A comparison of ABO blood group antibody titration methodologies and ABO IgG subclass measurement against graft survival in ABO-incompatible kidney transplant patients at Hammersmith Hospital

by

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Abstract

Background

ABOiRTx has the potential to increase kidney donor availability and access to transplantation. However, precise monitoring of ABO antibodies is critical in guiding treatment and assessing patient suitability for ABOiRTx. Haemagglutination titration assay is a semi-quantitative methodology commonly used for measuring ABO antibodies and the amount of PE required pre- or post-transplantation. Due to the variation in titration assays across institutions, this study was conducted to investigate for agreement/disagreement between these methods and suggest proposals for standardisation. 50 plasma samples were tested against the titration methods, Tube IAT, Tube DRT, Bio-Rad IAT, Bio-Rad DRT, Bio-Vue IAT and Bio-Vue DRT. 30 of these samples were from ABOiRTx patients with graft outcome data available while 20 were from ESRD patients awaiting transplantation. An ELISA method for the measurement of ABO IgG antibody subclasses (IgG1, IgG2, IgG3 and IgG4) was also developed and used to measure subclass levels in 9 patients.

All titration results showed overall statistically significant difference using ANOVA multivariate analysis which meant that the methods cannot used interchangeably. However, upon pairwise analysis, association was found between the following pairs of methodologies: Tube IAT/Tube DRT, Tube DRT/Bio-Rad IAT and Bio-Rad DRT/Bio-Vue IAT. The number of PE was closely linked to baseline but not post-transplant ABO antibody titres. The use of one operator did not improve the variability between the different titration methods. This suggests that all ABO antibody titration methodologies in current use globally cannot be used interchangeably, thus making it difficult to objectively manage patients pre-transplantation. From the study results, a reasonable choice of methodology to propose for standardisation across transplant centres is the Bio-Rad IAT column method which can be developed over time and therefore provide the basis for all comparisons.

Using the Indirect ELISA technique, most of the cases that experienced graft rejection (5/9) had a combination of IgG1 and IgG2 subclasses. No association was found between ABO IgG2, IgG3 and IgG4 as individual entities against graft outcome. IgG1 was the only antibody that demonstrated association with graft outcome. IgG2 was detected in 4/4 cases who did not experience graft rejection. IgG3 was detected in 4/5 graft rejection cases which supports its known feature of efficiency in activating complement. IgG4 was found in 1/9 cases, in a patient who experienced graft rejection. The sample size for this part of the study was too small, resulting in low statistical power. However, the Indirect ELISA technique developed in the study can be used to explore ABO IgG antibody subclass dynamics to further understand their role in ABOiRTx.

Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

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Abbreviations

AAMR	Acute Antibody Mediated Rejection
ABOc	ABO Compatible
ABOcRTx	ABO Compatible Kidney Transplant
ABOi	ABO incompatible
ABOiRTx	ABO incompatible Kidney Transplantation
AMR	Antibody Mediated Rejection
APC	Antigen Presenting Cells
CBT	Checkerboard Titration
CI	Confidence Interval
CIA	Coefficient of Individual Agreement
CMV	Cytomegalovirus
DBD	Donation after Brain Death
DCD	Donation after Cardiac Death
DFPP	Double Filtration Plasmapheresis
DRT	Direct Room Temperature Technique
DTT	Dithiothreitol
ELISA	Enzyme Linked Immunosorbent assay
ESRD	End Stage Renal Disease
GalNAc	α-D-N-acetylgalactosamine
GFR	Glomerular Filtration Rate
HAMR	Hyperacute Antibody Mediated Rejection
HAR	Hyperacute Graft Rejection
HAS	Human Albumin Solution
HLA	Human Leukocyte Antigen
HLAi	HLA incompatible
HLAiKTx	HLA Incompatible Kidney Transplant

HRP	Horse Radish Peroxidase
HSV	Herpes Simplex Virus
IA	Immunoadsorption
IAT	Indirect Antiglobulin Test
ICC	Intra-class Correlation Coefficient
IgG	Immunoglobulin G
IgA	Immunoglobulin A
IgM	Immunoglobulin M
IL	Interleukin
IVIg	Intravenous Immunoglobulin
MAC	Membrane Attack Complex
2-ME	2-Mercaptoethanol
MHC	Major Histocompatibility
NGP	Neoglycoprotein
NHSBT	National Health Service Blood and Transplant
NIH	National Institutes of Health
OD	Optical Density
OPD	Orthophenylenediamine
PBSS	Phosphate Buffered Saline Solution
PE	Plasma Exchange
PP	Plasma Pheresis
RAH-IgG	Rabbit Antihuman Globulin
RT	Room Temperature
SpA	Staphylococcal Protein A
SPR	Surface Plasmon Resonance
SD	Standard Deviation
SE	Standard Error

TCR	T-Cell Receptor
Th2	T-helper 2 lymphocytes
TPE	Therapeutic Plasma Exchange
UKNEQAS	United Kingdom National External Quality Assurance Scheme
UKNEQAS BTLP	United Kingdom National External Quality Assessment Scheme for Blood Transfusion Laboratory Practice
UNOS	United Network for Organ Sharing
VZV	Varicella Zoster Virus

Chapter One: Introduction

1.1 Overview of End Stage Renal Disease

End stage renal disease (ESRD) begins when the kidneys have failed and renal replacement therapy is initiated. In such cases of renal impairment, the patients are managed through the use of haemodialysis, peritoneal dialysis or transplantation (1).

ESRD significantly increases mortality and morbidity in patients and the incidence of this condition has doubled in the UK since 1995 and is a growing international problem (2). The incidence of patients with chronic (ESRD) is rapidly increasing worldwide as a result of an increase in patients with lifestyle associated diabetes, hypertension and advancement in diagnostic methodologies (3).

Most patients commencing dialysis for ESRD in low-income countries die or stop treatment within the first 3 months of initiating dialysis due to cost constraints. This is mainly because of the limited availability of haemodialysis and peritoneal dialysis facilities in these countries. However, kidney transplantation offers a better option for patients on dialysis by significantly reducing mortality and improving the quality and length of life for a third of the cost of haemodialysis. Although the initial cost of transplantation exceeds that of maintenance dialysis following transplantation, the subsequent cost is much reduced largely due to advances in cheaper immunosuppression regimens (4). As most patients would already be suffering from other underlying or co-morbid conditions such as hypertension or diabetes, undergoing transplantation procedure is taxing.

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Due to the scarcity of organ donors, consideration has to be made for ethical and financial issues associated with each transplantation procedure. How long the graft is expected to survive and how long the patient is expected to live are both critical elements in the decision-making process that governs management of ESRD. Factors that predict graft survival have been studied extensively in adults and children. Donor and recipient age, pre-existing donor hypertension and diabetes, prolonged cold storage time, re-transplantation, multiple blood transfusions and body mass index of both recipient and donor have demonstrated to play important roles in the post-transplant outcome along with a host of other factors.

Traditionally, ESRD patients would take a course of haemodialysis or peritoneal dialysis or both, possibly followed by one or more transplants. Increasingly, patients are opting for transplantation as the very first ESRD treatment modality, a choice labelled ‘pre-emptive transplant’. Much of the enthusiasm for pre-emptive transplantation stems from reports that they are advantageous for graft and patient survival. Increased time on dialysis is a predictor of negative short-term graft outcome and transplant recipient survival. The first successful attempt at kidney transplantation was in 1954 and as a result, since then, there has been an increase in the number of transplants being performed each year for a growing recipient population (5-7).

There is evidence of improved patient and allograft survival when pre-emptive or early transplantation is implemented before commencement of long-term dialysis or within 6-12 months of initiation of dialysis.

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Improved patient and allograft survival has been noted in cases of early transplantation (*i.e*. before commencement of long-term dialysis or within 6-12 months of initiation of dialysis) or pre-emptive transplantation (8, 9).

Advances in the field of kidney transplantation have led to an increased demand for donor organs which are in short supply worldwide. However, global access to transplantation is still limited, with Austria, USA, Croatia, Norway, Portugal and Spain having the greatest access and the USA, China, Brazil and India performing the most transplants (10). Most organs are donated from cadavers, following traumatic and non-traumatic brain injury. Cadaveric organs go to blood group matching recipients on a waiting list. **Figure 1** shows that, in the UK, according to the Organ and Transplantation activity report 2018/19 (11), the number of deceased kidney donors decreased by 2% in 2018-2019 compared to the previous year where the number of deceased donor kidney transplants increased by 3%. There has been a 2% increase in the number of deceased donors. In general, transplants and donors have increased with time and the total number of patients on the transplant list has decreased as a result.

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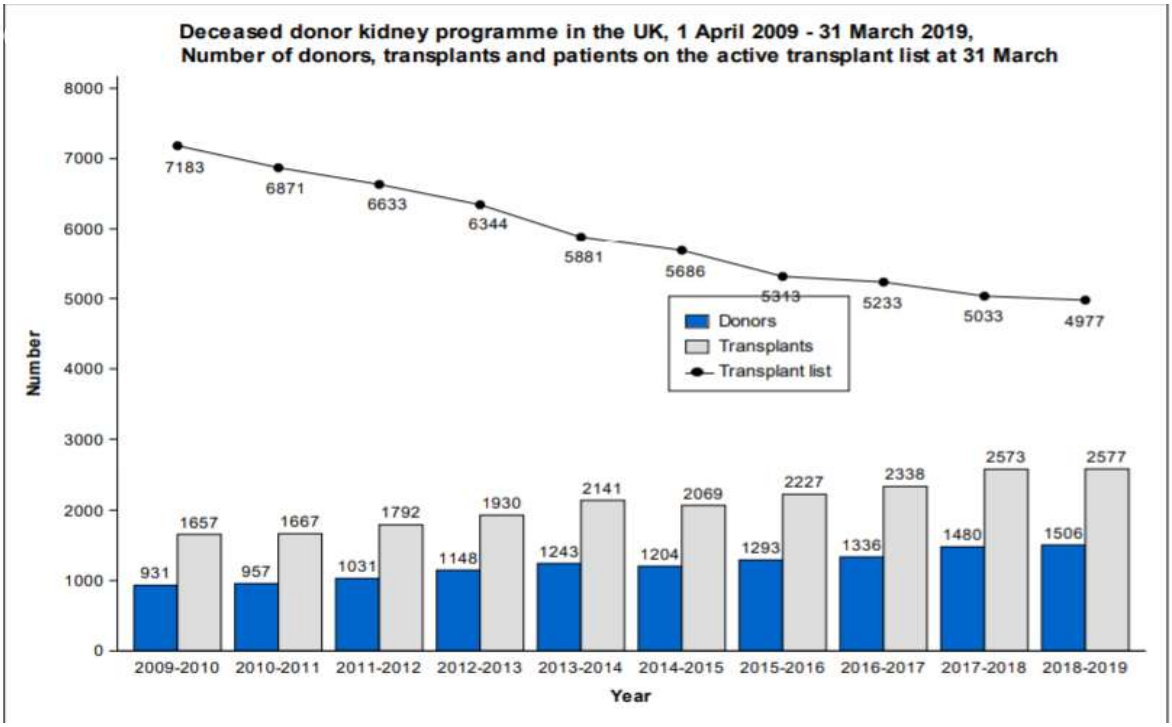

Figure 1 Number of deceased donors between periods 2009-2019 in the UK

This increasing demand for kidney transplantation requires innovation to expand the pool of potential kidney donors. For example, some initiatives have been devised to increase the donor pool *e.g*. the use of the Donation after Cardiac Death (DCD) criteria and Donation after Brain Death (DBD) which have contributed in reconciling supply and demand for organ donations (12). DCD refers to either the retrieval of an organ after cardiac arrest that is expected and from which the patient cannot or should not be resuscitated or retrieval of an organ following planned withdrawal of life support. DBD refers to retrieval of an organ where brain injury is suspected to have caused irreversible loss of the capacity for consciousness and respiration.

1.2 Attitudes towards transplantation

Ethnic minorities in different communities generally demonstrate lower donation rates (13). Reasons for this include lack of transplant awareness, mistrust of healthcare professionals and economic factors (14). In some countries, cultural beliefs and legal considerations amongst other factors hinder the development of deceased organ donation initiatives. Even in developed countries, where rates of deceased organ donation tend to be higher, organs obtained in this manner fail to satisfy the increasing patient demand.

Countries like Japan suffer a severe shortage of cadaveric grafts as a result of longstanding religious beliefs and therefore more work has been undertaken to develop and promote living donor kidney transplants. As a result, transplants of this nature have been performed since 1989 (15).

1.3 Live donor transplantation as a treatment option for ESRD

The considerable shortage of donor organs compounded by the increasing number of patients with ESRD on kidney transplant waiting lists has contributed to long waiting times for an appropriate organ allograft (16).

Most living donor transplants are ‘directed’ which means that the kidney is donated to a specific recipient known to the donor. However, there are several ‘non-directed’ living donor transplants (also known as altruistic donor transplants).

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When a potential living donor and recipient are biologically incompatible (blood group or tissue type wise) they may consider joining a list of others in the same situation with the hope that an exchange of kidneys between them can lead to a compatible living donor transplant. Between 2018 and 2019 in the UK, there were 100 paired living kidney donor transplants (97 adults and 3 paediatric recipients)(11).

Blood group incompatibility with regards to the ABO system has in the past been considered a contraindication to kidney transplantation(17) until advances in this area have enabled transplantation across this barrier. While kidney transplantation is best performed in the absence of ABO incompatibility, a large ESRD population and increasing organ shortage result in waiting times for deceased donor kidney transplant exceeding 5years in some countries such as Germany(18). Compared to DCD and DBD initiatives, directed or altruistic transplants cannot always be matched for ABO blood group compatibility. Consequently, this has led to the development of live donor ABO incompatible kidney transplantation (ABOiRTx) which is now being performed in renal centres worldwide at an increasing rate and has gone a long way in reducing the waiting time on transplant lists.

Kidney transplantation across the ABO blood group barrier has the potential to expand the pool of donors, increase the availability of transplantable organs and decrease the time on the waiting list for a kidney. In the UK, about 30% of any potential unrelated live kidney and recipient pairs are ABO blood group incompatible and most recipients are blood group O and B.

