4	0	6		
12 - 18	1	1	5 Match	10	1 or 2 Match	1	60-79	2	1	3		
> 18	0	Point	4 Match	0			40-59	1	2	0		
			< 4 Match	0			< 40	0	>2	0		

NOTE ;

1.Based on 1 years wait = 1 Point

2. HLA 6 & 5 matches carry "infinite" weight (10 points) DR matches (1 ro 2) gain 1 extra point. This avoids SA Racial bias but maintains some HLA matching benefit

3. Previous transplants are penalized exponentially per Tx

4. Children get extra points for 1st& 2nd Tx, not for 3rd or more

OTHER FACTORS:

6. No extended criteria kidneys into young (< 25 years)

7. Listing date is the date placed on this list and must be immediately ready for Tx call –up.

8. If suspended for any reason, waiting time continues to accrue

9. B group listed with O group after waiting more than 2 years

IF TWO ELIGIBLE SCORES ARE EQUAL, THE YOUNGER PATIENT GETS PREFERENCE

3.2 Patient population

This study comprised two different patient populations:

a) 43 samples from potential transplant recipients were analysed by both flow cytometry and luminex testing. 36 of these 43 samples were analysed by the CDC method.

b) Clinical follow up was performed on 50 patients. Of these 50 patients, Luminex antibody screening results were only available for 5 patients that were transplanted. Luminex antibody results are not available for the remaining 45 patients. Hence the clinical outcome of these patients were used as a measure of efficacy of the FCXM.

3.2.1 Laboratory validation of the FCXM

For the validation of the FCXM, patients who were called up for confirmatory CDC crossmatches during 2008 were selected. Stored sera from recipients were sent for Luminex antibody testing to the Anthony Nolan Trust Laboratory in Hampstead, UK. The standard kits used for Luminex antibody screening included Lifecodes:Lifescreen, Class I ID and Class II ID (Tepnel Lifecodes Corporation, Stamford, USA) assay kits as well as LABScreen Assay Kits :LS1PRA, LS2PRA, LSM12, LS1A01-LS1A04 and LS2A01 (One Lambda, Inc. Canoga Park, California, USA). This was done so that expected positive and negative results could be predicted by performing a 'virtual crossmatch' by looking at the antigen profile of the donor and the anti-HLA antibody specificities of the recipients. Hence, Luminex technology was used as the reference method for establishing the sensitivity, specificity, negative predictive value and positive predictive value for the flow cytometric crossmatch.

3.2.2 Clinical follow up

The clinical follow up was performed on a different population group. The rationale for using a different group of patients was largely due to the fact that a small number (n=6) of patients' who had FCXMs as well as Luminex antibody testing performed had received kidney allografts. In view of this, the clinical follow up was performed on a larger number of patients who had FCXMs performed but not necessarily Luminex testing.

3.3 Establishment of the Flow based Crossmatch assay

3.3.1 Blood samples and collection

Blood samples were collected from donors and potential recipients by the transplant co-ordinators in both the provincial and private sectors.

For Recipient samples:10mls of fresh serum (samples < 24hrs old from time of collection) was obtained from recipients. The samples were drawn in tubes that did not contain an anticoagulant (red top tubes).

For Donor Samples: Up to 70ml of peripheral blood was collected from donors in Acid Citrate Dextrose (ACD) tubes. Lymphocytes were isolated from the peripheral blood of the specific donors (using Ficoll preparation methodology as per our local standard operating procedure. Refer to Figure 11 in section "Preparation of PBMCs by Ficoll Separation"). These samples were maintained at room temperature until processing.

3.3.2 Instruments used for Flow Cytometry Crossmatch Testing

All flow cytometric analyses were performed on the Becton Dickinson LSRII and FACS CALIBUR flow cytometers (BD, San Jose, California) at the National Health Laboratory

Services, Haematology Laboratory at Charlotte Maxeke Johannesburg Academic Hospital. The quality control and maintenance for these instruments are performed according to the laboratory protocol as described in standard operating procedures (SOPs) HAE0032, HAE1357, HAE0045 (NHLS, 2008, 2009).

3.3.3 Flow Cytometric Crossmatch

Four hundred microlitres donor lymphocytes were incubated with 200µl of patients'/ recipients'serum. T and B cell populations were identified by flow cytometry using the following monoclonal antibodies – 5µl anti-CD3 APC BD (Becton Dickinson, California, USA, catalogue number: 345767) to identify T cells and 10µl anti-CD19 PE (Coulter Clone, Beckmann Coulter, USA, catalogue number: PNIM 1285U) to identify B cells. IgG antibodies were identified using 2µl FITC Polyclonal rabbit antihuman IgG (Dako, Denmark, catalogue number: F0056). Antibodies against B cells represent the presence of antibodies predominantly against HLA class II antigens. Antibodies against T cells represent the presence of antibodies against HLA class I antigens. The ratio of the mean fluorescence intensity (MFI) value of the patient compared to the mean fluorescence intensity of a negative control was used to determine positive and negative results (See figure 11 below).

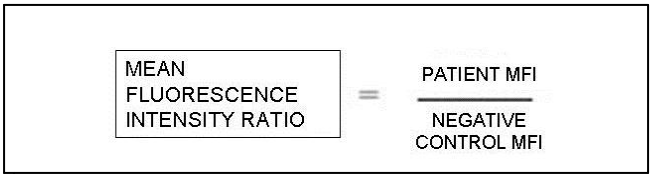


Figure 11: Equation used to determine mean fluorescence intensity (MFI) ratio

3.3.4 Preparation of samples for Crossmatch

Flow cytometric crossmatches were performed concurrently with CDC testing in all renal transplant patients. The protocol is based on the experience of the Anthony Nolan Trust, Hampstead.

Recipient sample preparation: Samples were centrifuged in the Beckmann Coulter Allegra X- 22 at 3500rpm for 15 minutes. Serum was removed and placed in plastic 5ml green top tubes.

Donor sample preparation: ACD tubes were gently agitated on an electric agitator/mixer for 20seconds. Peripheral blood mononuclear cells were prepared using Ficoll separation (described below).

Preparation of PBMCs by Ficoll Separation:

Five milliliters of Histopaque (Sigma, Aldrich, St Louis, USA and Steinham, Germany) was pipetted into a 15ml conical tube (Bibby, Sterilin Ltd, Stone, Staffs, UK) and the donor sample was gently added to the ficoll cushion. The sample was then centrifuged in the Beckmann Coulter – Allegra x-22 at 3500rpm for 15 minutes. The buffy coat containing the white blood cells was removed and washed twice with PBS (Phosphate Buffered Saline (PBS) (Dulbecco A, Hampshire, England). This method was carried out according to our local standard operating protocol HAE0066 (NHLS, 2005). See figure 12 for a graphical representation.

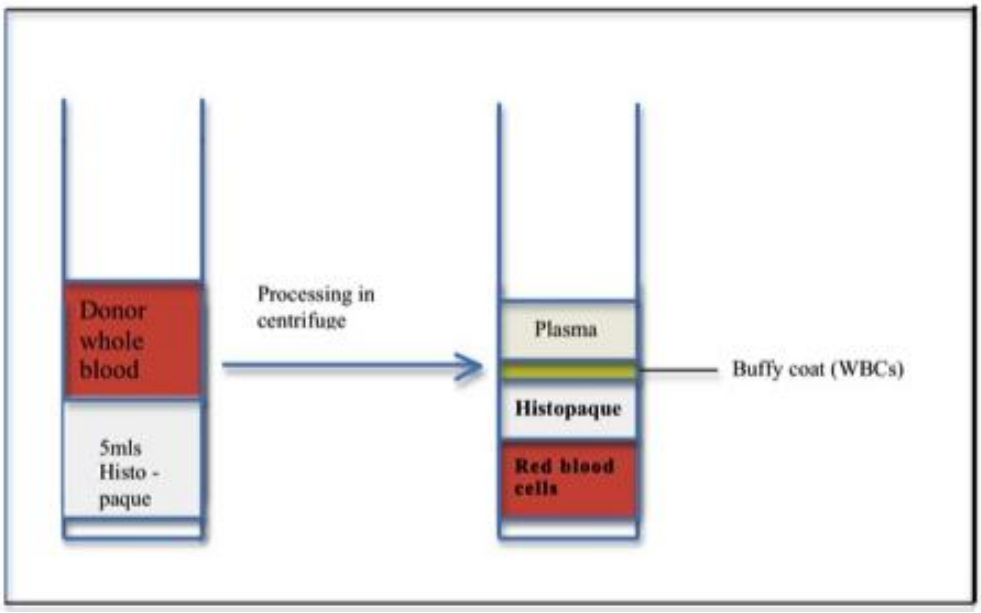


Figure 12: Diagrammatic depiction of Ficoll preparation A: Diagramattic representation of whole blood which has been added to Histopaque, B: After centrifugation, the whole blood sample separates by density gradient into a plasma layer, white blood cells (WBCs) or buffy coat. Histopaque and red blood cells remain at the bottom. The WBCs are removed only and used for the crossmatch as the lymphocytes are contained in this layer.

The buffy coat containing all white blood cells was removed and underwent further preparation for the crossmatch.

Washing of samples: Four milliltres of PBS was added to the buffy coat. The sample was centrifuged at 3000rpm for 3 minutes in the Rotofix 32 (Labotech). After centrifugation, the PBS was removed and the pellet resuspended. If the sample contained too many red blood cells after the first wash, the sample was further lysed with ammonium chloride (Saarchem, Merck Chemical, Gauteng, South Africa) before continuing with the second PBS wash. The sample was then ready to be used for the crossmatch.

3.3.5 Positive and Negative controls

Positive and negative controls were used in each run. The positive controls used in this study were multi-specific serum (Piazza, Adorno & Poggi, 1998) and anti lymphocyte globulin (ALG). Multi-specific serum is obtained from patients who are highly sensitized and have anti-HLA antibodies against a wide spectrum of HLA-antigens. ALG is the gamma globulin portion of serum derived from animals immunized against human lymphocytes. The serum from a healthy male volunteer with an AB positive blood group, who had no previous history of sensitization e.g. no previous blood transfusion, was used as a negative control (Piatosa, Rubik & Grenda, 2006),(Khodadadi, et al., 2006). This was kindly donated by South African National Blood Services (SANBS). Autologous crossmatching of the donor's sample (donor serum and donor lymphocytes) was also performed as an additional negative control.

3.3.6 Crossmatch Procedure

The donor cells were resuspended at a concentration of 1million cells/ ml in PBS. The cells were counted manually (using the Bright-Line, Hemacytometer, Hausser Scientific, Horsham PA, USA) in representative samples prior to resuspension to ensure the correct concentration. In four 5ml polystyrene round bottom tubes the following was added:

Tube 1: 400µl of the donor cells + 200 µl of positive control

Tube 2: 400µl of the donor cells + 200µl of the negative control serum

Tube 3: 400µl of the donor cells + 200µl of the multi-specific serum

Tube 4: 400µl of the donor cells + 200µl of donor serum (autologous negative control)

For recipients: Recipient tubes (depending on the number of recipients):400µl of donor cells + 200µl of recipient serum

All samples were incubated for one hour at 37ºC in the waterbath. After incubation in the waterbath, all samples were washed twice, vortexed and resuspended in 1ml of PBS.