The median waiting time for deceased donor kidneys in the UK is higher in these blood groups compared to blood group A (1381 days, 1329 days and 925 days respectively) (12). ABO incompatible kidney transplantation is becoming more commonplace as a result of improved knowledge and understanding of the associated immunologic mechanisms and effective immunosuppressive regimens (19). However, more work still needs to be done to ensure improved graft and recipient outcomes. ABOiRTx is further discussed in Section 1.5.

1.4 The ABO blood group system

The ABO blood group antigens remain of prime importance in transfusion medicine as they are the most immunogenic of all the blood group antigens. This blood group system is also critical in transplantation medicine as it plays a major role in histocompatibility. There are 4 antigens in the ABO blood group system; A, B, AB and H. Landsteiner, discovered blood types A, B and O (which he initially referred to as A,B and C) in 1901 and his co-workers, Adriano Sturli and Alfred von Decastello discovered blood group AB a year later in 1902.

1.4.1 Molecular basis of the ABO blood group system

The ABO gene locus encodes specific glycosyltransferases that synthesise A and B antigens on red blood cells. It is located on chromosome 9 at 9q34.1-q34.2 and contains 7 exons that span more than 18kb of genomic DNA. Exon 7 is the largest and contains most of the coding sequence. Exon 6 contains the deletion that is found in most O alleles which results in loss of enzymatic activity.

The A and B alleles each encode a glycosyl transferase that catalyses the final step in the synthesis of the A and B antigens respectively.

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A and B determinants are oligosaccharides carried on various glycoproteins and glycolipids and are not primary gene products but are synthesised by the action of the gene-encoded glycosyltransferases. In the production of these blood group antigens, a sequence of oligosaccharides that are conjugated either to polypeptides to produce glycoproteins, or to ceramide to produce glycolipids, is synthesised in a stepwise manner, where a specific glycosyltransferase catalyses the addition of each monosaccharide. There is a sequence of oligosaccharides that is common to all blood groups and is called the precursor substance (H-substance or H antigen). The addition of terminal monosaccharides to this precursor substance, determines the blood group of an individual.

The epitope of the H-antigen, the precursor of A, B, (and Le^a and Le^b) antigens of the ABO histo-blood groups is carried by at least 4 different core chains of internal carbohydrate backbones (types I, II, III and IV); the variation in these precursors generates antigenically distinct ABH epitopes. Localisation of ABH antigens carried on type I-IV chains varies among different tissues correlated with their embryonic and cellular differentiation (20).

The addition of fucose onto the precursor substance via an α-1-2 fucosyltransferase [FUT1] or H transferase, results in the production of the H antigen. This step is independent of the ABO system, but is central to subsequent blood group A and B production, which results from the addition of a terminal monosaccharide, α-D-N-acetylgalactosamine (GalNAc) in the case of blood group A, and α-D-galactose for blood group B.

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The A and B genes encode specific glycosyltransferase enzymes that catalyse the transfer of these sugars onto the H antigen.

For blood group O, the *O* gene does not produce an active transferase, with H substance effectively being blood group O. Fig 2 shows illustrations of the structures of A, B and O oligosaccharide antigens.

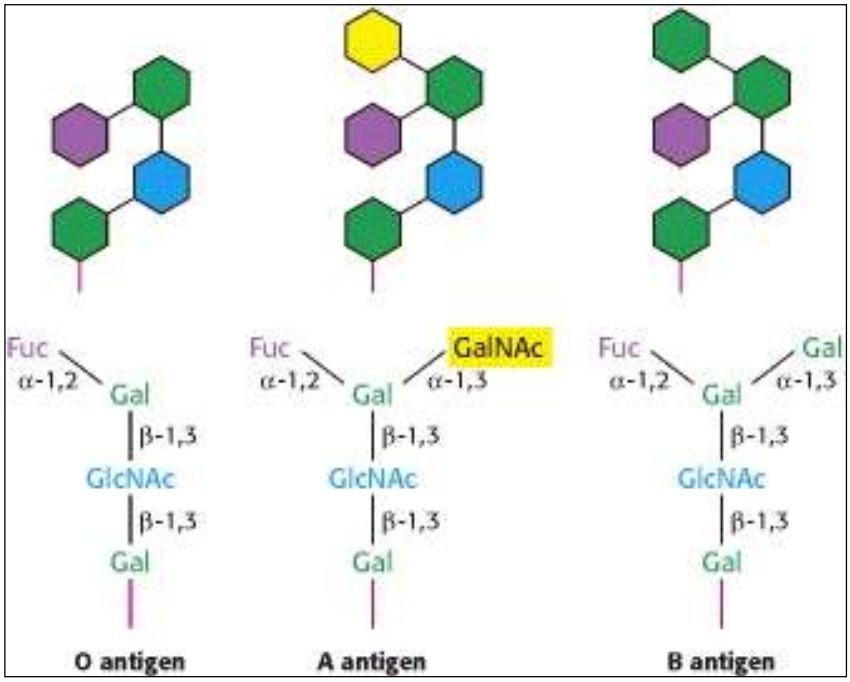


Figure 2 Structure of A, B and O oligosaccharide antigens

O, A and B Oligosaccharide antigens with terminal monosaccharides (Fuc, fucose; Gal, galactose and GalNAc, N-acetylgalactosamine)

In red blood cells, the enzyme that synthesises the H antigen is encoded by the H gene (FUT1). In saliva and other bodily secretions, the enzyme that synthesises the H antigen is encoded by the *Se* gene (FUT2).

The A/B polymorphism arises from several SNPs (Single Nucleotide Polymorphism) in the ABO gene which result in the A and B transferases that differ by 4 amino acids. As stated earlier, the O allele encodes an inactive glycosyltransferase that leaves the ABO antigen precursor (the H antigen) unmodified (21).

Thus, the ABH antigens are oligosaccharides which are phenotypically expressed as glycoproteins or glycolipids on cells and tissues and are produced through glycosyltransferase enzymes that progressively add monosaccharides to different precursor chains (22, 23). Depending on the carbohydrate moieties found on these precursor structures, ABH antigens can be classified as A types I-VI, B types I-VI and H types I-VI and at least 4 of these subtype chains have been known to carry ABH antigens in humans (22, 24, 25). These permutations in precursor structures create unique antigen epitopes (26).

1.4.2 Subtypes in the ABO blood group system

There are many variant ABO alleles that encode different variant ABO phenotypes, but they do not encode specific antigens other than the A and B antigens. For blood group A, these are often identified as the A1 or non-A1 antigens. The A1 subgroup can be identified directly by the *Dolichos biflorus* seed lectin binding in haemagglutination assays, which is specific for the A1 antigen determining carbohydrate N-acetyl-D-galactosamine. The weak non-A1 subgroups, such as A_2, A_3, A_x and A_{el} again express the A antigen and these subdivisions arise due to differences in both density and structure of the A antigen. (27, 28). About 80% of blood group A individuals are A1 which is differentiated by the concentration of the A antigen which is of clinical importance. Non-A1 individuals have fewer A antigen molecules on red cells and in the kidneys. This decreased A antigen density is due to reduced A glycosyl transferase activity in non-A1 blood groups (which is about 10% relative to A1).

Similarly, for blood group B, the major subgroup is Group B (B_1) while weaker subgroups such as B_3 and B_x also express the B antigen but differences again arise due to variation in antigen density and structure and B (B_1) is the main subgroup for Group B individuals (22, 23).

In blood group AB, both A and B antigens are co-expressed, however, the competitive action of their respective A and B transferases, against a fixed amount of cell surface H antigen, leads to an overall reduction in A or B antigen density.

1.4.3 General tissue distribution of ABO antigens

In general, the ABO blood group antigens are regarded as red blood cell antigens but they are also expressed on other body tissues such as most epithelial and endothelial cells including the kidney vascular endothelium, the convoluted distal tubules and the collecting tubules (29). These ABO antigens in an incompatible graft are the target for pre-formed natural immune ABO antibodies found in the serum of the recipient (Section 1.4.4). This explains why hyperacute graft rejection (HAR) almost always occurs after ABO-incompatible kidney transplantation and why kidney transplantation across the ABO barrier without a prior preconditioning protocol (removal of ABO antibodies plus appropriate immunosuppression) is considered an absolute contraindication (discussed further in Section 1.5).

Other blood cells, such as T-cells, B-cells and platelets, have ABO blood group antigens that have been absorbed from the plasma. Some individuals, called ‘Secretors’, produce a soluble form of the ABO blood group antigens which can be found in saliva and all other bodily fluids apart from cerebrospinal fluid.

1.4.4 Antibodies produced against ABO antigens

Anti-ABO specific antibodies are not present at birth and appear following exposure to environmental carbohydrate antigens (30). The presence of ABO-specific antibodies is explained according to Landsteiner's rule *i.e*. they develop against the A or B antigen absent in the individual as shown in Table 1 below. The quantity of antibody present or titre increases with early childhood immune development. The blood group O population has higher titres than blood group A or B populations although there is significant overlap, and the amount of antibody may be affected by age and dietary intake.

Table 1 Antigens and associated antibodies in the ABO blood group System

Blood group	**Antigens on Red Cells**	**Antibodies in Plasma**
A	A antigen	Anti-B
B	B antigen	Anti-A
O	Neither A nor B	Anti-A and Anti-B
AB	Both A and B antigens	Neither anti-A nor anti-B

ABO antibodies can be produced by 0.25% of circulating human B-lymphocytes. Anti-A and anti-B molecules occur as IgM, IgG, and IgA. Notably, group O individuals contain two separable antibodies, anti-A and anti-B and a cross-reacting antibody called anti-A,B which is predominantly IgG (27). ABO antibody levels show a normal distribution between individuals, depending on age, season and immunising events like infections, vaccinations and pregnancies.

1.5 ABO incompatible Renal Transplant (ABOiRTx)

In ABO incompatible (ABOi) transplantation, anti-A and anti-B become clinically significant as they bind to their respective specific antigens that are present on the vascular endothelia of the transplanted donor organ. The binding of these antibodies initiates a series of steps resulting in the loss of endothelial integrity and intravascular thrombosis (a process known as hyperacute rejection (HAR)). This process occurs within minutes of transplantation and is due to the activation of complement whereby complement components (C3a and C5b) acting directly or indirectly cause a decrease in graft function and irreversible graft loss as explained in section C3a has a pro-inflammatory effect, possessing chemotactic capability, attracting and activating granulocytes while C5b is instrumental in the formation of the membrane attack complex (MAC) via the recruitment of other complement components, C6, C7, C8 and C9. The MAC is responsible for cell lysis and necrosis.

Complement regulatory proteins influence complement activation to protect cells from damage. For example, C4 binding protein is a soluble regulatory protein that inactivates C4b and the classical pathway convertases. Membrane cofactor protein (CD46) and decay-accelerating factor (CD55) are expressed on endothelial cells and they control activity of the classical and alternative pathway convertases. Antibody mediated complement activation on endothelial cells may result in the upregulation of the expression of membrane cofactor protein CD59 which increases the resistance of cells to complement-mediated injury.

Introduction

The extent of complement activation by immune complexes is determined by multiple factors, including the isotype of the antibody, the concentration of the target antigen and level of immunoglobulin, local concentration of complement regulatory proteins or exposure to immunosuppressive drugs which affects antibody production.

ABO incompatible renal transplantation has long been considered a barrier to successful kidney transplantation. Over the last 25 years, increasing organ shortage necessitated research into overcoming the ABO antibody barrier. Currently, ABOiRTx has become a routine procedure with graft survival rates comparable to those of ABO compatible renal transplantation (ABOcRTx). Desensitisation (reduction and maintenance of the ABO antibodies to an acceptable level) is usually achieved by apheresis and B cell-depleting therapies accompanied by powerful immunosuppression (18). The A/B antibodies must be below a predetermined threshold at the time of the ABOiRTx and during the first 2 weeks post-surgery. Thereafter, a rebound of the anti-A/B antibodies does not appear to adversely affect the kidney transplant. This phenomenon is called “accommodation” and is not very well understood but is discussed in more detail in Section 1.10.

In contrast to adults, new-borns do not produce natural antibodies to ABH antigens, with isohaemagglutinin ontogeny generally beginning only after about 4-6 months of age. This developmental lag allows infants the opportunity to undergo safe ABOiRTx without the need for aggressive therapeutic interventions usually required for ABOiRTx in adults or additional immunosuppressive therapies.

1.5.1 Brief history of ABO incompatible Renal Transplantation

Humans have two strong transplantation antigen systems, the Human Leukocyte antigen (HLA) and ABO systems. In earlier studies Hume *et al* (17), Starzl *et al* (31) and Murray *et al* (32) expressed doubt regarding the feasibility of ABOiRTx. Although long-term survival of grafts was observed in some cases, overall experience indicated that hyperacute rejection (HAR) was still a possibility, therefore crossing the ABO barrier was generally considered an impossibility in the field of renal transplantation.

It is speculated that the idea of depleting anti-A/B antibodies was first introduced in 1981 following a renal transplant in a patient with major donor-recipient blood group incompatibility that was successfully treated for rejection through the use of plasmapheresis (32). In 1982 the first large study on ABOiRTx was conducted by Alexandre *et al* (33) in Belgium. They managed to achieve desensitisation through repeated plasmapheresis (PP), splenectomy, platelet transfusion and infusion of A or B trisaccharides coupled with heavy immunosuppression. Their approach resulted in a one-year graft survival of 75%. This development led to a wider utilisation and acceptance of ABOiRTx, first in Japan from the late 1980s, in the US from the mid-1990s and in Europe from the early 2000s (18).

In another study, Capellini's group showed that although A1 and B blood group skin was immediately rejected when grafted onto O recipients, blood group A2 skin grafts were rejected at a slower rate, comparable to O skin grafted onto O recipients. These findings provided a platform for attempting A2 incompatible kidney transplantation.

In 1987 Thielke *et al(34)* showed that 12 out of 20 transplants from a group A2 donors into O recipients maintained long term allograft function. The only major drawback is that this concept can only be used in a minority of kidney transplant cases owing to the low frequency of group A2 donors in most populations. Compared to blood group A1 and blood group B Individuals, blood group A2 recipients make up 20% of all group A Caucasians. The low expression of blood group antigen molecules (30-50%) on the surface of erythrocytes in blood group A2 donors, is believed to be responsible for the lower immunogenicity of organs donated by this group (35, 36). ABOiRTx with A2 organs has been accomplished with basic immunosuppressive therapy without any additional measures.

While, even today, kidney transplantation is best performed in the absence of major ABO incompatibility, the large ESRD population and an increasing organ shortage result in waiting times for a deceased donor kidney transplant exceeding 5 years in some cases. Therefore, transplantation across the ABO antibody barrier will theoretically increase the number of kidney transplantations from living donors by up to 30% consequently reducing the waiting time on the transplant list. With currently existing protocols, as many as 90% of patients with an ABOi living donor may effectively be desensitised and transplanted (18).

1.6 HLA and kidney transplantation

MHC class I and II proteins are cell surface glycoproteins. Class I expression is on most nucleated cells, whilst Class II is restricted to predominantly B lymphocytes, dendritic cells and some endothelial cells, but can be upregulated in inflammatory responses.

It is the specific role of MHC Class I and Class II molecules in presenting antigenic peptide to the T Cell receptor (TCR) that underlies their significance as barriers to transplantation.

The consequences of TCR engagement and co-stimulation are proliferation and differentiation of the T-cells into effector phenotypes and concomitant production of cytokines. The role of immunosuppression is to target signalling pathways responsible for T-cell activation, including TCR/CD3 engagement by the MHC, co-stimulatory pathways such as CD28 binding by CD80/CD86 and cytokine signalling pathways such as IL-2 binding to the IL-2 receptor including CD25.

Exposure to MHC proteins, otherwise called the HLA antigens, leads to the development of anti-HLA antibodies through B cell activation which requires T cell help. Anti-HLA antibodies are produced against both Class I and Class II proteins.

1.7 Transplant/Allograft Rejection

Allograft rejection can be hyperacute (HAR) (occurring within minutes after vascular anastomosis), acute (occurring days to weeks after transplantation) and/or chronic (occurring months to years after transplantation). Rejection can also be classified according to the pathophysiologic event; cellular and/or antibody mediated (AMR).

1.7.1 Antibody-mediated renal allograft Rejection (AMR)

Antibody mediated rejection can be hyperacute, acute or chronic as discussed in the sections below.

1.7.1.1 Hyperacute antibody mediated allograft rejection

Hyperacute antibody mediated rejection (HAMR) is characterised by immediate onset of thrombosis and occlusion of graft vessels due to preformed ABO antibodies directed against corresponding graft tissue.

Figure 3 shows the proposed pathogenesis of hyperacute rejection (HAR) induced by the binding of anti-A/B to antigens expressed on the endothelial cells of the ABOi graft followed by activation of the complement system. Subsequently, endothelial damage, inflammation and platelet aggregation can be provoked leading to vascular thrombosis, occlusion of blood supply and rejection.

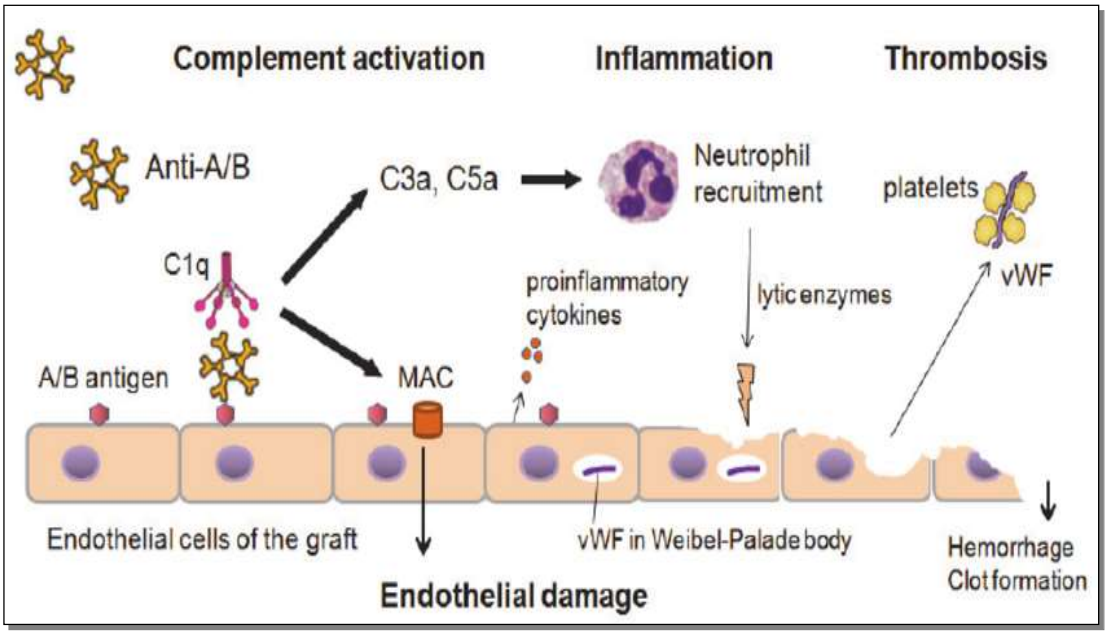


Figure 3 Proposed mechanism of hyperacute rejection in ABO incompatible kidney transplantation

Binding of anti-A/B to antigens expressed on the endothelial cells of the ABOi graft followed by activation of the complement system. Subsequently, endothelial damage, inflammation and platelet aggregation can be provoked leading to vascular thrombosis, occlusion of blood supply and rejection. (Adapted from Hur (2011) (37))

1.7.1.2 Acute antibody mediated rejection (AAMR)

Acute antibody mediated rejection (AAMR) is characterised by inflammation and leukocyte infiltration of graft vessels and is the most common type of rejection.

It can occur within weeks or months and is caused by specific recurrent antidonor antibodies against HLA, ABO or other antigens.

AAMR presents with an acute loss of graft function after transplantation but can also develop years after transplantation, often triggered by a decrease in immunosuppression (iatrogenic, non-compliance, or malabsorption) (38). Alloantibodies are now considered as essential mediators of acute rejection and chronic rejection as discussed in Section 1.7.1.3). Alloantibodies preferentially attack the peritubular and glomerular capillaries in contrast to T-cells which characteristically infiltrate tubules and arterial endothelium thus triggering inflammatory and cytotoxic effects against the graft (39).

Acute antibody-mediated rejection generally has a worse prognosis and requires a different form of therapy than the more common T-cell mediated rejection. Recognition of the clinical relevance of alloantibodies beyond their historical role as mediators of HAR has rested on a new diagnostic technique (C4d) that permits a definitive diagnosis of antibody-mediated rejection in a renal biopsy in the presence of high ABO antibody titres. C4d is a fragment of the molecule C4b, an activation product of the classic complement pathway (See Fig 4). Feucht *et al* (40) showed that peritubular capillary C4d deposition in renal transplant biopsies is strongly associated with a poor prognosis and raised the possibility that antibodies were responsible. C4d once formed remains in the tissue for several days after immunoglobulin and C1 have been released.

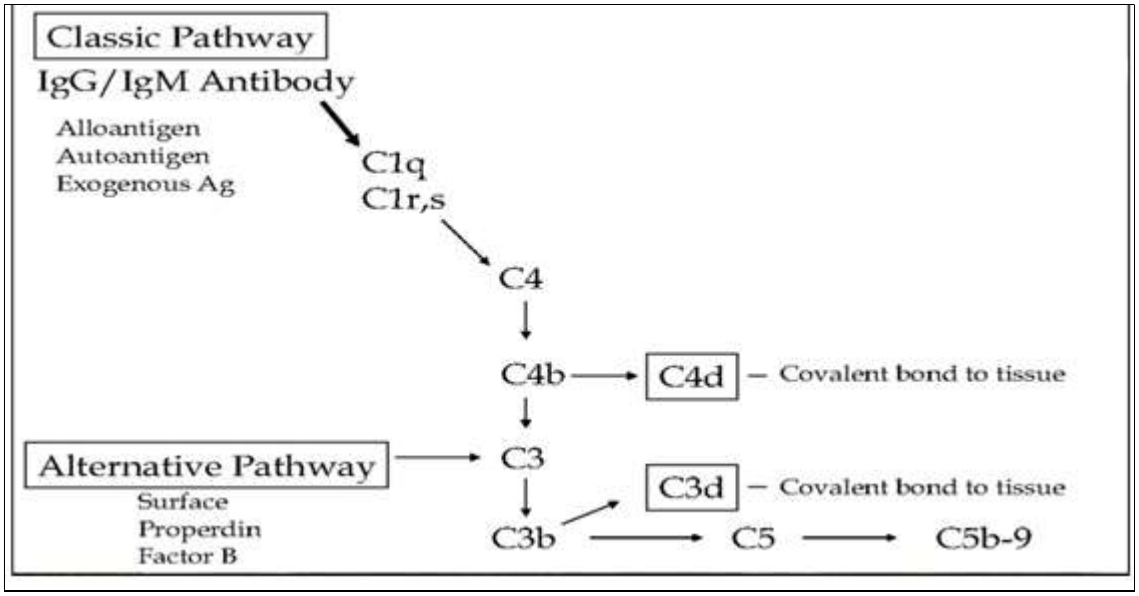


Figure 4 Complement activation pathways via the classic pathway in antibody mediated rejection

Due to improvements in desensitisation and immunosuppressive protocols, the incidence of AAMR has markedly decreased (41).

1.7.1.3 Chronic antibody mediated rejection (CAMR)

The National Institutes of Health (NIH) suggested diagnostic criteria for CAMR in ABOiRTx (42). Specifically, at least three of the following four lesions could be present: arterial intimal fibrosis, interstitial fibrosis/tubular atrophy, amplification of glomerular basement membrane or lamination of peritubular capillary basement membrane. AAMR in the early post-transplant period has an adverse impact on long-term graft survival and contributes to the development of transplant glomerulopathy in chronic rejection (43, 44).

The main feature of chronic graft rejection is the detection of antigens on the donor's tissues as foreign entities by the recipient's immune system.

However, the degree of immune reaction and thereby the degree and speed of graft rejection depends on the histocompatibility between donor and recipient as HLA matched grafts survive longer compared to HLA-mismatched grafts. The activation of the immune system involves 2 distinct pathways: the direct and the indirect pathways, as shown in Fig 5.

The direct pathway involves activation of CD4+ T-cells by the donor's antigen presenting cells (APCs). The indirect pathway involves the processing of the donor's graft antigens by the recipient's APCs that then activates immune cells. Indirect activation can stimulate the activity of activated B-cells, which then leads to the production of antibodies against the graft tissues. Cellular immunity has only a minor role to play in chronic allograft rejection.

The only role T-cells play in chronic rejection is via cytokines secreted by type 2 helper lymphocytes (Th2). Several studies have shown that cytokines secreted by Th2 lymphocytes *e.g.* IL-4, IL-5, IL-6, IL-10 and IL-13 are responsible for reactions such as tissue fibrosis and chronic rejection.

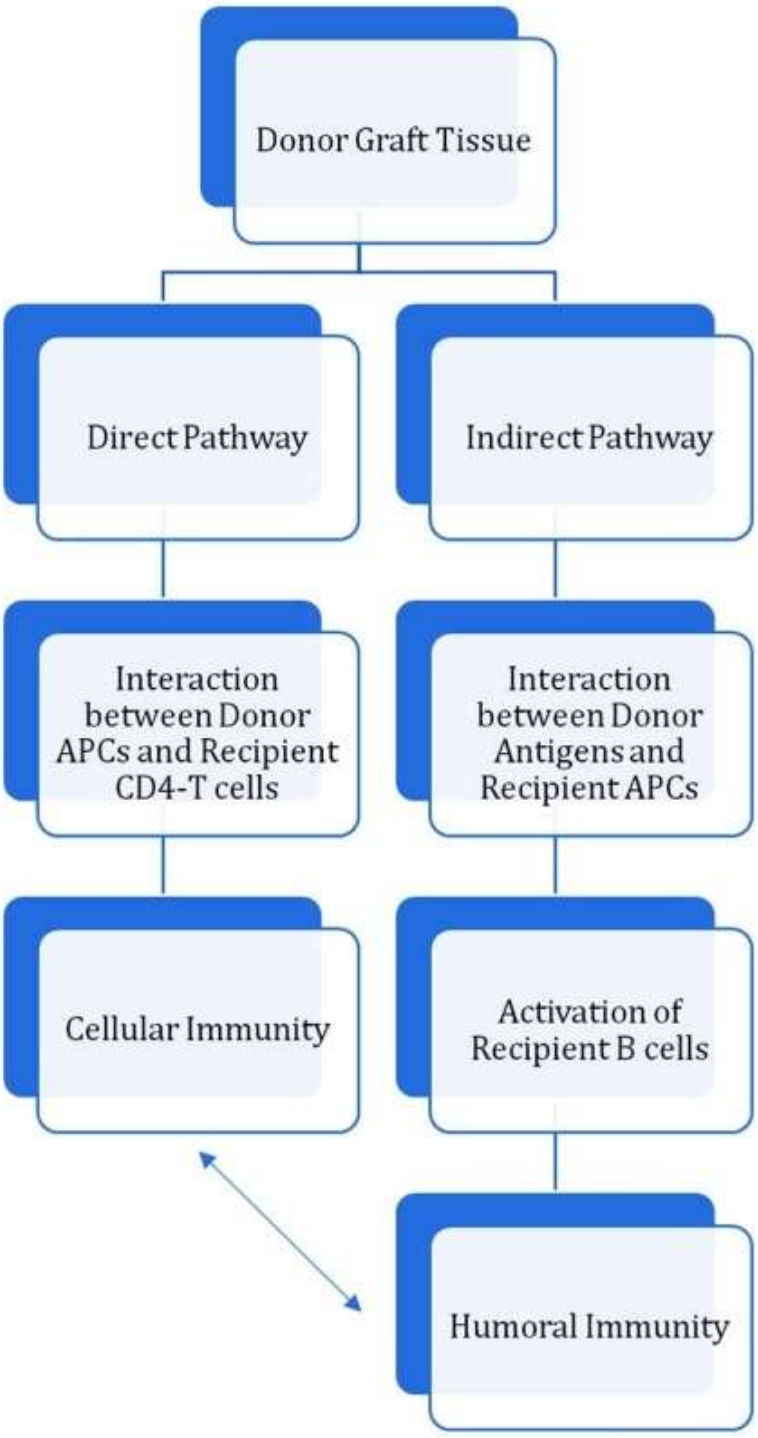


Figure 5 Direct and indirect pathways for allograft rejection

Adapted from (Bhatti, 2015)(45)

1.8 Preconditioning/ Desensitisation for ABOiRTx

The purpose of preconditioning/desensitisation protocols is to reduce the IgG and IgM antibody titres (Anti-A1 or anti-B) against the incompatible donor kidney.