Polyclonal rabbit antihuman IgG (Dako, Denmark, catalogue number: F0056) was vortexed prior to use. Two microliters of this anti rabbit IgG was added to each sample. The samples were then vortexed and incubated for 30 minutes at 4º C in the dark. This was followed by 2 washing steps as previously described. Five microlitres of anti-CD3 APC (Becton Dickinson, California, USA, catalogue number: 345767) and ten microlitres (10µl) anti-CD19 PE (Coulter Clone, Beckmann Coulter, USA, catalogue number : PNIM1285U) monoclonal antibodies were vortexed and added to the samples. The samples were vortexed again and left to incubate for 15 minutes in the dark at room temperature. The samples were washed once more and finally resuspended in 300µl of Facsflow (Becton Dickinson) before being acquired on the flow cytometer.

3.3.7 Instrument Settings

Instrument settings for all instruments were optimized using BD compensation beads (capture beads with anti-mouse IgG on their surface). Photo- multiplier tubes settings were established for a 4 colour experiment using 20ul each of FITC, PE, PerCP and APC antibodies. Compensation was set manually on the Calibur instruments and digitally on the LSRII using FACS DIVA. Results were acquired on the BD LSR II flow cytometer using FacsDiva software (Becton Dickinson). The following parameters were analysed:

FITC IgG against CD3APC BD

FITC IgG against CD19 PeCy7 BD

3.3.8 Flow cytometry acquisition

On the flow cytometers, the following parameters were analysed - FITC IgG against CD3 APC BD and FITC IgG against CD19 PECy7 BD. This was created using the following:

- Dot plots were created
- Based on forward and side scatter the lymphocyte population was gated (see Figure 13A)
- Of the lymphocyte population, CD3 positive cells (T cells) and CD19 positive cells B cells) were gated (see Figures 13B and 13C)
- The presence of IgG antibodies (against the B and T cells) were then identified by FITC positivity on the selected population. FITC mean fluorescence intensity was recorded on the population of interest.

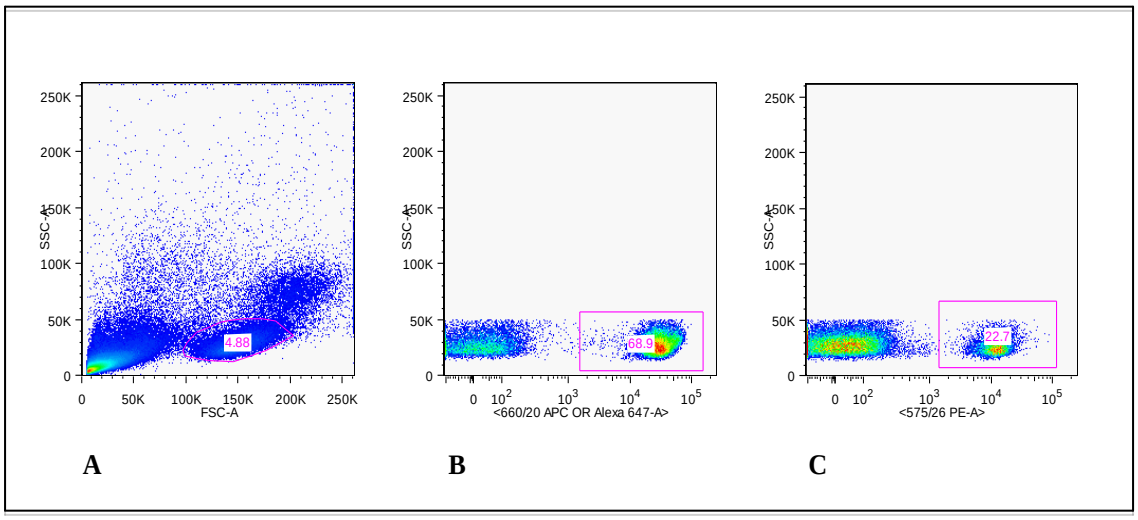


Figure 13: Representative flow cytometry dot plots (Flo Jo (Treestar, Ashland Oregon), Facs Calibur, BD) showing identification of lymphocytes by forward and side scatter characteristics (A), identification of T cells as a subset of lymphocytes by their expression of CD3 (B) and identification of B cells by their expression of CD19 (C)

3.3.9 Data Analysis

A sample was identified as positive or negative based on the ratio of the patient's MFI compared to the lowest negative control as defined previously (see Figure 11). FloJo (Treestar) software was used for data analysis.

The cut-offs described above for both the T and B cell crossmatches were based on the experience of the Anthony Nolan Trust (ANT) in Hampstead, United Kingdom (under the supervision of Dr Raymond Fernando and Dr Henry Stephens). The flow cytometer being utilized for FCXM at this centre in the UK is the Cytomics FC500 (Beckman Coulter (BC), High Wycombe, UK) with the CPX software program for analysis (ANT, 2006). BD and BC instruments use different platforms, BD instruments are reported to have wider pulse widths as well as pulse width extensions (https://www.beckmancoulter.com). This is thought to result in fewer, distinct events being analysed as compared to instruments with narrow pulse widths (e.g. BC instruments). The different platforms are thought to contribute to variation, particularly, in rare event analysis. Although the instruments used at our centre differs from that used in the UK, the difference in platforms is unlikely to affect thresholds for mean values obtained by the different instruments, as the flow crossmatch is not a rare event analysis and a minimum of 1million cells/ml PBS is a standard requirement.

B cell crossmatch:IgG antibodies (FITC labelled) that bind B cells (CD19 positive cells) were identified and the change in mean fluorescence intensity (MFI) was compared to that of the lowest negative control. If the change in MFI ratio was >5, the result was interpreted as positive (See Figure 14).

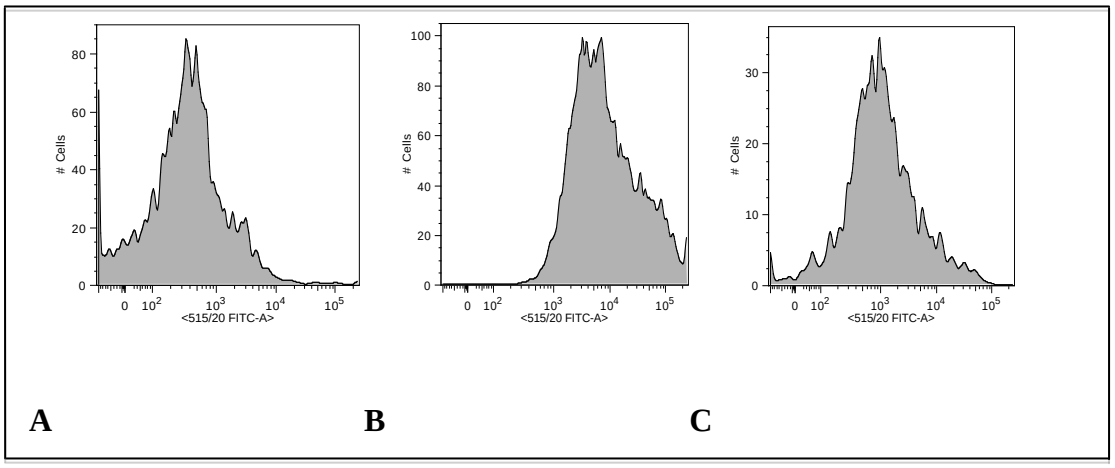


Figure 14: Representative histograms of a B cell negative control (MFI = 1056) (A), B cell positive control (MFI = 10500) (B) and an example of a patient with a negative B cell crossmatch (MFI= 2592)(C)

T cell crossmatch: IgG antibodies (FITC labelled) that bind T cells (CD3 positive cells) were identified and the change in mean fluorescence intensity (MFI) was compared to the lowest negative control. If the change in MFI ratio > 2, the result was interpreted as positive (see figure 15).

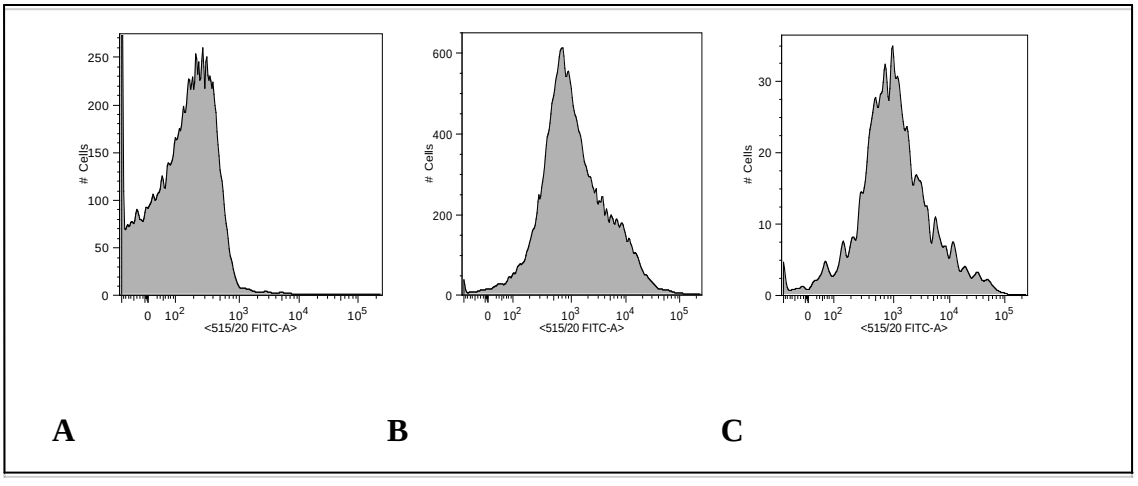


Figure 15: Representative histograms of a T cell negative control (MFI = 459) (A), T cell positive control (MFI = 1934) (B) and an example of a patient with a positive T cell crossmatch (MFI= 2260) (C)

3.3.10 Accuracy of Assay

Accuracy is defined as closeness of a result to the expected true result (Lewis SM, Bain BJ et al. 2006). To determine the accuracy of this assay, the results of the flow cytometric crossmatch were compared to virtual crossmatch results as determined by Luminex technology (using HLA antibody profiles of the recipients and the HLA profile of the donor).