Introduction

Although there is no generally accepted desensitisation protocol for ABOiRTx, all commonly used strategies employ common principles as shown in Fig 6.

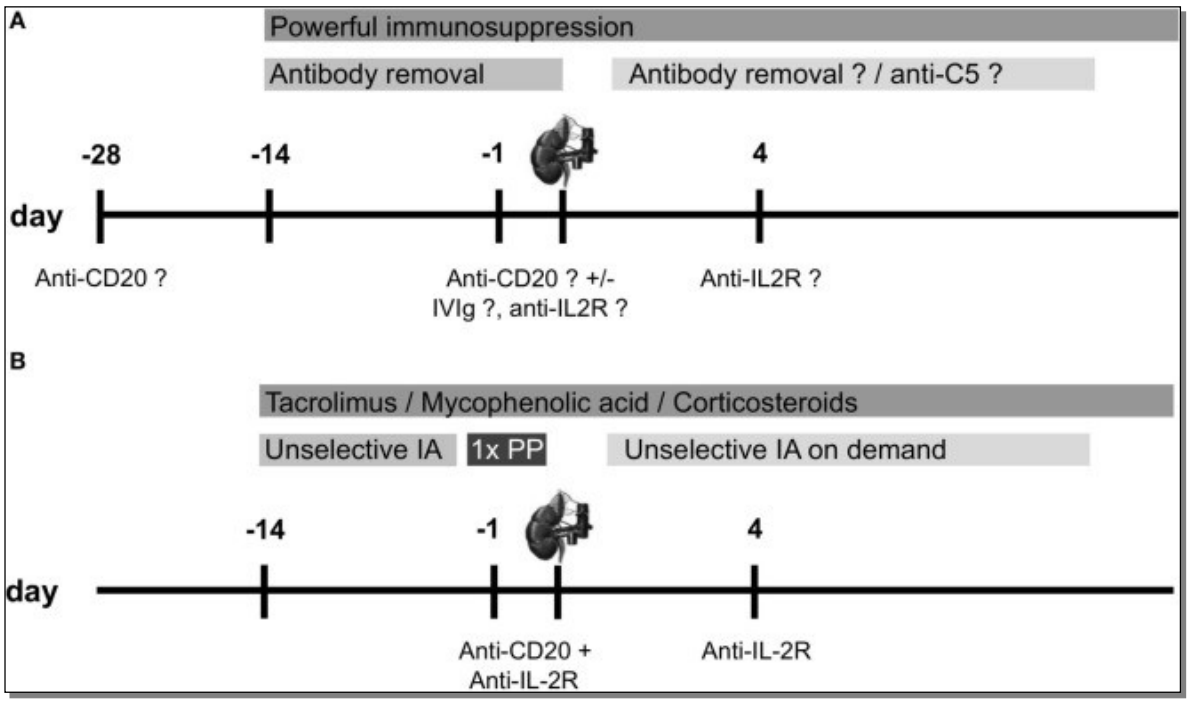


Figure 6 Overview of desensitisation protocols for ABOiRTx.

(a) Standard desensitisation protocol (b) Another example of desensitisation protocol. IA, Immunoadsorption; IVIg, Intravenous immunoglobulin; PP, plasmapheresis

These include, together with a powerful maintenance immunosuppression one or more of the following:

- Anti-A/B antibody depletion at the time of transplantation using Plasmapheresis, double filtration Plasmapheresis/membrane filtration or selective or unselective immunoadsorption (IA).
- Modulation of the recipient's immune system using intravenous immunoglobulins (IVIgs)
- Reduction of the B lymphocyte pool by splenectomy, or more recently by the anti-CD20 antibody rituximab.

- Prevention of the deleterious consequences of complement activation upon anti-A/B antibody binding to the graft endothelium (18).

The main purpose of desensitisation protocols is to ensure depletion and maintenance of anti-A/B antibodies in the immediate 2 weeks post transplantation period below a predetermined threshold that is considered safe. After this period, despite a rebound of anti-A/B antibodies at high levels, they do not cause damage to the transplant; a phenomenon called “accommodation” (18) -this is described in more detail in Section 1.10.

For transplant accommodation to occur, the offending ABO antibody has to be removed, and this can be achieved through use of techniques such as therapeutic plasma exchange (TPE), double filtration plasmapheresis (DFPP) and antigen specific immunoadsorption (IA) (46). These techniques are discussed in more detail in the following sections. However, there have been no clinical trials to evaluate the efficacy of antibody depletion procedures or a standardised monitoring protocol thus making it difficult to draw any conclusion on the relationship between these methods and their respective clinical outcomes (47). Alexandre *et al (33)* introduced an effective desensitisation protocol to achieve success in ABOiRTx. This protocol included pre-transplant repeated plasma exchange (PE) as a strategy to reduce the titres of anti-A and anti-B antibodies. A one-year graft survival of 75 % and a recipient survival of 88% were achieved in the 23 recipients (48). Due to the success of their protocol, it became the basis of the next desensitisation protocols for ABOiRTx.

These efforts were expanded in Japan because of the near absence of deceased donors and the only 0.15% of living A2 donors. In addition, the largest number of ABOiRTx since 1989, more than 1000 cases, has been performed in Japan (49). The percentage of ABOiRTx surgeries reached 14% of all living donor kidney transplants performed in Japan (34). Following the remarkable results reported in the Japanese centre utilising modern desensitisation techniques, together with the development of new immunosuppressive therapies, ABOiRTx began receiving new interest in Europe and the USA in the early 2000s (46).

Currently, there are many different induction immunosuppression regimens in HLA incompatible transplants (HLAiRTx) and ABOiRTx, using a variety of different approaches. No randomised controlled trials have been conducted to compare regimens in detail and as a result no recommendations have yet been made concerning the details of induction therapy. The table below summarises some of the most commonly used desensitisation modalities and a brief description of their mode of action.

Table 2 Prevention and treatment modalities for antibody mediated rejection

Category	Treatment	Major mechanism
I	Tacrolimus (FK506)	Indirectly inhibits B-cell proliferation secondary to reduced cytokine production by T-cells
I	Azathioprine	Inhibits DNA synthesis in dividing cells (T, B, and other dividing cells)
I	Mycophenolatemofetil (MMF)	Inhibits DNA synthesis in dividing cells mainly T and B cells.
I	Rituximab	Anti-CD20 (B cell surface marker) mAb, deplete B-cells. Anti-CD3 (T-cell surface marker) antibody, indirectly inhibits B-cell proliferation.
I	Antithymocyte globulin (ATG) and anti-lymphocyte globulin (ALG)	Anti-CD52 (Surface marker of thymocytes, T, B cells etc. Directly depletes B-cells
I	Campath-H	Anti-CD52 (thymocyte, B and T cell surface marker). Directly depletes B-cells.
I	Splenectomy	Surgical removal of site of functional maturation of B lymphocytes
II	Immunoadsorption	Remove antibody from periphery (blood group antigen-, protein A or anti-Ig antibody coated columns.
II	Plasmapheresis (PP) or plasma exchange (PE)	Remove antibodies and other humoral factors (complement, cytokines etc) from the periphery
II	Intravenous immunoglobulin (IVIG)	Anti-Idiotypic effects (Blocking of the antigen-binding sites of anti-donor antibodies and others)

I= Inhibition and depletion of antibody-producing cells
II= Removal or blocking of pre-existing or newly developed antibodies
(Adapted from Cai and Terasai 2005)(50)

1.8.1 Therapeutic plasma exchange

Therapeutic plasma exchange (TPE) is the most common, cheapest and simplest method for plasma antibody depletion (46). It also known as plasmapheresis (PP) and in this procedure, large volumes of patient's plasma are removed from the patient through filters and centrifugation and discarded but replaced with the same volume of colloid solutions such as human albumin solution (HAS) and/or fresh frozen plasma for regulation of haemodynamics (51-53). TPE is often used in combination with Immunoadsorption (IA) which is discussed in Section 1.8.2 below (54).

TPE is widely used to reduce antibody levels to predetermined acceptable levels both pre- and post-transplantation (55-57).

While TPE temporarily removes approximately 20% of the anti-ABO antibodies per session, it is not adequately selective and thus removes protective antibodies, coagulation factors (I, II, V, VII and X), antiviral and antibacterial immunoglobulin G (IgG) and Immunoglobulin M (IgM). This non-selectivity results in an increased risk of bleeding and/or infection (58). The number of TPE sessions required per patient depends on the initial antibody level/titre, the dynamics of antibody rebound and the clinical condition of the patient. On average it can take up to 2-8 exchanges to reduce the ABO antibody titres to the required level and this can take up to a fortnight.

1.8.2 Immunoadsorption (IA) columns

The selective technique of antigen-specific immunoadsorption (IA) is safe and more effective. Using IA, the plasma is processed through a Glycosorb ABO immunosorbent column, which selectively removes the ABO antibodies, and is then re-infused into the patient. No coagulation factors are removed with this technique and it allows for large volumes to be processed and no plasma volume discarded, thus making this technique more efficient than TPE (46). Since this technique does not affect the resultant plasma volume, there is no limit to the number of adsorption cycles (51).

1.8.2.1 Staphylococcal protein A column (SpA)

The Staphylococcal Protein A (SpA) is a protein found in the bacterial cell wall of *Staphylococcus aureus* and binds to most mammalian IgG with variable efficiency, by forming a strong bond with the Fc fragment of IgG subclasses (Subclasses 1, 2 and 4) and a less strong one with IgG3, IgM and IgA (59).

In ABOiRTx, SpA columns are used to remove anti-A and anti-B antibodies where patients undergo three or four sessions of IA until their anti-A IgG/IgM titres fall to the required levels (54). However, the use of SpA columns to remove antibody has been superseded by TPE which is widely accessible in most transplant centres.

1.8.2.2 Glycosorb columns

The protocol developed by Tyden and colleagues (60) introduced A/B antigen-specific antibody removal, in combination with rituximab therapy. They devised A/B antigen-specific antibody removal columns *i.e*. Glycosorb® ABO (Glycorex, Sweden). This involved the conjugation of A and B trisaccharides to Sepharose beads packed in a column. Plasma is then passed through these columns thus allowing for selective removal of ABO antibodies while leaving coagulation factors, complement, other immunoglobulins and albumin (61). Despite the ability of Glycosorb columns to selectively remove ABO antibodies, they also remove other anticarbohydrate antibodies, *i.e.* against *Pneumococcus* and *Haemophilus*, however, protein based immunity remains intact (62). The concept of the Glycosorb columns has recently been utilised in developing a pan-immunoglobulin technique that allows for concurrent removal of anti-ABO and anti-HLA antibodies. In this version of IA, sheep anti-human antibodies are used to remove human immunoglobulin while allowing for antibody elution and re-use of columns unlike conventional Glycosorb columns.

1.8.3 Splenectomy

The spleen is a multifunctional organ that produces lymphocytes, filters blood, stores red cells and removes those that are ageing.

Introduction

Performing splenectomy (removal of the spleen) in ABOiRTx recipients effectively removes a major site of functional maturation of lymphocytes, including antibody secreting B-cells, as the spleen plays a role in the production of anti-A and anti-B antibodies through B cells. However, splenectomy does not remove all antibodies as some will still be in circulation.

In the past, splenectomy was a necessity for the desensitisation protocol for ABOiRTx, due to its contribution in the reduction of the antibody producing B-cell pool (19). Splenectomy was replaced with rituximab (anti-CD20) monoclonal antibody for B-cell depletion – See Section 1.8.5.1 for a discussion on rituximab (19, 48).The effectiveness of splenectomy in ABOiRTx has not been unequivocally proven. For example, Sonnenday *et al* (48) did not find any significant difference in anti-ABO antibody levels post splenectomy between splenectomised and non-splenectomised patients. Gloor *et al* (63) also stated that splenectomy was not necessary even for patients with high baseline anti-ABO antibody titres. Takahashi (64) demonstrated that, due to splenectomy, significant numbers of memory B-cells exist in the bone marrow, splenectomy is not necessary to inhibit antibody production. Therefore, there is now a growing consensus that splenectomy in not necessary in ABOiRTx recipients (44).

The main drawback of splenectomy is that it associated with complications of immunodeficiency in children and the elderly who are susceptible to *Streptococcus pneumoniae* and *Haemophilus influenzae* infections (65). Several transplant centres have therefore developed protocols without splenectomy, which include antigen specific IA, rituximab and other immunosuppressive drugs.

1.8.4 Allograft Modification

A futuristic innovation that may be introduced involves the reduction of blood group antigen levels in the allograft by *ex vivo* infusion of endo-beta-galactosidase (Abase) (66). Recombinant endo-beta-galactosidase (ABase) releases A/B antigen and was produced in *E. Coli* BL-21.

This innovation is based on a study whereby Human A/B red cells were mixed with ABase and then subjected to flow cytometric analysis after incubation with human sera. ABase recovered 82% of A antigen and 95% of B antigen in human A/B red cells and suppressed anti-A/B antibody binding and complement activation effectively. *Ex vivo* perfusion of excised baboon kidney (group B) with ABase resulted in the reduction of B antigen expression in glomeruli to 6% after 4 hours (66). This development has created a possibility for the *ex vivo* removal of A and B antigens before kidney ABOiRTx and this might pave the way for removal of the requirement for antibody reduction and immunosuppressive protocols.