3.3.10.1 External Quality Assurance

In addition, accuracy of the procedure was also determined by performance of the flow cytometric crossmatch in an external quality assurance (EQA) program, the UK NEQAS for Histocompatibility and Immunogenetics (http://www.wtail.org.uk/neqas/docs). This quality assurance program distributes samples for testing 5 times per year. With each distribution, 4 test human sera samples preserved with sodium azide and 2 anticoagulated whole blood samples diluted in tissue culture medium are forwarded to the participating laboratories (http://www.wtail.org.uk/neqas/docs). Performance and reporting of the T cell crossmatch only is required with this EQA program.

3.3.11 Precision of Assay

Precision refers to the reproducibility of an assay (Lewis, Bain & Bates, 2006). To determine the precision of this assay, sera known to cause positive and negative results with different donor lymphocytes respectively, were analysed.

Serum A was obtained from a previously pregnant (two previous pregnancies), volunteer whoseserum was found to result in a positive crossmatch with a specific volunteer's lymphocytes.

Serum B was obtained from a non transfused, non pregnant donor volunteer. This serum was found to result in a negative crossmatch result with another volunteer's lymphocytes. These samples were run in replicate on 3 different occasions.

3.4 Luminex anti HLA antibody testing

Luminex results were reported as antibodies likely to result in a positive crossmatch, antibodies that may result in a positive crossmatch and antibodies unlikely to result in a positive crossmatch (see Appendices 5 and 6). Only those antibodies reported as likely to result in a positive crossmatch were used to perform the virtual crossmatch as the Luminex method is very sensitive. Using the HLA-antigen profile of the donor (performed by SANBS) and anti-HLA antibodies (performed by reference laboratory) present in the sera of patients, a virtual crossmatch was performed and rates of prediction of positive and negative results was therefore possible and used as a gold standard.

3.5 Retrospective clinical analysis of transplanted patients

Following the laboratory validation of the assay, a clinical follow up was performed on patients who were transplanted. The method of follow up included retrospective, record review from outpatient files and databases and/or consultation with the treating nephrologist. This record review documented at least 3 months of clinical follow up post transplant.

Written informed consent was obtained from patients (See Appendix 4 for consent form). As only 6 of the patients were transplanted from the list of crossmatches that were compared to the Luminex data, we extended our patient pool by including patients who had FCXMs done and were transplanted but did not necessarily have Luminex testing performed.

Hyperacute rejection and acute humoral rejection were considered the primary clinical complications post-transplantation. Acute humoral rejection was defined clinically by a rapid deterioration in serum creatinine level or a failure of the kidneys to function primarily, by renal biopsies and by the clinical impression of the treating nephrologist.

Protocol biopsies are performed on all adult patients, locally, at 3 months (irrespective of the clinical condition of the patient) so that early pathologic changes can be detected. Biopsies are performed earlier if acute rejection is suspected. Biopsies can be associated with complications (e.g. haematoma formation, arterio-venous fistula)(Schwarz, Gwinner & Hiss, 2005).

In the paediatric population, current practice at the Charlotte Maxeke Johannesburg Academic Hospital, does not include the performance of protocol biopsies. Laboratory parameters such as creatinine levels, erythrocyte sedimentation rate and C-reactive protein are monitored. Changes from baseline values or lack of improvement of these parameters warrant further investigation or treatment.

Clinical parameters such as poor or no urine output post transplantation are also assessed. If rejection is suspected, a dose of intravenous steroids is administered and the patient is monitored looking at biochemical results. A biopsy is performed if there is a lack of or no response to these

measures. Other investigations, e.g. nuclear scans are also performed for assessment of perfusion through the transplanted kidney.

Data used for clinical follow up in this study include creatinine levels, renal biopsy results and immunosuppressive drugs used. The FCXM and CDC crossmatches were negative in all patients transplanted.

4.0 Results

4.1 Laboratory Testing

The initial aim of this study was to compare the performance of the flow cytometric crossmatch to the CDC method of crossmatching. However, during the study it was found that ~16% (7/43) of the CDC crossmatch samples yielded no result owing to poor lymphocyte viability. Hence, the results from the virtual crossmatch using the data from Luminex anti HLA-antibody profiles and the HLA typing was used as the reference method for establishing the sensitivity, specificity, negative predictive value and positive predictive value for the flow cytometric crossmatch (and not the CDC method).

4.1.1 Results of Flow Crossmatch compared to the Luminex Testing

B cell crossmatch: Of the 43 samples, analysed by flow cytometric crossmatching, 5 samples (Samples 33, 36, 39, 42 and 43 in Table 5 below) were found to be positive and 38 were found to be negative for the B cell crossmatch. Virtual crossmatch results predicted by Luminex technology predicted 2 positive and 41 negative results for the B cell crossmatch. The flow cytometric crossmatch predicted the 2 results found positive as predicted by Luminex. In addition, the flow crossmatch revealed 3 false positive B cell crossmatch results. The two methods agreed for 40 of the samples. See Table 5.

T cell crossmatch: Of the 43 samples, analysed by flow cytometric crossmatching, 10 samples were positive and 33 were negative. Virtual crossmatch results predicted by Luminex technology revealed 6 positive and 37 negative results for the T cell crossmatch. The flow cytometric crossmatch predicted 4 of the 6 positive results (as predicted on Luminex). In addition, the flow

cytometric crossmatch revealed 6 false positive results. The two methods were concordant for 35 (81.4%) of the 43 samples (See Table 7). Two samples with clearly negative results on flow cytometry were predicted to be positive by Luminex testing.

Table 5: Results of CDC, Luminex and FCXM. The Luminex results were used as the gold standard. Pos: positive result, Neg: negative result, Nr:No result

Subject No	CDC T	CDC B	Luminex T	Luminex B	T cell FCXM	B cell FCXM
1	Neg	Neg	Neg	Neg	Neg	Neg
2	Neg	Neg	Neg	Neg	Neg	Neg
3	Neg	Neg	Neg	Neg	Neg	Neg
4	**Nr**	**Nr**	Neg	Neg	Pos	Neg
5	**Pos**	Neg	Neg	Neg	Neg	Neg
6	Neg	Neg	Neg	Neg	Neg	Neg
7	Neg	**Pos**	Pos	Neg	Pos	Neg
8	**Nr**	**Nr**	Neg	Neg	Neg	Neg
9	**Nr**	**Nr**	Neg	Neg	Neg	Neg
10	**Nr**	**Nr**	Neg	Neg	Neg	Neg
11	Neg	Neg	Neg	Neg	Neg	Neg
12	Neg	Pos	Pos	Neg	Neg	Neg
13	Neg	Pos	Neg	Neg	Neg	Neg
14	**Nr**	**Nr**	Neg	Neg	Neg	Neg
15	Neg	Neg	Neg	Neg	Neg	Neg
16	Neg	Neg	Neg	Neg	Neg	Neg
17	Neg	Neg	Neg	Neg	Neg	Neg
18	Neg	Pos	Neg	Neg	**Pos**	Neg
19	Neg	Pos	Neg	Neg	Neg	Neg
20	Neg	Pos	Neg	Neg	Neg	Neg
21	Neg	Neg	Pos	Neg	Neg	Neg
22	Neg	Neg	Neg	Neg	Neg	Neg
23	Neg	Neg	Neg	Neg	**Pos**	Neg
24	Neg	Neg	Neg	Neg	Neg	Neg
25	Neg	Neg	Neg	Neg	Neg	Neg
26	Neg	Neg	Neg	Neg	Neg	Neg
27	**Nr**	**Nr**	Neg	Neg	Pos	Neg
28	Neg	Neg	Neg	Neg	Neg	Neg
29	Neg	Neg	Neg	Neg	Neg	Neg
30	Neg	Neg	Neg	Neg	Neg	Neg
31	Neg	Neg	Neg	Neg	Neg	Neg
32	Neg	Neg	Neg	Neg	Neg	Neg
33	Pos	Pos	Pos	Pos	Pos	Pos
34	Neg	Neg	Neg	Neg	Pos	Neg
35	Neg	Neg	Neg	Neg	Neg	Neg
36	Pos	Neg	Pos	Neg	Pos	Pos
37	Neg	Neg	Neg	Neg	Neg	Neg
38	Neg	Pos	Neg	Neg	Pos	Neg
39	Neg	Neg	Neg	Neg	Neg	Pos
40	Neg	Neg	Neg	Neg	Neg	Neg
41	Neg	Neg	Neg	Neg	Neg	Neg
42	Neg	Neg	Neg	Neg	Neg	Pos
43	**Nr**	**Nr**	Pos	Pos	Pos	Pos

4.1.2 Sensitivity and Specificity Tables FCXM

B cell crossmatch: Using the Luminex method as the gold standard, the flow cytometric crossmatch showed a sensitivity of 100%, a specificity of 92.7%, a negative predictive value (NPV) of 100% and positive predictive value (PPV) 40%. There was 93% agreement in the nominal result. (See Table 6)

Table 6: Two by two (2x2) Table of B cell FCXM compared with Luminex

	Virtual Crossmatch positive	Virtual Crossmatch negative	
FCXM positive results	2	3	Total number of positive tests (5)
FCXM negative results	0	38	Total number of negative tests (38)
	Total who are actually crossmatch positive (2)	Total who are actually crossmatch negative (41)	Total population (43)

There was good agreement between the flow cytometric crossmatch results and the results as predicted by the Luminex method. These results were deemed to be acceptable particularly in view of the internationally well recognized variability in the B cell crossmatch assay.

T cell crossmatch: Using the Luminex assay as the gold standard, the flow cytometric crossmatch showed a sensitivity of 66.7%, a specificity of 83.8%, a PPV of 40% and NPV of 93.9%. There was 81.4% agreement in the nominal result. See Table 7.

Table 7: 2x2 table of T cell FCXM compared with Luminex

	Virtual Crossmatch positive	Virtual Crossmatch negative	
FCXM positive results	4	6	Total number of positive tests (10)
FCXM negative results	2	31	Total number of negative tests (33)
	Total who are actually crossmatch positive(6)	Total who are actually crossmatch negative (37)	Total population (43)

4.1.3 FCXM compared to existing technologies (CDC crossmatch)

CDC crossmatch results were only available for 36 of the 43 samples. The other 7 samples yielded no results due to poor lymphocyte viability and hence could not be interpreted.

4.1.3.1 Sensitivity and Specificity Tables CDC

B cell crossmatch: Using the Luminex method as the gold standard, the CDC B cell crossmatch showed a sensitivity of 100%, a specificity of 80%, negative predictive value (NPV) of 100% and positive predictive value (PPV) of 12.5%. There was 80.6% agreement in the nominal result. (See Table 8)

Table 8: 2x2 Table of CDC B cell crossmatch compared with Luminex

	Virtual Crossmatch positive	Virtual Crossmatch negative	
CDC positive results	1	7	Total number of positive tests (8)
CDC negative results	0	28	Total number of negative tests (28)
	Total who are actually crossmatch positive (1)	Total who are actually crossmatch negative (35)	Total population (36)

T cell crossmatch: Using the Luminex assay as the gold standard, the CDC T cell crossmatch showed a sensitivity of 40%, a specificity of 96.8%, a PPV of 66.7% and NPV of 90.1%. There was 88.9% agreement in the nominal result. See Table 9.