1.8.5 Immunosuppressive regimens

There are slight differences in preconditioning formalities, depending on the transplant centre, in order to ensure transplant success. Despite these differences, most use a combination of pre-transplant TPE, intravenous immunoglobulin (IVIg) and tacrolimus-mycophenolate-based immunosuppression. Splenectomy or rituximab (anti-CD20) administration is used selectively.

Close monitoring of the anti-ABO antibody titre is undertaken for a minimum of two weeks post ABOiRTx and, if necessary, TPE is performed in order to prevent or minimise any antibody rebound (19, 67).

1.8.5.1 Anti-CD20 Monoclonal antibody (Rituximab)

As a major limitation of splenectomy is suppressed immunity against certain pathogens, the anti-CD20 monoclonal antibody, Rituximab has now replaced this procedure.

This drug directly inhibits B-cell proliferation and induces cellular apoptosis through the binding of complement which then mediates antibody dependent cell-mediated cytotoxicity and subsequent cell death (68). Rituximab is generally considered as a form of “chemical splenectomy” because of its ability to deplete the B-cell compartment (69). The main advantage of Rituximab over splenectomy is that it removes the B-cell during the period where there is greatest risk of AMR and thereafter allows restoration of the humoral immune system without the need to remove the spleen (70). Fuchinoue *et al* (71) reported that patients who received Rituximab induction had a lower incidence of AMR and better outcome of 5-year graft survival compared to those in ABOcRTx or ABOiRTx recipients who were not treated with Rituximab.

1.8.5.2 Intravenous immunoglobulins

Intravenous immunoglobulins (IVIg) are administered by some transplant teams before ABOiRTx to prevent the anti-A/B antibody rebound in the immediate phase after transplantation. IVIg infusion is also thought to reduce complications due to infections by substituting depleted immunoglobulins.

It is important to note that IVIg preparations contain IgG antibodies directed against A/B antigens and can falsely increase anti-A/B antibody titres thus confounding the interpretation and consequently wrongly influencing clinical decision making (72, 73).

Therefore, it is important that all clinical details are collected before undertaking ABO antibody titrations in order to take into account factors that can influence the results.

1.9 Acceptable anti-blood group antibody titre at time of transplantation

Since the removal of anti-ABO antibodies is crucial to the success of the transplant, optimal target levels of these antibodies need to be established for prophylactic purposes against AMR. Figure 7 below shows the impact of anti-ABO antibody starting titres on AMR.

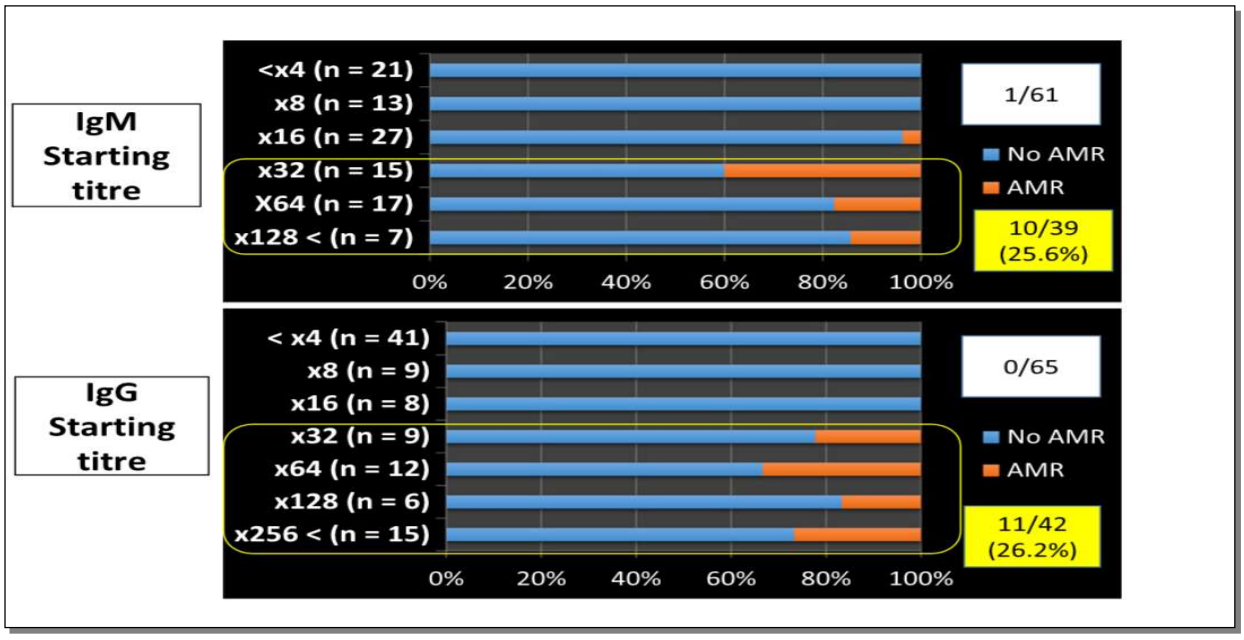


Figure 7 Impact of anti-A/B antibody starting titre on active antibody-mediated rejection episodes

Introduction

Before the initiation of preconditioning, the baseline anti-ABO antibody titre is well established as a significant predictor of the severity of AMR as well as graft survival (44, 74).

From the data shown in Fig 7, it can be postulated that an anti-ABO antibody titre of both IgG and IgM class >32 just before transplant is a strong predictor of AMR.

Therefore, in general it can be subjectively suggested that desensitisation is necessary to reduce anti-ABO blood antibodies to a titre of ≤16. The subjectivity of the suggestion stems from the differences in immunosuppressive regimens used and antibody titration methodologies by each transplantation team.

The titre level at which a renal transplantation can safely be performed has not been unequivocally established. Welsh *et al* (75) claimed that in their study, the results were better if a titre <64 (in A2 to O cases) was reached. Other authors aim at titre levels ≤ 16 in major (A1 and B cases)(76). Some institutions use the antiglobulin IgG antibody titre endpoint as the critical titre when assessing patients before and after transplantation. Others consider both IgM and IgG antibody titres (47).

Antibody reduction in ABOiRTx is regarded as the key to improving outcomes, in patients at high risk of both hyperacute and acute rejection. The target titre levels of equal to or less than 8 on the day of transplant have been shown to improve outcomes (77).

The importance of lowering antibody titres to less than 8 to prevent negative outcomes with higher anti-graft antibodies is logical as early clinical outcomes in Japan showed that higher pre-operative IgG levels were associated with worse clinical outcomes both in graft survival and humoral rejection (78).

Early rejection of transplanted kidneys is most likely to be humoral, involving antibody binding to renal vascular endothelium. This antibody-mediated process may not initially be accompanied by elevated antibody levels as adsorption of antibodies in the kidney may keep levels constant, until saturation of the binding sites leads to antibody titre levels rising. Antibody titres of greater than 128 have been successfully transplanted with post-transplantation titres of 16 being maintained, without antibody-mediated rejection occurring (79).This might mean that pre-transplantation ABO antibody titres might not be as important if levels are reduced post- transplant through plasma exchange and immunosuppression.

1.10 Accommodation

The presence of alloantibody is usually associated with tissue injury, however, not all allografts are adversely affected despite the presence of alloantibody. This phenomenon is called “accommodation” and was first described in animal models of xenotransplantation when xenograft loss was not observed despite the presence of anti-donor antibody (80). Accommodation was first described in human ABOiRTx whereby biopsies of functionally healthy grafts showed persistence of ABO antigens on endothelial surfaces (81, 82) despite the presence of anti-ABO antibodies. This discovery inspired the idea that a different antibody and ABO antigen interaction with no concurrent graft injury existed.

In order to understand the process of accommodation, molecular markers of immunological injury/activation are now being used to demonstrate activation of the immune system, despite good allograft function and no chronic histological injury in light microscopy. With the accumulation of evidence showing immunological activity against the graft, it can therefore be hypothesised that accommodation may represent a dynamic process of slow graft rejection resulting in either immunological tolerance or graft injury.

Park *et al* (83) defined the criteria of accommodation in ABOiRTx to include (a) detectable anti-ABO antibody in the recipient's serum, (b) normal graft histology according to light microscopy, (c) the presence of A or B antigen in the graft and (d) renal function similar to that of ABO compatible patients (GFR greater than 45mL/min at 1 year after transplant). In 2006, the American Society of Transplantation also established that accommodation occurred when complement component (C4d) deposition was detected with normal function and structure of graft (84).

1.10.1 Mechanism of accommodation

A few mechanisms have been proposed to explain accommodation. These include reduction in antigen-antibody interaction due changes in function of anti-donor antibody or graft antigen, difference in antigen expression, decreased susceptibility of injury to endothelium and complement components, acquired resistance of allograft through the expression of an anti-apoptotic gene or an expression of complement regulatory protein(s).

Ishida *et a*l (41) demonstrated that functional or structural changes in anti-blood type antibodies greatly contributed to successful accommodation after ABOiRTx. They showed that anti-A antibody showed strong binding activity against donor erythrocyte membranes pre-accommodation, but this activity was suppressed once accommodation was established. In addition, the presence of diffuse C4d in the absence of capillary endothelial damage or inflammation suggests that accommodation may be due to resistance to the terminal effectual part of the complement cascade in ABOiRTx (39). Another concept was introduced by Griesemer *et al* (84) suggesting that upregulation of a complement regulatory protein such as CD59 may be involved in accommodation through interference with the formation of the membrane attack complex (MAC) on the accommodated graft.

Kirk *et al (85)* suggested that accommodation is due to a switch between the IgG subclasses to favour the IgG2 subclass that is less effective at activating complement and that competitively inhibits the binding of the more cytotoxic subclasses.

Park *et al* (83), Delikouras and Dorling (86) discovered that the Bcl and Bcl-xl anti-apoptotic molecules were found in the accommodated ABO incompatible kidney graft. Conversely, Bax, a pro-apoptotic marker, was not detected. Salama *et al* (87) demonstrated upregulation of Bcl-xl in microvascular endothelial cells of accommodated grafts.

This evidence seems to support the hypothesis that the initial exposure of the endothelium of the kidney allograft to low levels of anti-ABO antibody will trigger series of protective changes in the graft that manifest thus effecting accommodation.

1.11 Antibody rebound post transplantation

The association between antibody levels and graft function has not been clearly demonstrated. For example, good graft function has been observed despite ABO antibody titres of up to 256 (13). The key to clinical outcome seems to be the transplanted graft rather than the presence of antibody.

1.12 Clinical outcomes in ABOiRTx

Single centres report good outcomes for patients receiving ABOiRTx, equivalent to ABOcRTx cohorts within centre. This comparability between ABOiRTx and ABOcRTx could be attributed to advances in desensitisation techniques resulting in improved graft survival rates (18). Fig 8 shows cumulative incidence of death censored graft survival compared to patient survival in recipients of ABOiRTx.

Introduction

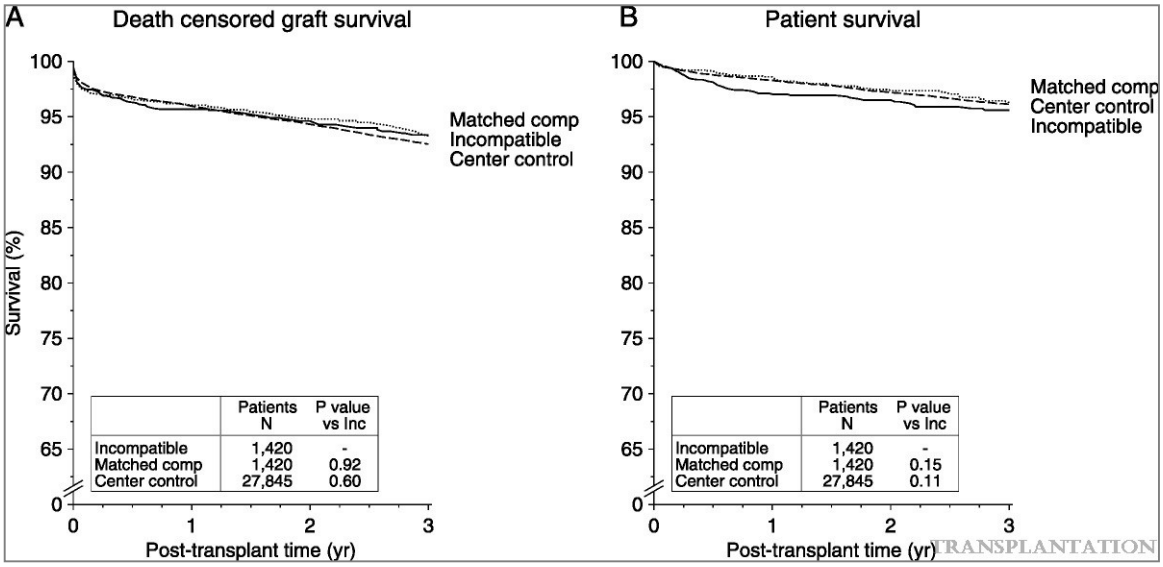


Figure 8 Cumulative incidence of (A) death censored graft survival and (B) patient survival in recipients of an ABO-incompatible living donor graft and matched controls receiving an ABO-compatible living donor graft(18)

Gernberg *et al* (88) reported that ABOiRTx had no negative impact on long-term graft function compared to that of ABOcRTx in terms of patient survival, graft survival or incidence of acute rejection after a mean follow-up of three years. Tanabe (69), summarised the outcomes of 851 ABOiRTx performed in 82 institutions in Japan between 1989 and 2005. The five-year graft survival in their study was 79% with patient survival at 90%. Fuchinoue *et al* (71) , reported the five-year outcome of ABOiRTx as a graft survival rate of 100% whereas Ishida *et al* (49), achieved a graft survival of 57% and patient survival of 89% at ten years postoperatively for more than 130 cases of ABOiRTx.

In the analysed United Network for Organ Sharing (UNOS) data of Gloor and Stegall (2007)(44), they concluded that a long-term immunological response against ABO incompatibility had little effect on graft survival with current immunosuppressive protocols and patient monitoring.

For instance, in the study of Tyden *et al* (60), recipients with a baseline anti-A or -B IgG titre of up to 1:128 were successfully transplanted with no episode of acute rejection. Montgomery (67) reported one-year patient and graft survivals of 96.3% and 98.3% respectively, in a cohort of 60 consecutive ABOiRTxs using a variety of protocols. Oettl *et al* (89) demonstrated a 100% survival rate of both patients and grafts at one-year after transplant.

1.13 ABOiRTx and infection

A higher frequency of viral infections such as Cytomegalovirus (CMV), Herpes Simplex Virus (HSV), Varicella Zoster Virus (VZV) and BK virus as well as *P. jirovecii* pneumonia, wound and severe urinary tract infections have been described in ABOiRTx patients (90, 91). A higher incidence of BK virus replication and BK virus associated nephropathy was also observed by Becker *et al* (92). Bentall *et al* (93) hypothesised that different blood group antigens may influence binding of viral pathogen receptors to sialic acid on renal tubular cells.

For example, it is thought that ABOiRTx recipients may be more susceptible to BK virus allograft nephropathy compared to HLA incompatible recipients even though HLA incompatible recipients have the most immunosuppression (94). Infections and other comorbid conditions may affect the survival of the graft and the recipient and this will affect survival analysis data.

1.14 Cost-benefit analysis of ABOiRTx

As ABOiRTx requires protocols for antibody reduction and immunosuppression which require utilisation of resources. The cost of ABOiRTx also includes the surgical procedure, cumulative total length of hospital stay and total number of pre- and post-operative hospital visits.

Therefore, the justification for ABOiRTx in the National Health Service (UK) is based upon both clinical and cost effectiveness.

A review of standard transplantation revealed that it is cost effective by a margin of about £15000 per annum over a 10-year period. A meta-analysis of papers published between 1968 and 1998 indicate a cost saving of US$55000-80000 per life year by each centre in haemodialysis, compared to a cost of US$10,000 per life year saved by transplantation. Overall, this yields a cost saving for transplantation of US$45000-70000 per patient per year (13).

The Mayo Clinic, USA, estimated that ABOiRTx was approximately US$38000 more expensive than ABOcRTx, but the cost effectiveness was realised overtime(95).

Transplantation is not only beneficial for the individual but also represents value to the greater health economy. The first year of care after a kidney transplant costs around £17000 and £5000 for every subsequent year, whereas the average cost of dialysis is £30800 (96). After transplantation, many patients can return to work and therefore become less dependent on state support.

1.15 ABO antibody measurement

Despite good outcome data achieved in most centres, the need to provide reliable care through accurate and reproducible titre levels continues to be a goal that remains to be achieved. The number of plasmapheresis cycles is based on accurate antibody measurement, and this has an impact on patients, staffing and ultimately the cost of the process of transplantation (95, 97).

Introduction

Antibody measurement after a clinical event such as graft rejection does not adequately benefit clinical management compared to that which can predict the risk of the clinical event in order to pre-emptively guide patient management.

Currently several methods for this purpose exist and they include: Titration (by either column or tube techniques) , Flow Cytometry, Enzyme Linked Immunosorbent Assay (ELISA)(98) and Surface Plasmon Resonance (SPR) (99). SPR is a platform that allows molecular interactions (antibody- antigen binding affinities) to be assessed as association and dissociation constants. This is a concept that looks not at the quantity of antibody but rather the quality of antibody in terms of binding and hence can be used to investigate anti-ABO specific antibody binding to A/B antigens.

Of all these methods, 'titration' meets most of the attributes of the ideal method *i.e.* it is simple to perform, already in routine use, cheap and fast. Krishnan *et al* (100) demonstrated correlation between flow cytometry and haemagglutination titre in clinical cases with anti-ABO specific antibody and monitored changes in levels over time in response to clinical therapies. Although flow cytometry has been known as a more sensitive method than agglutination for antibody detection such as HLA antibodies, however, there is not much evidence to support its use in measuring ABO antibodies(101).

Development of all these methods will assist in the formulation of reliable and reproducible assays to measure anti-ABO antibodies.

Improved reproducibility of anti-ABO antibody measurement is an important step towards:

- Prospective studies across centres comparing various desensitisation protocols including TPE and immunosuppression. This will subsequently allow for more meaningful meta-analytical inferences as antibody measurement methodology will no longer be considered a variable due to standardisation.

- Risk stratification based upon antibody quantification, *i.e*. the ability to identify patients likely to develop early allograft loss or AMR who may be offered alternative treatment strategies such as paired exchange/kidney swap with another donor.

It may also be possible to differentiate antibodies on the basis of isotype, subclass or other characteristics associated with likelihood to cause organ damage. This is covered in Section 1.16.

1.15.1 ABO antibody titration (Haemagglutination Assay)

While anti-HLA antibody measurement has progressed overtime, the measurement of anti-ABO specific antibodies is still largely based on a technique reported by Landsteiner over 100 years ago. This haemagglutination technique is a serial doubling dilution of patient's plasma until no reaction is obtained against cells with the corresponding antigen. The titre is then defined as the inverse of the last dilution to give a positive reaction. There are different variations of this technique, but all are based on the same principle of the last serial dilution to give a positive reaction.

However, not only are the conditions at which reactivity is assessed different between laboratories, but the ability to reproduce them precisely has also been recognised as problematic. For example, it has been the practice for a long time to save a sample to be retested alongside a subsequent one to ensure that an apparent change in strength was not due to different application of the technique. While such duplicate testing mitigates the problem of imprecision within the laboratory, variation in technique makes application of uniform guidelines problematic across laboratories and time and contributes to the difficulty in predicting the clinical impact of antibody strength.

An ideal anti-A or anti-B titre before ABOiRTx is defined differently dependent on the centre and, to further compound this issue, the titration methods vary across different centres. As a result, interventions to allow for ABOiRTxs to be successful vary widely resulting in some patients being over- or underprepared.

Data from an exploratory exercise carried out by UK NEQAS (102-104), showed a wide variation in practice and consequent variation in antibody levels (titres) obtained even when the same technology was used on the same set of samples. Bentall *et al* (105) on behalf of UK NEQAS ABO titration group reported results of a survey of 14 UK centres undertaking ABOi transplantation and the median IgG haemagglutination titres that clinicians would accept patients onto the ABOi program for treatment was 512 (range, 128-4096). The median target for acceptable titre on the day of transplantation was 8 (range, 2-16).

These centres had up to 5 dilution titre difference when comparing in-house techniques in the respective laboratories which was reduced to 1 dilution difference when a reference technique was used. The variation in results across the centres is shown in Fig 9.

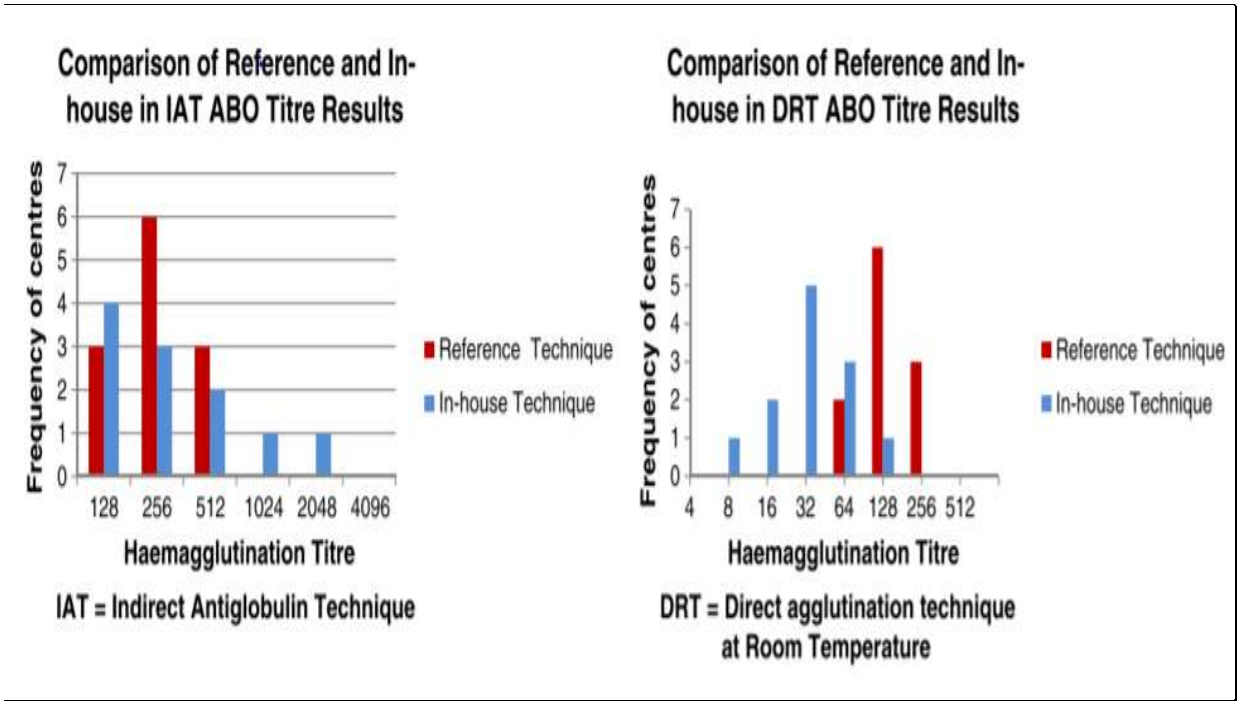


Figure 9 A comparison of reference and in-house Indirect Agglutination technique (left) and Direct Agglutination room temperature technique (DRT) (right)

This demonstrates a need to standardise methodology and formulate national guidelines for ABO titrations. The non-standardisation of titration protocols causes problems where titration results are shared between transplant centres for decision making. Several other surveys and studies have shown the differences in detailed protocols and results (102-104, 106). This implies that a technique for determination of ABO antibodies must be available and must be ideally simple to perform, readily available, cheap, fast reliable and reproducible.

In terms of ABO antibodies, “titre” is defined as the maximum dilution of the patient’s plasma (which contains either anti-A and/or anti-B) causing agglutination with a defined suspension of A1 or B cells (29).

Most laboratories perform ABO antibody titration by a haemagglutination technique, and it is known that that this method is not perfect, with considerable inter-and intra-laboratory variation, although there is a degree of standardisation with gel-card based haemagglutination assays (107). This makes it hard to define an upper limit of antibody level at which transplantation is high risk. Although it can occur with any pre-transplant level, AMR is more likely when the ABO titre is greater than 256 (108, 109).

1.15.2 Standardisation of ABO antibody titration

Antibody titration is difficult to standardise due to several factors as discussed in the following section. Several surveys have been undertaken to understand and solve the problem of differences in titration protocols to enable comparison between different centres or studies. In these studies, the main sources of method variation included (76):

- Different technologies (*e.g*. tube or column methods)
- Differences in the definition and visual interpretation of the titration endpoint
- Different reagents used *e.g*. type of diluent, Coomb’s reagent and test red cells
- Use of plasma or serum
- Use of different Incubation times
- Reagent cells used (Commercial or Donor)
- Repeated Freezing and thawing of sample

- Absence of a reference standard

The presence of so many sources of variation makes standardisation difficult to achieve as all the differences must be reconciled and a consensus reached on a specific detailed method for either column or tube methods.

The final method chosen should provide minimal susceptibility to technical (reagents and equipment) and subjective (human judgement) influence. These factors are described in more detail in the following sections.

1.15.3 Use of controls

Currently there are no standard controls that can be used to monitor the precision of titration methods. The parallel use of a frozen aliquot of a previously run sample during each titration may prove beneficial in providing an internal reference against which the current procedure could be referenced (106). This represents an internal control to ensure reproducibility of results but may not be ideal for reducing inter-laboratory variability of titrations unless the same control is used across all institutions. The sensitivity of this technique in detecting procedural variability would need to be determined on larger scale as there have not been any studies conducted to that effect. In addition, repeated freezing and thawing of the control may introduce error by affecting antibody activity but this can be circumvented by aliquoting the control to avoid refreezing.

It may be necessary to establish an international standard (definitive control) against which each laboratory carrying out its chosen method of titration will rationalise or standardise their result to cater for the variation across institutions.

A regular quality control survey may be necessary to improve the accuracy of titration; however, standardisation of anti-A/B antibody titration is essential before this can be done.

1.15.4 Titration endpoint and grading reactions

The titration endpoint is defined as the last dilution of plasma to give a positive reaction with a given reagent cell. This begs the question, “What reaction grade should be considered as positive?” since titration involves visual interpretation which is prone to subjectivity. Reproducible grading is well known to be problematic even among staff in the same laboratory. The difference between a 1+ and a 2+ grading may be less discernible to some individuals than that between a weak (W+) and a 1+. The effect of such differences in judgement was demonstrated by Aubuchon *et al* (106) who showed that inter-laboratory variation of titration results may be reduced by using a weaker end-point grading (W+) instead of the usual 1+. Therefore, there should be a consensus on the grading of the titration endpoint and perhaps a more objective way of reading the endpoint should be devised to reduce inter-observer variability.

Before initiation of preconditioning, the baseline anti-ABO antibody titre is well known as a significant predictor of the severity of antibody-mediated graft injury as well as graft survival. Although a few recent reports have shown that a high baseline antibody titre is no longer predictive of poor graft outcome in patients that received tacrolimus- or mycophenolate mofetil-based immunosuppression, antibody removal should be as complete as possible (110).

Different groups use the antiglobulin IgG antibody titre endpoint when performing the pre- and post-transplant assessments. Others consider both IgM and IgG titres. It is also uncertain which isotype of anti-ABO antibody between IgM and IgG, is associated with antibody mediated graft damage (111).

1.15.5 Reagent red cells used

Given the extent of variability in antibody titration techniques, it is necessary to use the same method for consistency between different centres. This would mean using the same reagents (*e.g.,* test red blood cells) and conditions (like incubation temperature and centrifugation speed). However, it is impossible to use the same test/reagent red cells worldwide due to logistical problems, therefore, it has been suggested that kidney donor red blood cells (*i.e*. red cells from the respective kidney donor) be used as test red cells for A/B antibody titration in ABO-incompatible kidney transplantation (51). This is based on the assumption that expression of ABO antigens on the red cells is similar to the expression in the ABO-incompatible organ to be transplanted.