Table 9: 2x2 Table of T cell CDC crossmatch compared with Luminex

	Virtual Crossmatch positive	Virtual Crossmatch negative	
CDC positive results	2	1	Total number of positive tests (3)
CDC negative results	3	30	Total number of negative tests (33)
	Total who are actually crossmatch positive(5)	Total who are actually crossmatch negative (31)	Total population (36)

4.1.4 Sensitivity, Specificity, NPV and PPV of FCXM and CDC crossmatch (using the Luminex results as gold standard)

B cell crossmatch: Similar, high sensitivities and negative predictive values were documented for both assays. The FCXM however demonstrated higher specificity, positive predictive value and percentage agreement with the nominal results (compared to the CDC method).

T cell crossmatch: A higher sensitivity and negative predictive value was noted using the FCXM. The CDC crossmatch showed a higher Specificity, Positive Predictive Value and percentage agreement with the nominal results.

Table 10: Sensitivity, Specificity, NPV, PPV, % agreement - looking at 43 samples FCXM and 36 samples CDC

	FCXM T cell	CDC T cell	FCXM B cell	CDC B cell
Sensitivity	66.7%	40%	100%	100%
Specificity	83.8%	96.8%	92.7%	80%
NPV	93.9%	66.7%	100%	100%
PPV	40%	90.1%	40%	12.5%
% agreement	81.4%	88.9%	93%	80.6%

4.1.5 External Quality Assurance FCXM

Results of the UK NEQAS for Histocompatibility and Immunogenetics for external quality assurance (QA) assessment revealed an acceptable level of performance. Crossmatch results are deemed acceptable if it is in with the consensus in 85% or more of the samples tested. Eight (8) samples were provided for crossmatch assessment and the laboratory achieved an 87.5%

crossmatch result that was in agreement with the consensus. The EQA program does not require the reporting of results for the B cell crossmatch (See Appendix 7).

4.1.6 Precision of the assay FCXM

Precision of the flow crossmatch assay was assessed using sera from volunteers known to result in a positive and negative crossmatch, respectively, with known donor cells (from specific volunteers as described in the Methods section). Serum from volunteer A was known to result in a positive crossmatch result for both T and B cells. Serum from volunteer B was known to result in a negative crossmatch result for both T and B cells.

4.1.6.1 Positive crossmatch:

Samples from these volunteers were obtained and crossmatches performed. This was done on 3 different occasions. See Appendix 8which demonstrates the results for the B and T cell crossmatch.

Summary of Intravariabillity for positive crossmatch:

- **B cell results:**

  During the first run, 7 crossmatches were performed and all 7 analyses consistently yielded the expected positive results. During the second run, 6 crossmatches were performed. One sample revealed an unexpected negative result. During the third run, 7 crossmatches were performed. Two samples revealed unexpected negative results.

- **T cell results**:

  Samples were run on 3 different occasions. In total 20 samples were analysed,

  and all 20 revealed expected positive results for the T cell crossmatch,

consistently.

4.1.6.2 Negative crossmatch:

Samples from volunteer B were run 3 times. See Appendix 9 which shows the results of the crossmatch runs for this volunteer and donor cells.

Summary of Intravariability for negative crossmatch

- **B cell results**:

  With each run, 3 crossmatches were performed. All 9 crossmatches produced expected negative results consistently

- **T cell results**:

  With each run, 3 crossmatches were performed. All 9 crossmatches produced expected negative results consistently.

4.1.6.3 Intervaribility

Summary of Intervariability for positive crossmatch

To assess inter-variability between runs on different days, 20 crossmatches on the serum sample from volunteer A were run against the same donor over a period of 3 days and the results were compared (See Appendix 10).

- **T cell crossmatch**

All T cell results were correctly consistently positive.

- **B cell crossmatch**

  3/20 samples (15%) yielded unexpected negative results for the B cell crossmatch.

Summary of Intervariability for negative crossmatch

The data from volunteer B were also analysed simultaneously. All 20 samples revealed true negative results for both the T and B cell crossmatches.

4.2 Clinical Follow up

4.2.1 Results of clinical follow up

Of the 60 patients transplanted over the period of review, a full dataset was available for 50 patients. Flow cytometric crossmatches were not performed on 4 patients and clinical data was not available for 6 patients. This lack of data on the 6 patients was due to patients being followed up at centres outside Gauteng as well as due to a lack of feedback from the treating physicians.

4.2.1.1 Patient Characteristics

In this study population, 88% (44/50) of patients transplanted were adult patients and 12% (6/50) patients were paediatric patients. The age range of the adult population was 20-80 years. In the paediatric population the age range was 13-20 years. In the adult group, the majority of patients transplanted were male (79.5%) (35/44 patients). The most commonly listed aetiology of underlying renal disease in the adult population included chronic diseases such as diabetes mellitus (6/44 patients), hypertension (4/44 patients), unknown aetiology (3/44 patients) but for the majority the cause of renal failure was not documented in the medical records (27/44). Other causes of renal failure included glomerulonephritis (2/44 patients), obstructive uropathy (2/44 patients), nephrotic syndrome (1/44 patients) and focal segmental glomerulosclerosis (1/44

patients). In contrast, causes of renal failure in the paediatric group included dysplastic kidneys (2/6 patients), cirrhotic kidneys (1/6 patients) and familial nephrotic syndrome (1/6 patients).

Table 11: Clinical follow up – Patient characteristics (CMJAH- Charlotte Maxeke Johannesburg Academic Hospital, CHB- Chris Hani Baragwanath Hopsital, HJH- Helen Joseph Hospital). Patients from CMJAH, CHB, HJH and Private Hospitals were all adult patients.

	Number of patients	Male	Female
Paediatric State	5	3	2
Paediatric Private	1	1	0
CMJAH	16	12	4
CHB	10	4	6
HJH	1	1	0
PRIVATE	17	14	3
Total	50	35	15

4.2.1.2 Results of hyperacute, acute rejection

Features of rejection were based on the availability of results such as histology results and creatinine levels or the clinical impression of the treating doctor. At their most recent follow up, 82% (41/50 patients) demonstrated no overt features or signs of any rejection.

In this study, none of the patients demonstrated features of hyperacute rejection. Six of the fifty patients (12%) did have features highly suggestive of possible acute humoral rejection (See Table 11). The clinical parameters of these 6 patients are described in greater detail in Table 12. Three of fifty patients (6%) of patients demonstrated features of possible acute cellular rejection or other causes for graft dysfunction. The clinical parameters of these patients are described in table 13.

Table 12: Results of hyperacute and acute rejection

Patient characteristics	Number of patients
Patient with features suggestive of hyperacute rejection	0
Patients with no overt evidence of acute cellular or humoral rejection and no overt hyperacute rejection	41
Patients with possible acute humoral rejection (AHR) or overt features of AHR	6
Other (acute cellular rejection, loss of graft functioning)	3

Table 13: Clinical data on the 6 patients with features worrying for acute humoral rejection. MMF – Mycofenalate Mofetil, CYA- Cyclosporin

Patient Number	Chemical parameters	Histology Results	Medication used	Outcome
Patient 1	**Creatinine levels**: 3 month follow-up: 158 µmol/l At 4 month biopsy: 141µmol/l At 12 month biopsy: 162 µmol/l	**4 month biopsy**: Features of acute cellular rejection, acute tubular necrosis. Large venule with intraluminal blood cells- concern for possible humoral rejection. **12 month biopsy**: Non-specific acute tubular interstitial nephritis, borderline suspicious changes for acute rejection.	MMF, Imuran, CYA Prednisone	14 months post transplant, patient has reasonable,stable creatinine levels (121 µmol/l)
Patient 2	2 months post Transplant: 1266 µmol/l 3 months post transplant (nephrectomy done): 726 µmol/l	**2 month biopsy:** Interstitial tubulitis. Borderline changes suspicious for acute cellular rejection. Possible drug effect, possibly infection related **3 months post transplant**: Nephrectomy done – histology revealed acute cellular and acute vascular rejection	MMF Imuran Solumedrol/ Prednisone CYA Simulect	Nephrectomy done and8 months post transplant patient has persistently high/ worsening creatinine levels: 935 µmol/l
Patient 3		**Biopsy**: Revealed thrombosis due to possible acute rejection	Data not available	Lost graft due to thrombosis

Patient 4	6 months post transplant: 111 µmol/l. But patient had severe proteinuria (in nephrotic range)	**6 months post transplant**: Features of thrombotic microangiopathy. Acute humoral rejection but differential diagnosis includes drug effect, anticardiolipin syndrome & recurrent haemolytic uremic syndrome. **7 months post transplant**: More active thrombotic microangiopathy.	MMF Imuran Solumedrol Prednisone CYA OKT3	The patient was dialysed and had plasmapheresis. Improvement in urine output was noted with no signs of graft tenderness. 6 days post later patient was passing urine,creatinine level of:380 µmol/l
Patient 5	10 days post transplant: 511 µmol/l 2 months post transplant: 232 µmol/l 4 months post transplant: 256 µmol/l	**10 days post transplant**: Possible acute humoral rejection. No features of hyperacute or acute cellular rejection. **4 months post transplant autopsy done**: bilateral ESRD & hydronephorosis. No macroscopic evidence of acute rejection or sepsis.	MMF Solumedrol/ Prednisone CYA Simulect	The patient demised 4 months after transplantation. The autopsy, however did not show overt features of acute rejection.
Patient 6	2 weeks post transplant: 164 3 weeks post transplant: 927 5 weeks post transplant: 547	**3 weeks post transplant**: Parenchymal necrosis involving 20% of the core tissue with vascular change in keeping with acute antibody mediated rejection. **5 weeks post transplant**: Biopsy sample was inadequate.	MMF Prednisone CYA	Donor specific antibody (DSA) testing performed did not reveal the presence of any DSA. The patient has shown improvement with creatinine levels of 87 4 months post transplant.

4.2.1.3 Clinical data on the 6 patients with possible/overt features of acute humoral rejection

In this study, patients' demonstrated features of rejection between 10 days and 6 months post transplant. Patient 1 had a protocol biopsy performed 6 months post transplant which showed features more suggestive of acute cellular rejection, rather than acute humoral rejection. However, on repeat biopsy these features were found to be less prominent and the patient improved with a stable creatinine level 14 months post transplant. Whether this patient had a definitive episode of acute humoral rejection is not entirely clear. This patient was the only one in this group who had Luminex antibody testing done on a pre-transplant sample. The virtual crossmatch as suggested by Luminex revealed negative results for both the T and B cells.

Three patients (identified as patients 2, 5 and 6) were reported to have features suggestive of acute humoral rejection on biopsy, however as C4d staining was not performed on any of these patients' biopsies, definitive diagnoses could not be made according to the Banff criteria.

The remaining 2 patients had possible alternative diagnoses for poor graft functioning including graft thrombosis which may or may not be associated with an immunological injury (patient 3) as well as drug-related microangiopathy(patient 4). Although patient 4 showed features of severe thrombotic microangiopathy on renal biopsy, 6 months post transplant, it was felt that while the thrombotic microangiopathy was most likely drug related, acute humoral rejection could not be excluded completely.

4.2.1.4 Clinical data on other patients with poor graft function

In addition to patients with suspected acute rejection, 3 patients demonstrated poor graft function and other complications including sepsis, persistently high creatinine levels and possible atypical cell-mediated rejection (See Table 14).

Table 14: Patients with poor graft functioning or demonstrated features of acute cellular rejection. All T and B cell crossmatcheswere clearly negative at cut-offs of 2 and 5, respectively. *ATN – acute tubular necrosis

Patient	Clinical follow up		
	Post transplant biopsy/ histology	Other clinical data	Flow cytometry results
Patient 1	Possible haemophagocytic syndrome on bone marrow analysis	Persistently high creatinine levels post transplant	B cell: negative T cell: negative
Patient 2	Biopsy at 3 months: borderline acute cellular rejection. Biopsy at 8 months: focal mild ATN *	9/12 post transplant developed sepsis. Patient demised due to sepsis.	B cell: negative T cell: negative
Patient 3	Biopsy at 6 months: atypical form of acute cellular rejection cannot be excluded. Last creatinine level: 404 μmol/l	Creatinine level at most recent follow up: 404 μmol/l (6 months post transplant)	B cell: negative T cell: negative

5.0 Discussion

The implementation of a successful transplant program relies on a multidisciplinary approach and team (composed of laboratory personnel, transplant co-ordinators, nephrologists and surgeons), from both a clinical and laboratory perspective. The overall goal is to provide a patient with an improved quality of life by giving them a functioning graft for the maximum period of time. In order to achieve this goal, the best possible donor-recipient match is critical. This is largely dependent on accurate laboratory testing, the availability of donors and optimal immunosuppressive therapy.

The flow cytometry crossmatch has various advantages over the existing CDC method of crossmatching. It is more sensitive than the CDC method or the AHG-enhanced CDC method(Fettouh, et al., 2006),(Wen, et al., 2006) and this study addresses the establishment and validation of this assay in the routine laboratory setting.

The set up of the FCXM at our centre was based on the flow cytometry method used by the ANT. The cut-offs used were also based on the ANT method, however different cytometric platforms were utilized. This FCXM was validated in our local setting using Luminex analysis and virtual crossmatching as the gold standard i.e. the FCXM in our local setting (based on the ANT method) was validated against Luminex technology. Our FCXM results were also compared to existing technologies e.g. CDC crossmatching. The clinical outcomes of patients who underwent FCXM were also assessed to determine the clinical utility of the FCXM with respect to rate of rejection (especially acute and hyperacute rejection).

5.1 The development of the FCXM

The initial aim of this study was to compare the performance of the FCXM to the existing CDC method of crossmatching. This analysis was limited by the number of CDC samples which were not resulted because of poor lymphocyte viability (16% or 7/43 samples). High lymphocyte viability is a well-known requirement for an accurate CDC crossmatch result (Eng & Lefell, 2011). Furthermore, detection of low titre antibodies and non-complement binding antibodies, missed on a CDC crossmatch, is possible with the FCXM. In the absence of a reliable gold standard and in view of the limitations associated with the CDC method of crossmatching it was felt that validation of the FCXM should be compared to a more sensitive technique viz. Luminex Technology.

In this study the key components for the development of the flow cytometric crossmatch assay, included: establishing cut off values for positive and negative results, precision analysis, determining the sensitivity, specificity, negative and positive predictive values as well as comparing the available CDC crossmatch results to Luminex technology as a comparison to the FCXM.

One of the biggest dilemmas was establishing cut-off values for determination of positive and negative values for the FCXM since recommendations and values varies from centre to centre and there is no standardization between centres (Martin, et. al., 1994), (O'Rourke, et. al., 2000),(Karpinski, et al. 2001), (Haririan, Nogueira & Kukuruga, (2009). Cut-off values have been based on the mean or the median obtained from sera obtained from control patients ± 2 or 3 standard deviations (Pelletier, et al 1999), (Billen, Voorter & Christiaans, 2008). This does pose difficulties and it is recommended that the cutoff values and interpretation of results should be

interpreted in conjunction with the rates of acute rejection and graft loss for the local population. In this study, we based our initial cutoff values on the experience of a well- established Histocompatibility Laboratory (Anthony Nolan Trust in Hampstead, United Kingdom under the supervision of Dr Raymond Fernando and Dr Henry Stephens). The outcome of patients transplanted in our centre, however, is carefully monitored which will, in future, allow for further evaluation of the assay and adjustment should this be necessary.

During the precision analysis, 15% false negatives were detected on analysis of the B-cell crossmatch (using the serum from volunteer A). In our setting, any patient that has a positive crossmatch result (either B or T cell) will not be transplanted. The significance of a positive B cell crossmatch and graft survival remains unclear (Kotb, Russell & Hathaway, 1999),(Bryan, et al., 2007). In the literature, cases of renal transplantation have been performed in the setting of a positive B cell crossmatch and negative T cell crossmatch with recommendations for close post transplant antibody monitoring and/or use of desensitization protocols (Pollinger, Stegall & Gloor, 2007), (Burns, Cornell, Perry, 2008). There were no false negatives in the T cell crossmatch precision analysis.

The data from volunteer B (i.e. the negative crossmatch) were also analysed simultaneously. All 20 samples revealed true negative results for both the T and B cell crossmatches.

The results of the B cell flow cytometry crossmatch showed better concordance with the Luminex results as compared with the results of the T cell crossmatch. The T cell crossmatch had a sensitivity of 66.7% with a specificity of 83.8% and negative and positive predictive values of 93.9% and 40%, respectively. The B-cell crossmatch had a sensitivity and negative predictive

value of 100% with a specificity of 92.7% and a positive predictive value of 40%. A similar study of 354 patients (Ho, Vascilescu & Colovai, 2008), examined the sensitivity, specificity and clinical relevance of the CDC crossmatch, flow crossmatch and virtual crossmatch (using a solid phase assay such as Luminex assessment of DSA). Graft loss was compared with the presence of DSA. Graft loss without DSA constituted a false negative and graft loss in the presence of DSA a true positive. DSA without graft loss was a false positive result. In this study the sensitivity of the flow crossmatch was 17.1%, the specificity was 85.9%, the PPV was 12.8%, the NPV was 89.6% and accuracy was 78.5%(Ho, et al., 2008). Compared to this study, our FCXM revealed a higher sensitivity, a specificity that is comparable (T cells: 83.8% and B cells: 92.7%), a higher PPV and a similar NPV. The clinical analysis in our study demonstrated that the accuracy of our test is comparable (as ~82% of patients did not demonstrate possible evidence of acute humoral rejection – see section below) to that stated in the above study for the FCXM (accuracy of ~85%).The CDC crossmatch results from our centre had higher sensitivities (40% for the T cell crossmatch and 100% for the B-cell crossmatch compared with 5.3%) and comparable specificities (96.8% and 80% compared with 99.1%).

The FCXM in our centre had a higher sensitivity and negative predictive value for the T-cell and B-cell crossmatch compared with the CDC crossmatch. This is consistent with the findings in other centres which report a positive FCXM in 13-21% of cases where the CDC crossmatch was negative (Karpinski, et al., 2001), (Vascilescu, Ho & Colovai, 2006). The T-cell FCXM has a lower specificity and PPV compared with the CDC crossmatch. For the B cell crossmatch similar, high sensitivities and negative predictive values were documented for both assays. The FCXM however demonstrated higher specificity, positive predictive value and percentage agreement with the nominal results (compared to the CDC method).

Discrepant results were noted between the FCXM and Luminex method. The FCXM detected 4 of the 6 expected positive results by Luminex. In view of the small number of positive results found in our study (six), the 2 discrepant results by FCXM had a large impact on our pool positive of results. The results of the CDC crossmatch were also analysed for these 2 false negative results and concurred with flow results. This suggests that other factors could have contributed to the discrepancy in results such as the FCXM picking up non-HLA antibodies. On clinical follow up, one of these patients (who was FCXM and CDC crossmatch negative but Luminex positive) was transplanted. One year post transplantation, this patient did not demonstrate any features of rejection. This suggests that Luminex technology is very sensitive and detects very low titre antibodies that may not be clinically relevant will not result in a positive crossmatch. Single antigen bead technology is a more sensitive method of detecting donor specific anti-HLA antibodies(Gebel, et al., 2009),(Taylor, Kosmoliaptsis & Summers, 2009). In a recent study, the density of HLA molecules on the bead as well as the strength of the anti-HLA antibodies can influence the results of luminex testing (Warner P 2009). A bead with low density HLA epitopes in the presence of a strong anti-HLA antibody results in a positive result, whereas a bead with high density HLA molecules in the presence of a weak antibody would result in a negative result. Large numbers of clinically insignificant antibodies, directed against commonly found epitopes (public epitopes) on T-cells, may be detected (Bielmann, Honger & Lutz, 2007). Reactivity to altered epitopes present on the beads but not present on the patient's HLA molecules may result in false positive results (El-Awar, Lee & Terasaki, 2005), (Warner P 2009) and poor integrity of the single antigen beads with poor antibody binding may result in false negative results (El-Awar, et al., 2005). Rare or uncommon HLA specificities may not be represented in the single antigen bead array and may result in false negative results (El-Awar, et al., 2005).

While being a very sensitive test, the flow crossmatch is also described as being less specific and subject to interferences due to non- specific binding of IgG antibodies (Lobo, Isaacs & Spencer, 2002). The FCXM can also detect non HLA antibodies (e.g. anti-phospholipid antibodies) (Nanni-Costa, Scolari & Iannelli, 1997). The specificity of the FCXM is, therefore, poorer when compared to other methods like the Luminex technology which has been developed for the sole detection of anti-HLA antibodies (Gebel, et al., 2009). Possible modifications to increase the specificity of the test include the use of an enzyme pronase to digest the Fc IgG receptors on lymphoctyes prior to crossmatching (Lobo, et al., 2002) or simultaneous performance of the CDC and flow crossmatch (Won, Jeong & Kim, 2006).

The limitation of this analysis was the discrepant results of the 3 assays used. The luminex virtual crossmatch, CDC crossmatch and FCXM are subject to different biological interferences and limitations**.** In view of the inherent differences between the 3 methodologies used in this study, clinical follow-up was considered essential. The FCXM showed high predictive values when compared with the rejection rate in this study.