However, there is no absolute correlation between ABO antigen expression on red blood cells and in kidneys (76).

In the current study, commercial red cells, supplied by the National Blood Service will be used as they are readily available for other purposes in our laboratory and inter-batch variability can be monitored whenever new batches are supplied to exclude the “type of reagent red cells” used as a variable.

1.15.6 Absence of a reference standard

The classical diagnostic accuracy paradigm involves a comparison of the results of the test under evaluation (index test) with the results of the reference/gold standard (*i.e*. the best available method to determine the presence or absence of the condition or disease of interest). Accuracy measures agreement of test under evaluation with the outcome of the reference standard. In our study, there is no acceptable reference standard as all methods to be investigated are in routine use worldwide regardless of their differences. In the absence of an acceptable gold standard, application of the accuracy paradigm becomes impossible as there is nothing to compare the methods against.

Antibody titres are conventionally measured against reagent cells of donor ABO type and not against donor red cells, and IgG levels, rather than IgM levels are important in terms of defining risk of failure. There are differences in the methods for measuring anti-A and anti-B antibodies that may make comparison from different studies and countries difficult. Titres lack absolute precision so, even with a similar method, significantly different titre levels can be obtained. This is supported by a comparative study performed in 3 centres in Sweden and Germany that showed that, even though laboratories used the same methods, there were considerable differences between the results obtained in different laboratories where the same sample differed by a median of 3 (range 0-6) titre steps using gel and tube techniques (112).

1.15.7 External quality assurance scheme

Whichever method is used to measure ABO antibody levels, it is recommended that there is regular quality control of the method, for example the National External Quality Assurance Scheme (NEQAS) for Blood Transfusion Laboratory practice allows for the comparison of results obtained in different laboratories (13). This will allow individual laboratories to compare their results with other laboratories to assess for consensus and therefore formulate the basis for introducing internal adjustments until results are comparable.

1.16 Antibody subclasses in ABOiRTx

1.16.1 Significance of ABO IgG subclasses in renal transplantation

IgG is the most common antibody isotype in humans and it exists as IgG1, IgG2, IgG3, IgG4 subclasses which are robustly stable. The differences between the subclasses are mainly in the constant regions especially in the hinges and upper CH2 domains. These are the parts that are involved in binding to IgG-Fc receptors (FcγR) and complement CR1.

The basic structure of all immunoglobulin molecules is a unit of 2 identical light (L) polypeptide chains and 2 identical heavy (H) polypeptide chains linked together by disulphide bonds. The class and subclass of an immunoglobulin is determined by its heavy chain type.

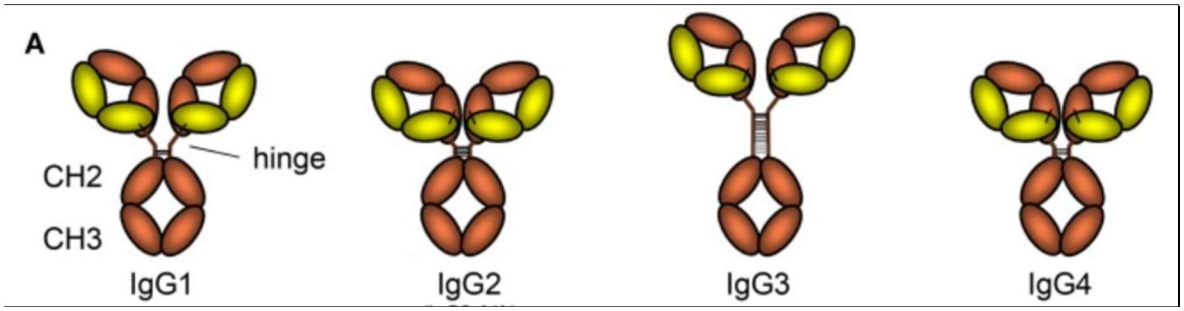


Figure 10 Schematic layout of IgG subclasses

(Adapted from Vidarsson et al 2014) (115)

Figure 10 shows how the heavy and light chains are linked, the length of the hinge and the number of disulphide bridges connecting the heavy chains. These properties result in the differential effector functions in terms of *e.g*. complement activation in graft rejection (113).

The role of IgG subclasses in graft rejection has not yet been fully investigated. Human IgG3 followed by IgG1 is the most potent complement binder of the subclasses. Human IgG4, the subclass present in the least concentration, is predominantly expressed in association with chronic antigen stimulation where it inhibits complement (C1q) binding.

In their study Lee *et al* (114) used flow cytometry and obtained results that suggested that IgG1 and IgG2 were the dominant IgG subclass in ABOiRTx recipients. In ABOiRTx, the IgG isotype is clinically significant in predicting the outcome and risk of transplant rejection. Using a flow cytometric analysis, Stussi *et al (98)* reported that anti-ABO specific antibody subclass distribution of healthy individuals was composed mainly of IgG2 subclass. IgG1 and IgG3 subclasses constituted a small fraction and IgG4 was not detected.

Introduction

The range of subclass detected in an earlier study by Kay(115) , using haemagglutination with subclass specific Antihuman globulin (AHG), demonstrated that subjects of all ABO groups had the capacity to produce IgG antibodies with each subclass, but those of group O produced the broadest spectrum of IgG subclasses and greatest strength of reactions. Another study suggested that IgG1 and IgG2 were the dominant subclasses in ABOiRTx recipients and the baseline levels of these subclasses were found to correlate with the baseline total IgG titre (114).

Immunoglobulin subclasses (IgG1, IgG2, IgG3 and IgG4) differ in their ability to activate the complement cascade and trigger rejection. The Fc portion of IgG1 and IgG 3 confer the ability to fix complement which then plays a role in graft rejection as described in Section 1.7(115).

Among the IgG subclasses, IgG3 was found to recover relatively soon and seemed to be resistant to the effects of rituximab. The IgG3 level in the ABOiRTx patients recovered quickly from the effects of double filtration plasma pheresis, suggesting that IgG3 producing B-cells were resistant to rituximab (116).

FcγR expression profiles of B-cells have been found to affect the dynamics of B-cell subset depletion through targeting CD20. Fortunately, IgG3 is rarely induced in situations of ABO incompatibility and predominant subclass has been shown to be IgG2 rather than IgG3 (117) .

The assessment of IgG subclass antibodies has not been extensively studied in ABOiRTx.

To risk-assess patients, their IgG subclass profile was investigated before desensitisation in order to describe the respective distribution of subclasses and correlate these with graft outcome.

1.17 Scope of this thesis

1.15 Scope of this thesis

In order to make informed clinical decisions in the preparation and subsequent management of ABOiRTx patients, accurate and reproducible information of antibody levels and antibody activity is crucial. Therefore, the aim of this study is to:

1. Compare commonly used ABO antibody titration methods and assess these for agreement/disagreement between them.
2. Compare antibody titres obtained by each titration method against transplant outcome to determine association with graft survival.
3. Develop an ELISA (Enzyme Linked Immunosorbent Assay) for reproducible measurement of anti-ABO IgG antibody subclasses.
4. Determine association between anti-ABO IgG subclasses (as measured by the developed ELISA method) and ABOiRTx outcomes.

Chapter Two: Antibody Titration Methods

This chapter presents the methods and outcomes for the Method Comparison study undertaken and subsequent evaluation of ABOiRTx patient samples. The first part of this study centres on the comparison of 6 ABO titration methods, which were then used to measure antibody titres in the samples from ABOiRTx patients.

2.0 Study setting

The study was carried out at Imperial College Healthcare NHS Trust (Hammersmith Hospital Blood Transfusion Department and Leslie Brent Laboratory). This is a teaching hospital and is one of the largest renal transplantation units in Europe, which made it ideal for this study.

2.1 Study population and kidney donor demographics

The whole study consisted of 50 samples taken from ESRD patients of which 30 had ABOiRTx. The other 20 samples were used to make up the optimal sample size as Calculated for the study but could not be used for calculations involving graft outcome data. as these patients had not yet had their transplants and therefore did not have graft outcome data.

ABOiRTx transplant patients were recruited into the study by reviewing the Register for ABO-Incompatible Renal Transplant patients kept in the Blood Transfusion Laboratory at Hammersmith Hospital. The Register consisted of Special Forms showing the patient and donor blood groups and information on the recommended blood group of plasma required for Therapeutic Plasma Exchange. Only patients whose plasma samples were frozen from when they were transplanted and whose clinical history was easily accessible were selected into the study. A total of 30 patients were selected into the study, 12 females and 18 males. Mean age of participants was 50.42 years.

Average weight at transplantation was 76.9kg. 27% (n=8) of the study population were on both Rituximab and Daclizumab induction immunosuppressive treatment and 73% (n=22) on Rituximab only. 13% (n=4) of the recipients were onto their second kidney transplant and 11 subjects had pre-emptive transplant performed while 19 were on dialysis while waiting for transplantation. Fig 11 shows the primary causes of ESRD in the study patients.

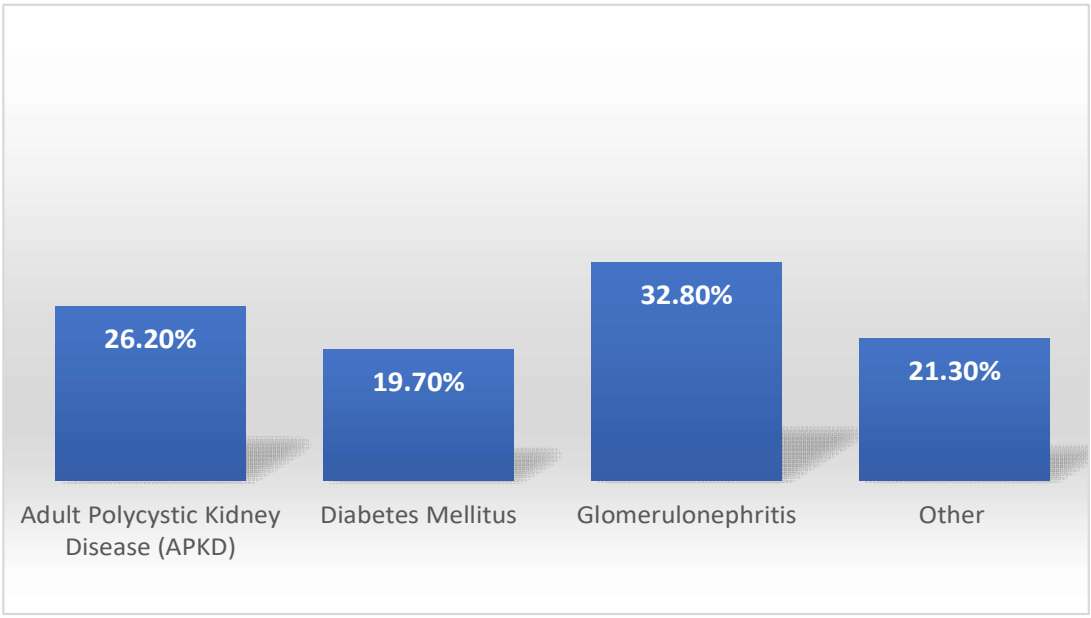


Figure 11 *The primary causes of ESRD in study patients*

The mean age of the donors was 48.2 years and 59.4% of donors were male while 40.6% were female. The ethnic background distribution of donors that donated kidneys to study patients are shown in Fig 12.

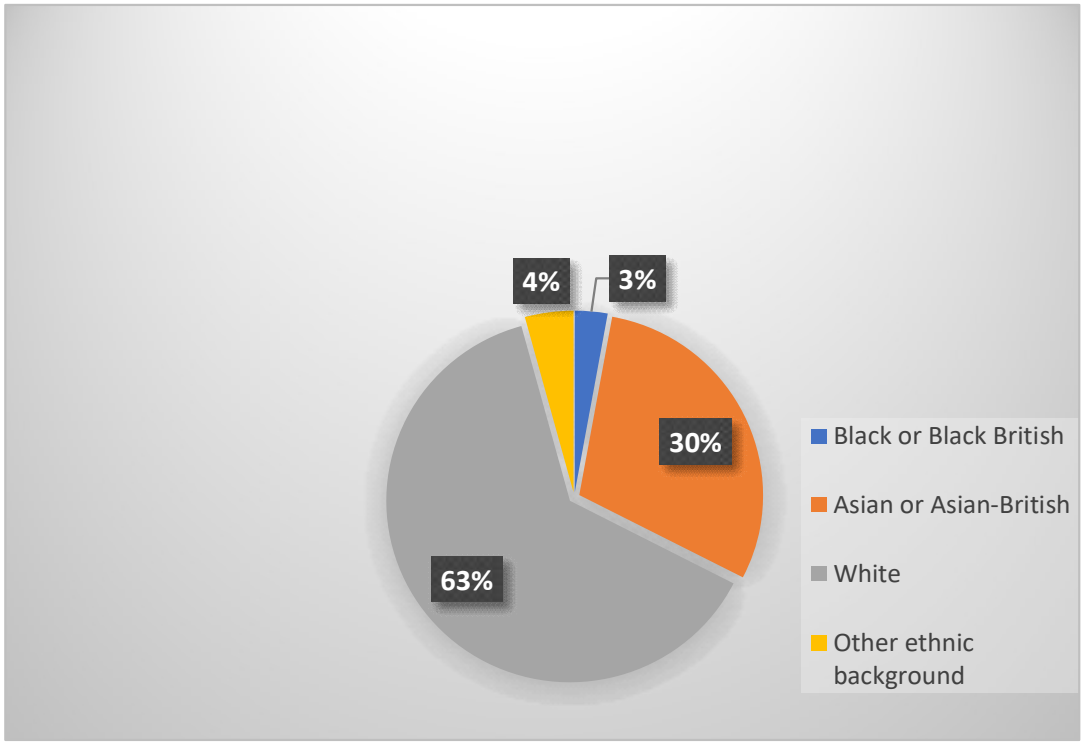


Figure 12 Ethnic background distribution of donors that donated kidneys to study patients.