5.2 Retrospective Clinical Analysis

The second objective was to assesss the clinical follow up of patients transplanted at the Johannesburg centre. In this study all patients who were transplanted had negative T and B cell crossmatch results as defined by both FCXM and CDC methods. Clinical follow up was performed using outpatient records, patient databases and clinical feedback from the treating nephrologists. This study revealed a need for the standardization of data collection ideally with clinical reports from the treating nephrologist and surgeon and formal follow-up. Eighty two percent (41/50) of the patients transplanted did not reveal any overt signs suggestive of acute

rejection at follow up. Importantly, none of the patients showed features suggestive of hyperacute rejection, reinforcing the clinical utility of the flow cytometric crossmatch assay. Despite this, 12% of patients showed features suggestive of acute rejection. This finding is consistent within the literature (acute rejection rates have been reported to vary between (23%-35%) possibly as a result of test inadequacy (Fettouh, et al., 2001),(Nickel, et al. 2004),(Scornik, Guerra & Schold, 2007).

Protocol biopsies post transplantation is not performed by all centres and remains a point of controversy,(El-Amm & Gruber, 2009). When performed, protocol biopsies are valuable tool for the assessment of rejection. Conflicting views exist in literature on the implementation of paediatric protocol biopsies for the early detection of subclinical rejection. Concerns regarding safety as the procedure may pose the risk of graft loss, psychological effects on the child and family, lack of expertise as well as infrastructure and the lack of clinical benefit have been cited as reasons for not incorporating protocol biopsies into routine pediatric transplant programs (Birk & Rush, 2006), (Shapiro& Starzl, 2006). Birk et al have however, reported that in a cohort of 21 children aged between 8.2 and 16.2 years, that 21% low risk patients demonstrated evidence of subclinal acute rejection on protocol biopsy and that changes in creatinine levels were an unreliable predictor of rejections. Furthermore, there were no serious adverse events or fatalities related to the performance of these biopsies, reported in this study (Birk, et.al., 2006).

Routine histologic examination usingof special stains (viz C4d stain) and testing for the presence of DSA provide a definitive diagnosis of rejection. At present, C4d staining is only performed by one laboratory service in the Private sector in South Africa. C4d staining is an integral component in the diagnosis of antibody mediated rejection.and with demonstration of DSA forms part of the internationally recognized criteria for assessment of acute humoral rejection. No C4d

staining results were available for the6 patients suspected to have acute humoral rejection. Although the histological features were suggestive of acute humoral rejection in these patients, definitive diagnosis was not possible. DSA testing had not been validated at our centre at the time of this study although it has subsequently been implemented. Studies by Bohmig, Bartel and Regele (2009) show discrepancies between C4d staining and DSA positivity which may result from absorption of DSA by the graft, presence of non HLA antibodies or IgM antibodies, low titres of antibodies and false positive C4d staining. In addition, a graft may accommodate (tolerate immune-mediated injury despite circulating DSAs) and give discrepant C4d and DSA results (Cecka, Zhang& Reed, 2005). Accommodation can occur if DSAs accumulate slowly post-transplantation, with antibody absorption by the graft or because of immunoregulatory strategies including inactivation of the membrane attack complex of complement or upregulation of protective factors like anti-inflammatory cytokines (Dehoux & Gianello, 2009). Timing of post-transplantation biopsies may also influence the histological picture(El-Amm, et al., 2009)

Antibodies that target other non HLA molecules may play a role in graft dysfunction (Amico, Honger & Bielmann et al., 2008). These antibodies, including anti-angiotensin antibodies and anti-MIC A antibodies, may target renal endothelial cells. Amico et al (2008), showed an acute rejection rate of 2.3% of patients (10/433) who were both CDC and virtual crossmatch negative and suggested that this may be the result of non-HLA, endothelial-directed antibodies. Three of the 10 patients demonstrated anti-MIC-A antibodies directed at donor antigens. Zou et al (2007) showed that the presence of anti-MICA antibodies predicted poorer graft survival at 1 and 5 years in a group of 1910 first-time renal recipients. Anti-MICA antibody testing was not performed in our setting at the time of this study but implementation of the test will be useful in cases of acute rejection where anti-HLA antibodies against the donor cannot be demonstrated.

For adults, the local regimen for induction immunosuppression, includes the use of Basiliximab (Simulect), Mycophenolate Mofetil, Solumedrol and a calicneurin inhibitor such as Cyclosporin or Tacrolimus. Prednisone replaces basiliximab or solumedrol in maintenance immunosupression and the other drugs are used at lower doses than at induction. The protocols used are similar to that described in literature (Panigrahi, et al., 2007), (Cinti, Pretagostini & Lai, 2009). In this MMED study, it was found that 5 of 6 patients presenting with possible acute rejection, had easily accessible data regarding their immunosuppressive regimens. These 5 patients were on similar immunosuppressive therapy (viz. Corticosteroids, Cyclosporin and Mycophenolate Mofetil). Two of the patients, in addition, were started on either OKT 3(a monoclonal antibody used in cases of suspected acute rejection) or Simulect (which is Basiliximab, a monoclonal antibody against IL2- receptor (anti-CD25)). This suggests that an immunological mechanism was not responsible for the graft failure in all of these cases although this could not be confirmed in the absence of C4d staining. Two patients of the 6 showed response to the immunosuppressive therapy with graft recovery.

With limited resources at hand, the scope of testing in this setting should include efficacious, low cost testing. The cost of the FCXM assay is R2150.00 per donor (for related living donors) and R4000.00 for cadaver crossmatches while virtual crossmatching using Luminex is R4,179.80. This is a minimal expense in the overall setting of renal care and renal replacement therapy. Data from a leading, medical aid insurer in South Africa, Discovery Health, reveal that the cost of a single renal transplant procedure is approximately R430,000.00 (<http://www.discovery.co.za/discovery/health /health_benefit_trakcer_april.pdf>, updated April 2011). The cost of hemodialysis and peritoneal dialysis is estimated to range between $6,000.00 and $40,000.00 per annum (Dirks & Levin, 2006).

6.0 Conclusion

Laboratory testing forms an integral component in the work up of a transplant patient. In any setting, identifying the recipient with the lowest immunological risk for rejecting an allograft is the principal objective.

In this study, the flow cytometric crossmatch test was chosen as it is internationally accepted to have a higher sensitivity than the CDC assay (currently being used locally) and is not limited by biologic variability (as the CDC is affected by poor lymphocyte viability) .

In this study, the FCXM did not reveal a higher sensitivity compared to the CDC method of cross-matching. The CDC method, however, had a greater number of false positive results. The FCXM assay was validated based on its high negative predictive value (T cell crossmatch negative predictive value= 93.7% and B cell crossmatch negative predictive value= 100%) obtained, when compared to the results as predicted by Luminex technology. However, as illustrated in the results and discussion section, there is no gold standard method to which cross-matching can be compared to. The Luminex technology while described as a very sensitive method of antibody screening for HLA antibodies, is vulnerable to its own biologic interferences and can be influenced by operator subjectivity and the experience of the laboratory performing tests. Furthermore, Luminex technology may detect antibodies that are not clinically significant.

Although laboratories may establish their own cutoff values for the B and T cell crossmatch, the lack of standardization of these cutoff values internationally and locally also poses another point of difficulty.

At the time of this study current local transplant practice, involved initial CDC crossmatching of a large number of recipients on the transplant list using preplated sera. Following this analysis, the top 10 patients were called in for fresh specimen collection and hence performance of CDC and flow cytometric crossmatching on current sera. FCXM is not a practical way of crossmatching large sample numbers (as is done for the initial CDC crossmatch on preplated sera).

For the various reasons mentioned, continued performance both the FCXM and CDC assays represent the safer method for selection of potential recipients. Current clinical practice in Gauteng, requires potential recipients to be B and T cell crossmatch negative by both CDC and FCXM assays.

Clinical follow up was performed on patients to determine the presence of clinical features of humoral rejection. While this represents the ultimate end point of this study, it is also subject to constraints in our setting. The absence of adequate resources in the state sector and especially the absence of the C4d stain hampers accurate diagnosis of humoral rejection. Nonetheless, the rate of acute rejection found in this study is comparable to that reported in literature. Furthermore, no confirmed cases of hyperacute rejection were documented.

Recommendations arising from this study:

1) Continued use of both the CDC and Flow cytometry crossmatch assays.

2) Detailed accumulation of data on sensitization events in patients on the transplant list as well as their clinical progress after transplantation. Closer liaison is required

between treating physicians, surgeons and laboratory personnel.

3) Introduction of C4d staining on routine biopsies

4) With the introduction of sensitive HLA antibody screening techniques (e.g. using Luminex technology) and higher resolution HLA typing and sophisticated databases, virtual crossmatching on all patients may become a viable option. Stemming from the outputs of this evaluation, the validation of Luminex HLA-antibody detection has recently been completed at our centre and virtual crossmatching recently introduced.

5) Testing for the presence of donor specific antibodies in all patients suspected to have acute rejection should be implemented nationally

6) Introduction of MIC-A antibody testing. The clinical relevance in terms of graft outcome will have to be determined for the local population.

7) Assessing the clinical utility of tests associated with the cell mediated immunity in the setting of renal transplantation e.g. soluble CD30 and Immuknow assays (Susal et al, 2007)

7. 0 Appendices

Appendix 1: Banff criteria for renal allograft biopsies – 2009 update

Table 1: Banff 97 diagnostic categories for renal allograft biopsies—Banff '09 update

1. Normal
2. Antibody-mediated changes (may coincide with categories 3, 4 and 5 and 6)
   Due to documentation of circulating antidonor antibody, C4d,[1] and allograft pathology

C4d deposition without morphologic evidence of active rejection

   C4d+, presence of circulating antidonor antibodies, no signs of acute or chronic TCMR or ABMR (i.e. g0, cg0, ptc0, no ptc lamination (<5 layers by electron microscopy), no ATN-like minimal inflammation). Cases with simultaneous borderline changes are considered as indeterminate

Acute antibody-mediated rejection[2]

   C4d+, presence of circulating antidonor antibodies, morphologic evidence of acute tissue injury, such as (Type/Grade)
   I. ATN-like minimal inflammation
   II. Capillary and or glomerular inflammation (ptc/g >0) and/or thromboses
   III. Arterial – v3

Chronic active antibody-mediated rejection[2]

   C4d+, presence of circulating antidonor antibodies, morphologic evidence of chronic tissue injury, such as glomerular double contours and/or peritubular capillary basement membrane multilayering and/or interstitial fibrosis/tubular atrophy and/or fibrous intimal thickening in arteries

3. Borderline changes: 'Suspicious' for acute T-cell mediated rejection (may coincide with categories 2 and 5, and 6)
   This category is used when no intimal arteritis is present, but there are foci of tubulitis (t1, t2 or t3) with minor interstitial infiltration (i0 or i1) or interstitial infiltration (i2, i3) with mild (t1) tubulitis
4. T-cell mediated rejection (TCMR, may coincide with categories 2 and 5 and 6)

Acute T-cell mediated rejection (Type/Grade:)

   IA. Cases with significant interstitial infiltration (>25% of parenchyma affected, i2 or i3) and foci of moderate tubulitis (t2)
   IB. Cases with significant interstitial infiltration (>25% of parenchyma affected, i2 or i3) and foci of severe tubulitis (t3)
   IIA. Cases with mild to moderate intimal arteritis (v1)
   IIB. Cases with severe intimal arteritis comprising >25% of the luminal area (v2)
   III. Cases with 'transmural' arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells with accompanying lymphocytic inflammation (v3)

Chronic active T-cell mediated rejection

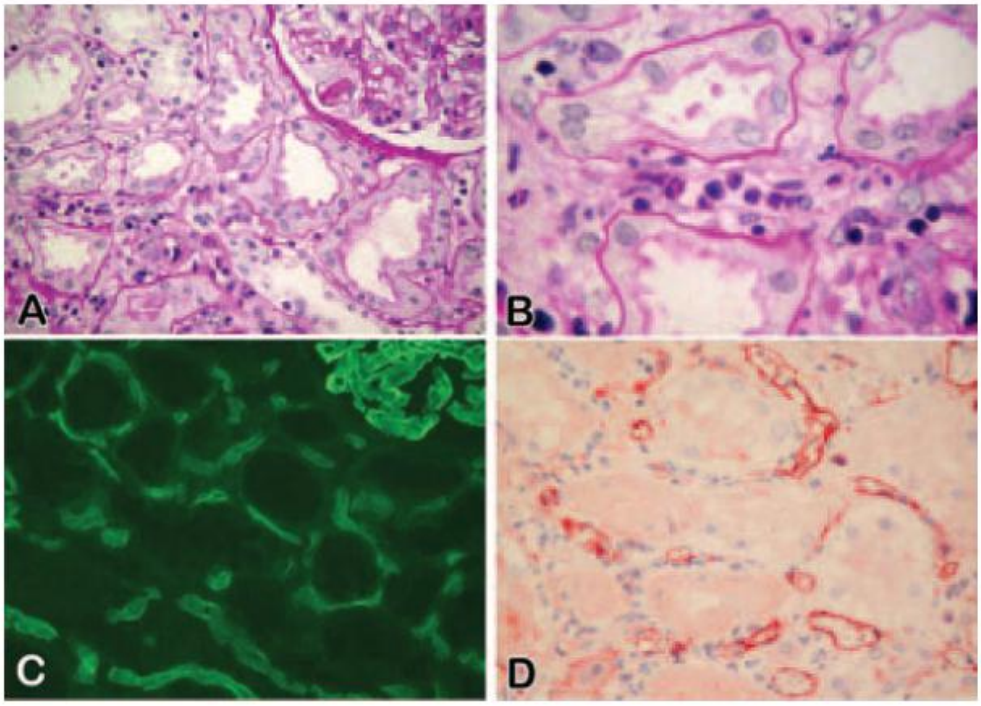
   'chronic allograft arteriopathy' (arterial intimal fibrosis with mononuclear cell infiltration in fibrosis, formation of neo-intima)

5. Interstitial fibrosis and tubular atrophy, no evidence of any specific etiology
   (may include nonspecific vascular and glomerular sclerosis, but severity graded by tubulointerstitial features)
   Grade
   I. Mild interstitial fibrosis and tubular atrophy (<25% of cortical area)
   II. Moderate interstitial fibrosis and tubular atrophy (26–50% of cortical area)
   III. Severe interstitial fibrosis and tubular atrophy/ loss (>50% of cortical area)
6. Other: Changes not considered to be due to rejection- acute and/or chronic (For diagnoses see table 14 in (49); may include isolated g, cg, or cv lesions and coincide with categories 2, 3, 4, and 5)

ATN, acute tubular necrosis.
The 2009 updates are underlined. All existing scoring categories (g, t, v, i, ptc, cg, ct, ci, cv, ah, mm) remain unchanged (45, 49).
[1]Please refer to Banff 2007 classification paper (45).
[2]Suspicious for antibody-mediated rejection if C4d (in the presence of antibody) or alloantibody (C4d+) not demonstrated in the presence of morphologic evidence of tissue injury.

American Journal of Transplantation 2010; 10 464-471: Banff 09 Meeting Report: Antibody Mediated Graft Deterioration and Implementation of Banff Working Groups, Sis, Mengel, Haas.

Appendix 2:Picture displaying histologic features of Acute Antibody Mediated Rejection



Picture 1: - Histologic features of AMAR

- A- the peritubular capillaries which show the presence of polymorponuclear cells and mononuclear cells. In the top right hand side of the picture is glomerular capillaries which demonstrate some polymorphonuclear cells, mononuclear cells and fibrin deposition.
- B- In addition to the presence of polymorphonuclear cells, interstitial oedema is noted
- C- Monoclonal C4D antibody staining using immunofluorescence microscopy
- D- Polyclonal C4D staining of peritubular capillaries on immunohistochemistry

Solange Moll and Manuel Pascual, Humoral Rejection of Organ Allografts, American Journal of Transplantation 2005; 5: 2611–2618,

Appendix 3: Ethics approval of original protocol and revised protocol

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Ramparsad

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M080218**
PROJECT	Establishment of a Flow Cytometric Assay for T and B cell crossmatching in the setting of Renal Transplantationo
INVESTIGATORS	Dr N Ramparsad
DEPARTMENT	Molecular Med & Haematology
DATE CONSIDERED	08.02.29
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 08.04.02 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof W Stevens

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical)

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

16 March 2009

Dr Narisha Ramparsad
Department of Haematology and Molecular Medicine
National Health Laboratory Services
University

Dear Dr Ramparsad

RE: Amendments of Protocol M080218

This letter serves to confirm that the Chairman of the Human Research Ethics Committee (Medical) has reviewed and approved the revised protocol and consent form on the abovementioned protocol.

Thank you for keeping us informed and updated.

Yours sincerely,

Anisa Keshav
Secretary
Human Research Ethics Committee (Medical)

Appendix 4: Consent Form

Consent Form

Good day

Kidney disease is a serious problem worldwide. One of the ways of treating kidney disease includes kidney transplantation. All over the world, there are a larger number people needing kidney transplants and the number of donations is not able to meet the demand, hence many people are on a transplant waiting list. To help prevent complications and failure of kidney transplantation, good screening and testing methods must be established. One of the ways to improve this, is to provide good testing methods. Before a transplant can take place, a variety of tests are done on both the recipient (i.e. patient on the renal transplant list) and the donor. At present, the Complement Dependent Cytotoxic assay is a type of test that is being used to determine compatibility between donor and recipient. Methods to improve testing are being looked at so that the appropriate renal patients are given the best possibly matched kidney. We, at the National Health Laboratory Services, Department of Haematology and Molecular Medicine are investigating a test called a flow cytometry crossmatch test. In addition to the routine test (i.e. the CDC test) that is currently being used , the flow cytometric testing is also being performed just before a transplant and helps in determining compatibility between patients and prospective donors . For this test to be validated and eventually become part of routine testing (as is done internationally), blood samples from both donors and potential recipients are being tested with both the CDC and the flow cytomteric crossmatch methods.

The sample is used for this test only and not for any other purpose. The data and results obtained from this testing remain confidential and access to this information is limited to health-care workers involved in the transplant.

To help us in the establishment of this test, clinical information regarding, you, the recipient is required. This may include information regarding the cause of kidney failure, transfusion history, results of blood tests etc . This information is not anonymous but will remain confidential. For this purpose, consent will be required from you.

I , the undersigned (patient / guardian) _____,
hereby agree for clinical information (i.e. information in my hospital file and results of investigations performed) on (patient/ guardian)
_____ , to be accessed for the purposes of follow up in assisting in the validation of the flow cytometric crossmatch testing in kidney transplantation.

Signature (patient/guardian) :_____
Date:_____

Witness : _____
Date:_____

For further information or queries please contact one of the following people:

Narisha Ramparsad: 0845565914

Elizabeth Mayne :0823376349

Appendix 5:Results of Luminex testing : Class I anti HLA antibodies

	Hosp No	Sample ID	Ab specificities likely to cause a positive x-match: Strong positive	Ab specificities that may cause a positive x-match: Positive	Ab specificities that are unlikely to cause a positive x-match: Weak Positive
Negative	Control				
	210254	1		B42 A25 A26 A66 A31 A32	B81
	260387	2		A80 CW1	A43 CW4
	280241	5	NEGATIVE on sAg		
	040254	6		A31	
	260788	7	All BW6 B Ag: B44 B82 B62 B35 B42 B75 B64 B71 B8 B72 B27 B76 B39 B18 B67 B7 B45 B50 B61 B54 B56 B65 B81 B61 B78 B48	B41 A1 B60	B51
	030554	9		A2 CW17	
	141166	13		A24 A23 B35	
	010793	14	B60 B48 B81 B8 ?BW6	B7 B76 B41 B72 B 65 B82	B18 B42 B56 B71
	281147	15	A25 A32 B51 B38 B49 B27 B55 B52 B77 A24 B37 B59 A23 A26 B42 B53	A69 B81 CW18 A66 B57 B63 CW17 A68 B50 B57 B7 B58 CW5 B13 B62 A34 A34 B13	A33 CW2 B61 B72 B44 CW6 B56 B61 B67 B76 B47 B48 B71 CW15 CW4 A33 B82
	280757	17			CW9 CW10 B75 B82 A68
	141166	19	A23 A24		
	280241	20	NEGATIVE on sAg		
	200956	21	A68 A3 A74 B53 A11 B44 A69 B27 A24 A33 B38 B49 A29 A23 B59 B37 A29 A80 A30 B77 B57 A34 A31 A66 B58 B57 B51 A32 B45 A26 B63 A33 B47 B51 A30 A43 B52 A66 A36 B64 A1 A25 A34 B50 B61 B13	B61 B75 B65 B62 B48 CW10	CW1 B60 B39 B82 CW15 B72
	010159	22	NEGATIVE on sAg		

281147	23	A25 A32 B51 B38 B49 B27 B55 B52 B77 A24 B37 B59 A23 A26 B42 B53	A69 B81 CW18 A66 B57 B63 CW17 A68 B50 B57 B7 B58 CW5 B13 B62 A34 A34 B13	A33 CW2 B61 B72 B44 CW6 B56 B61 B67 B76 B47 B48 B71 CW15 CW4 A33 B82
070492	24	A29 A43	B27	B76
110575	25	B62 B50 B71 A2 B51 B57 A2 B58 B35 B75 B53 B37 B49 B77	B63 B56 B72 B51 B78 A68 A69 B81 A24 B48 B52	A24 B46 CW7
170771	27		B35 B51	
260788	31	All BW6 B Ag: B44 B82 B62 B35 B42 B75 B64 B71 B8 B72 B27 B76 B39 B18 B67 B7 B45 B50 B61 B54 B56 B65 B81 B61 B78 B48	B41 A1 B60	B51
100362	32			A25 A26 A66
160982	33		B62	
260172	35	A24 A23 A34 A69 A33 A68 A2 A11 A3 A34 A29 B82 B45 B76	A66 A31 A30 A74 B44	
120293	36		A2 A24 A31 A29 A23	A33 B37
160864	39			A66 A26 A25
080774	40	B57 A2 B58	A23 A24	B38 B63 B62 B53
070492	41	B43 A29	B27 B76	
200956	42	A68 A3 A74 B53 A11 B44 A69 B27 A24 A33 B38 B49 A29 A23 B59 B37 A29 A80 A30 B77 B57 A34 A31 A66 B58 B57 B51 A32 B45 A26 B63 A33 B47 B51 A30 A43 B52 A66 A36 B64 A1 A25 A34 B50 B61 B13	B61 B75 B65 B62 B48 CW10	CW1 B60 B39 B82 CW15 B72
171263	44	A31 A30 A80 A33 A30 A74	B82 A66 A34 A68	CW10 A3
04972	46			B57 ?A2
261085	48	NEGATIVE on sAg		
150372	50	B51 A2 B53 A25 B76 A34 A26 B35 A69 A66 A74 A33 B8 B18 A43 A29 A68 A11 A31 A32 B37 B46 B52	A36 A1 B75 B63 B45 CW7	B59 B38 B64 CW5
260387	51		A80	CW1
160864	53			A66 A26 A25 A43

280554	54		A2 B75	A30
280554	55		A2 B75	A30
210262	56	B56 B49 B52 B57 B78 B77 B53 B63 B58 B51 B72 B16 B46 CW10 B35 B50 B75 B45 B51 B54 B71 B76 CW9 B38 B62 B64 B37 B44 B39 B8 B55	B13 A80 A66 B48 A3 B59 B82 A25 B61 A24, A24	A11 A1
080774	57	A2 B57 B58		A23 A24
210262	58	B56 B49 B52 B57 B78 B77 B53 B63 B58 B51 B72 B16 B46 CW10 B35 B50 B75 B45 B51 B54 B71 B76 CW9 B38 B62 B64 B37 B44 B39 B8 B55	B13 A80 A66 B48 A3 B59 B82 A25 B61 A24, A24	A11 A1
240560	59		B44 B45 B7 A66 B81 B37	B27

Appendix 6: Results of Luminex testing: Class II anti HLA antibodies

	Hosp No	Sample ID	Ab specificities likely to cause a positive x-match: Strong positive	Ab specificities that might cause a positive x-match: Positive	Ab specificities that are unlikely to cause a positive x-match: Weak Positive
Negative	Control				
	260387	2			DQ7 DR4
	260788	7	DR17 DR1 DR12 DR16 DR15 DR13 DR12 DR103 DR8 DR13 DR14 DR11 DR4 DR51 DR18 DR52 DR9 DR10	?DQ7	
	030554	9			DP2,14 DR9
	141166	13	DR53		?DQ7,8
	281147	15	DR53 ?DP9,3,17	DR4	
	010748	16			DQ7,8,9
	141166	19	DR53		
	200956	21			?DP2,4
	010159	22	NEGATIVE on sAg		
	281147	23	DR53 ?DP9 ,3,17 DR4		
	070492	24		DR51, ?DQ7 DR103 ?DP10,14,13 DR1,9,4	
	110575	25	DR12 DR15 DR13 DQ7 DR103 DR16 DR15 DR51 DR11 DQ9 DR4 DQ8	DR1 DR7 DR9	DQ4 DQ5
	260788	31	DR17 DR1 DR12 DR16 DR15 DR13 DR12 DR103 DR8 DR13 DR14 DR11 DR4 DR51 DR18 DR52 DR9 DR10	?DQ7	
	100362	32			DR17
	260172	35	DR103 DR1 ?DP9 DP5 DR11 DQ6 DQ4 DR51 DR10	DR9	DQ7
	070492	41		DQ7 DR51 ?DP10,14	
	200956	42			DP4,2
	261085	48		DR13	
	150372	50	DQ6 DR9 DR103 DR53 DR51 DR10 DR1 DQ2 DR4 DR15 DR14 DR7 DR16	DQ5	
	260387	51	NEGATIVE on sAg		
	210262	56	DR1 DR10	DR13 DR103 DQ4	DQ7 DQ9
	210262	58	DR1 DR10	DR13 DR103 DQ4	DQ7 DQ9
	240560	59			DR103 DR18 DR4

Appendix 7: Certification of Participation in External Quality Assurance



UKNEQAS for HISTOCOMPATIBILITY and IMMUNOGENETICS
UNITED KINGDOM EXTERNAL QUALITY ASSESSMENT SERVICE

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CERTIFICATE OF PARTICIPATION – JANUARY – DECEMBER 2008

This document confirms that

NH LAB SERVICE - SOUTH AFRICA

Haematology Department
National Health Laboratory Service
PO BOX 1038
Johannesburg – 2001
SOUTH AFRICA

has participated in the
UK NEQAS for Histocompatibility and Immunogenetics
and has returned results for the following analyte(s):

CROSSMATCHING BY FLOW CYTOMETRY

SUSAN CORBIN - SCHEME MANAGER

Appendix 8: Reproducibility runs with serum A

Donor cells crossmatched against Serum A (First run)

Negative Controls		MFI B cells		MFI T cells			
Neg control autologous		52.2		16.7			
Neg control male AB serum		784.0		484.0			
Cut- Off B cells:	261.0						
Cut-Off T cells:	33.4						
Postive Control ALG		6595.0		1027.0			
Samples						Ratio B	Ratio T
1		9858.0	Pos	690.0	Pos	188.9	41.3
2		10644.0	Pos	744.0	Pos	203.9	44.6
3		14397.0	Pos	873.0	Pos	275.8	52.3
4		8033.0	Pos	808.0	Pos	153.9	48.4
5		8349.0	Pos	523.0	Pos	159.9	31.3
6		7432.0	Pos	599.0	Pos	142.4	35.9
7		7008.0	Pos	645.0	Pos	134.3	38.6

Donor cells crossmatched against Serum A (Second run)

Negative controls		**MFI B cells**		**MFI T cells**			
Autologous		73.1		10.5			
Male AB		48.8		10.7			
Cut-Off B cells	244.0						
Cut-Off T cells	21.0						
Positive control ALG		1058.0		392.0			
Samples						Ratio B	Ratio T
1		**231.0**	**Neg**	96.0	Pos	4.7	9.1
2		316.0	Pos	107.0	Pos	6.5	10.2
3		422.0	Pos	144.0	Pos	8.6	13.7
4		325.0	Pos	128.0	Pos	6.7	12.2
5		904.0	Pos	248.0	Pos	18.5	23.6
6		383.0	Pos	136.0	Pos	7.8	13.0

Donor cells crossmatched against Serum A (Third run)

Negative controls		**MFI B Cells**		**MFI T cells**			
Autologous		4164.0		402.0			
Male AB serum		3427.0		403.0			
Cut-Off B cells	17135.0						
Cut-Off T cells	804.0						
Positive control ALG		36691.0		13870.0			
Samples						Ratio B	Ratio T
1		**97774.0**	Pos	1300.0	Pos	28.5	3.2
2		**14451.0**	**Neg**	4657.0	Pos	4.2	11.6
3		18554.0	Pos	4387.0	Pos	5.4	10.9
4		**103000.0**	**Pos**	5312.0	Pos	30.1	13.2
5		18182.0	Pos	5376.0	Pos	5.3	13.4
6		**16355.0**	**Neg**	5421.0	Pos	4.8	13.5
7		25165.0	Pos	4216.0	Pos	7.3	10.5

Appendix 9: Reproducibility runs with serum B

Donor cells crossmatched against Serum B (First run)

Negative Controls		MFI B cells		MFI T cells				
Neg control autologous		110.0		7.0				
Neg control male AB serum		19.7		5.1				
Cut off B	98.5							
Cutt off T	10.2							
Positive control ALG		718.0		174.0				
Samples						Ratio B		Ratio T
1		44.6	neg	4.5	Neg	2.3		0.9
2		45.7	neg	6.5	Neg	2.3		1.3
3		54.3	neg	4.7	Neg	2.8		0.9

Donor cells crossmatched against serum B (Second run)

Negative Controls		MFI B cells		MFI T cells				
Neg control autologous		24.6		4.1				
Neg control male AB serum		15		4.3				
Cut off B	75.0							
Cut off T	8.2							
Samples						Ratio B		Ratio T
1		9.7	neg	4.5	Neg	0.6		1.1
2		20.6	Neg	4.9	Neg	1.4		1.2
3		6.0	Neg	3.9	Neg	0.4		1.0

Donor cells crossmatched against Serum B (Third run)

Negative Controls		MFI B cells		MFI T cells				
Neg control autologous		1445.0		95.7				
Neg control male AB serum		1187.0		118.0				
Cut off B	5935.0							
Cut off T	191.4							
Samples								
1		1072.0	neg	94.9	neg	0.9		1.0
2		4120.0	neg	101.0	neg	3.5		1.1
3		2818.0	neg	121.0	neg	2.4		1.3

Appendix 10: Results of all crossmatches performed with serum A and serum B

Results of all crossmatches performed with Serum A

Sample	Run				
		Ratio B	Result	Ratio T	Result
1	1	188.9	Pos	41.3	Pos
2	1	203.9	Pos	44.6	Pos
3	1	275.8	Pos	52.3	Pos
4	1	153.9	Pos	48.4	Pos
5	1	159.9	Pos	31.3	Pos
6	1	142.4	Pos	35.9	Pos
7	1	134.3	Pos	38.6	Pos
1	2	4.7	Neg	9.1	Pos
2	2	6.5	Pos	10.2	Pos
3	2	8.6	Pos	13.7	Pos
4	2	6.7	Pos	12.2	Pos
5	2	18.5	Pos	23.6	Pos
6	2	7.8	Pos	13.0	Pos
1	3	28.5	Pos	3.2	Pos
2	3	4.2	Neg	11.6	Pos
3	3	5.4	Pos	10.9	Pos
4	3	30.1	Pos	13.2	Pos
5	3	5.3	Pos	13.4	Pos
6	3	4.8	Neg	13.5	Pos
7	3	7.3	Pos	10.5	Pos

Results of all crossmatches performed with Serum B

Samples	Ratio B	Result	Ratio T	Result
1	2.3	Neg	0.9	neg
2	2.3	Neg	1.3	neg
3	2.8	Neg	0.9	neg
4	0.6	Neg	1.1	neg
5	1.4	Neg	1.2	neg
6	0.4	Neg	1.0	neg
7	0.9	Neg	1.0	neg
8	3.5	Neg	1.1	neg
9	2.4	Neg	1.3	neg

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