The majority of the donor population consisted of white and Asian or Asian British individuals while Black or Black British and other ethnic minorities constituted the remainder of the donor complement and this population distribution is reflective of this part of London where a significant portion of the population are Asian or Asian-British and White.

For the method comparison aspect of this study, plasma samples (n=50) obtained from both ABOiRTx pre-transplant ESRD patients routinely attending the Renal unit at the Hammersmith Hospital were used (Section 2.8). Of these 50, 30 had already had their ABOiRTx while 20 were ESRD patients awaiting transplantation as states in Section 2.1.

2.3 Ethical consideration

The study was approved by the Imperial College Healthcare NHS Trust Joint Research and Compliance Office at Hammersmith Hospital on the 8th of July 2013.

It was excluded from the ethical approval process as it was considered to be a Quality Assurance exercise as opposed to Research according to the HTA (Human Tissue Authority) definition. No new method was developed in this study but rather a comparison of well-established methodologies already available for operational use. In addition, no samples were collected prospectively but residual samples from the original method used at Imperial for ABO antibody titre measurement for clinical care during transplant were used. Plasma samples from all ABO incompatible transplant patients were frozen from 2005 to 2015 and were available for analysis in this study.

From the HTA (2004) (part 1 section point 10a)(118) and Royal College of Pathologists (RCPath) documents (Page 13 point 12)(119); it follows that as this is for Quality assurance purposes with no need for new specimens, no specific patient consent nor patient anonymity is required.

Regarding patient sensitive information, the Renal team overseeing care of the patients reviewed the patients' notes and relayed requested outcome data relevant to the study.

2.4 Study design

The cornerstone of many method evaluations is "Method Comparison" in which a set of specimens is assayed by both an existing method and the new method followed by comparison of results (120). Ideally in a method comparison study design, several methods are compared against a gold standard which if absent complicates this type of design. There is no gold standard method for ABO antibody titration therefore methods will have to be compared against each other for agreement. Four main points were considered in order to provide guidance in conducting the comparison of these methods(121):

- Selection of the titration methods
- Timing of the measurement

- Number of measurements
- Conditions of measurement

2.5 Selection of titration methods

There are 2 main methodologies which are commonly used for ABO antibody titration *i.e*. conventional tube and column methods. Due to the use of different variations of these methods, all available methods were gathered, and the most common ones were selected and assessed for agreement. All institutions performing ABOiRTx in the UK and a few representative external laboratories were contacted to obtain their antibody titration protocols.

Most titration methods were obtained from the UK National External Quality Assurance Scheme (NEQAS) who had record of individual hospital titration methodologies. The methods were then entered into a spreadsheet and checks were carried out for procedural similarities and differences after which methods with the greatest differences were selected into the study to ensure a fair representation of each method variation. A total of 6 methods were selected into the study.

2.6 Timing of measurements

As the main objective of this part of the study was to determine if the titration methods under investigation could be used interchangeably to measure antibody levels, the testing should ideally have been carried out at the same time to guarantee consistency of assay conditions.

However, this was not possible as one individual conducted all tests in the study to exclude inter-observer variation.

Multiple small batches (*e.g*. 5 samples) were ~~run~~ tested at the same time over a month to accommodate between-day variations. To monitor “between/inter day variations”, a previously tested sample was run in parallel with a current batch of samples to ensure repeatability by checking if the same result was produced.

2.7 Number of measurements

For the Method Comparison study, an initial pilot study was conducted on 10 renal patient samples in order to generate preliminary data so the number of samples required for the full method comparison study (referred to as the main study) could be determined. Paired titrations were performed on each sample to reduce chance findings and also to facilitate application of the statistical method chosen for data analysis.

Large numbers of sets of paired measurements add precision to the results and cause the data to approach a normal distribution thus enabling the use of bias and precision statistics.

Duplicate titrations were performed on both the pilot and main study samples on each sample to allow for agreement statistics to be applied. These 2 sets of data were analysed and presented both separately and combined.

2.8 Sample inclusion criteria

Samples used for the method comparison study were from ESRD patients pre-transplant. All samples met the minimum acceptable demographics for Blood Transfusion *i.e*. First name, surname, Hospital number, date of birth and gender.

Samples were also checked for adequacy to ensure enough volume for all measurements to cover all 6 methods compared. Only samples with readily accessible clinical outcome data were selected into the study.

Samples with other red cell antibodies apart from the naturally occurring ABO antibodies were titrated against reagent cells that did not have the corresponding antigen to avoid false elevation of the titre. 3 samples were processed in this manner.

2.10 Comparison of haemagglutination antibody titration methodologies

2.10.1 Preparation of serial dilutions

For each plasma sample tested, dilutions (Master dilution (Neat), 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/2048) were carried out in Phosphate Buffered Saline (P.B.S.S) (Inverclyde Biologicals®, United Kingdom). To achieve this serial dilution, 1 volume of P.B.S.S was added to each of the dilution tubes labelled 1/ 2 up to 1/2048.1 volume of neat plasma was added to 1 volume of P.B.S.S in the tube marked 1/ 2 and mixed. 1 volume of this dilution was then transferred to the next tube marked 1/ 4 and the process repeated until the last tube marked 1/2048.

With each transference of plasma, a new pipette tip was used to avoid cross-contamination between individual dilutions due to carryover which may systematically cause a falsely elevated antibody titre. An automated calibrated pipette was used to ensure consistency in volumes through elimination of pipetting technique as a variable.

2.10.2 Dithiothreitol (DTT) for the inactivation of IgM antibodies

DTT (Dithiothreitol) predominantly degrades IgM by reduction of intermolecular disulphide bonds, and is therefore useful to determine the presence of IgG antibodies in the haemagglutination assay (98).

Plasma samples are first treated with 0.1 M DTT reagent (NHSBT) in a 1:1 ratio then incubated for 30 min at 37°C, and this allows for measurement of only IgG class antibodies after destruction of IgM by the DTT. Titration is then performed as described above in section 2.10.1. DTT is preferred as it is more resistant to oxidation in air and more efficient as a reducing agent than 2-Mercaptoethanol (2-ME).

DTT acts by cleaving the disulphide bonds of pentameric IgM thus abolishing agglutinating and complement binding activities. This allows the detection of IgG antibodies in plasma. The negative control for the titration of anti-A and anti-B, IgM and IgG methods was the serial dilution of serum against blood group O cells, since blood group O has neither A nor B antigens and therefore no agglutination is expected.

2.10.3 Conventional tube Haemagglutination assay for the quantitation of IgM/IgG antibodies

One volume of 3% A1, B or O blood group cells (National Health Service Blood and Transplant (NHSBT) reagents United Kingdom) was then added to each serial dilution of plasma.

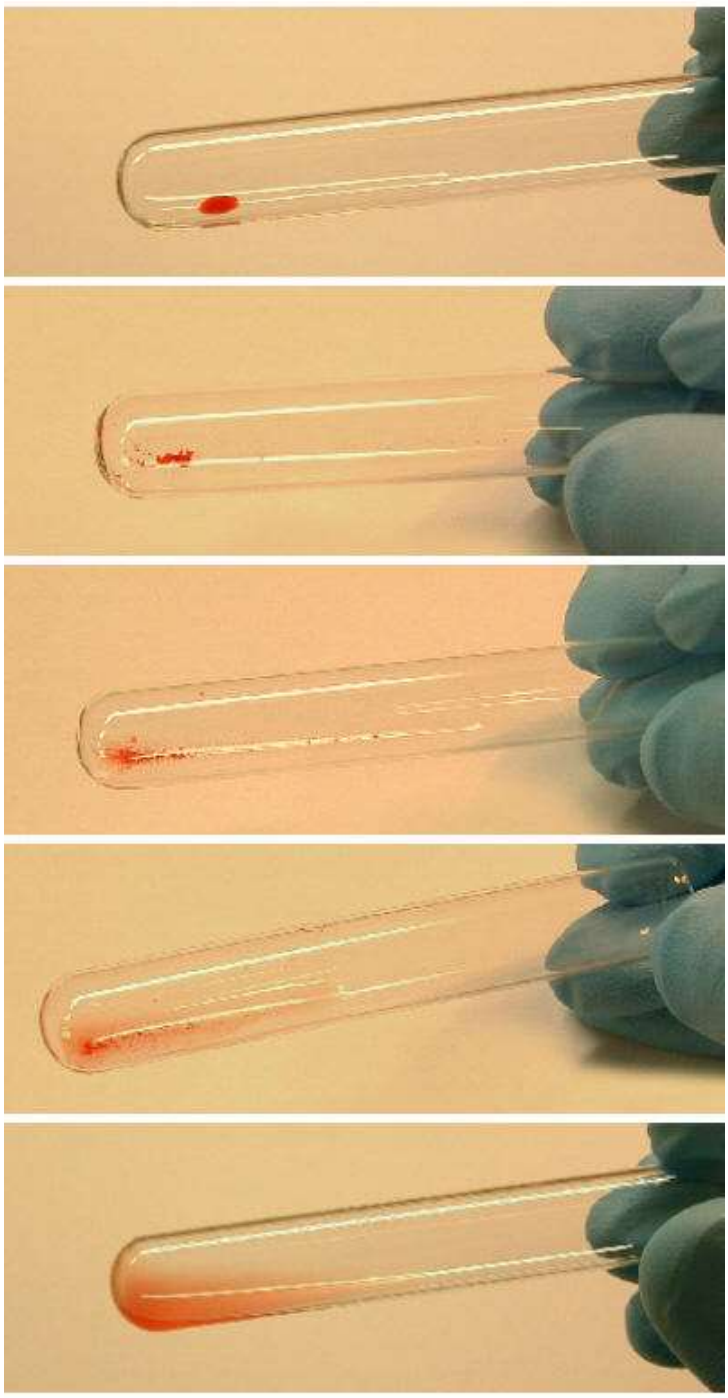
All tubes were incubated (37°C, 45 mins) then centrifuged at 1000 x *g* for 30s.

All dilutions were then washed with P.B.S.S using an automated cell washer (Medion 150) and after the 4th wash, polyspecific anti-human-globulin (AHG) (Biotest ®, Germany) was added to each sample and centrifuged for a further 30s at 1000 x *g*. Each tube was then examined macroscopically for agglutination using the grading shown in Fig13. The inverse of the highest dilution producing agglutination was recorded as the IgM or IgG titre for that sample.

Red cells were obtained from NHSBT supplies (NHSBT Liverpool) for column method; A1rr (product code PR014); B*rr* (Product code PR035) in CellStab suspension, 0.8 ± 0.2% concentration/ML. 3% red cell suspension in Alsevers was also obtained from NHSBT Liverpool for manual tube method.

All samples with a recent transfusion history were screened for antibodies using 3 set of screening cells with a homozygous expression of the most commonly encountered alloantibodies whose presence in the patient's plasma may falsely elevate antibody titre due to their IgG nature.

DTT treatment was also used in this study in order to objectively measure the titre of IgG anti-ABO antibodies (as stated above in Section 2.10.2)



4+ agglutination: Red cell button is a solid agglutinate: Clear background.

3+ agglutination: Red cell button breaks into several large agglutinates: Clear background.

2+ agglutination: Red blood cell button breaks into many medium-sized agglutinations: Clear background; No free red cells.

1+ agglutination: Red cell button breaks into many small clumps barely visible ground macroscopically: background is turbid: many free red blood cells.

Negative: No agglutinated red cells present. Red cells are observed flowing off the red cell button.

Figure 13 Grading of Tube Haemagglutination reactions

2.10.3 Column agglutination

2.10.3.1 Bio-Rad technique

Bio-Rad IAT cards were used for the IAT titration of IgG anti-ABO antibodies while Bio-Rad NaCl cards were used for the room temperature direct agglutination technique for determination of IgM anti-ABO antibodies.

The Bio-Rad IAT cards were labelled with each patient's unique identifier, where appropriate and 50µl of the required ABO group Diluent 2- or Cellstab-suspended red cells were dispensed into appropriately labelled wells of the Bio-Rad IAT or NaCl cards. 25µl of each plasma dilution was added to the appropriate well of the IAT or NaCl card. The NaCl cards were incubated at room temperature for 15 minutes (Room temperature maintained between 20◦C and 24◦C and controlled by AAW control systems). The IAT cards were incubated at 37◦C for 15 minutes. Following incubation, the cards were centrifuged in a Diamed centrifuge for 30s at 85 x *g*. The cards were then manually read on a light box, the results were graded, and the titre recorded.

In cases where the end point was not reached, the titration was extended by performing further dilutions until a negative result was obtained.

Reagent red blood cells were obtained from NHSBT Liverpool: Arr, Brr and OR1r, in Cell stab 0.8±0.2% concentration/ml.

For the DTT technique, 300 µl of plasma was diluted with 300µl of 0.1M DTT reagent (NHSBT) and incubated for 30 minutes at 37°C. PBS was then used for serial dilutions of plasma using the doubling dilution method (Section 2.10.1). The Indirect agglutination using antiglobulin technique (IAT) used 50µl of red cells suspended in ID Diluent 2 to each microtube and then 25µl of the DTT treated plasma serially diluted from 1:1 with ID Diluent 2.

Gel cards containing anti-IgG in the columns were used (004025, Coombs Anti-IgG, Bio-Rad, UK). This was incubated at 37°C for 15 minutes and then centrifuged at 85 x *g* and read as per the previous procedure.

Direct agglutination at room temperature (DRT) was performed using neutral Bio-Rad cards (005015, NaCl, Enzyme Test and cold Agglutinins, Bio-Rad, UK) with 50µl of RBCs suspended in ID Diluent 2 to each microtube. 50µl of each plasma dilution was added to the corresponding microtube and incubated at room temperature for 15 minutes. The gel cards were centrifuged (85 x *g*) for 10 minutes in DiaMed (1-Centrifuge 12S-II, Bio-Rad, UK). The gel cards were analysed over a light box and the last well giving a positive result of agglutination was recorded as the titre result for that sample (Fig14).

A single user performed all haemagglutination assays for historic control samples in parallel with study samples during each run to check for reproducibility of technique.

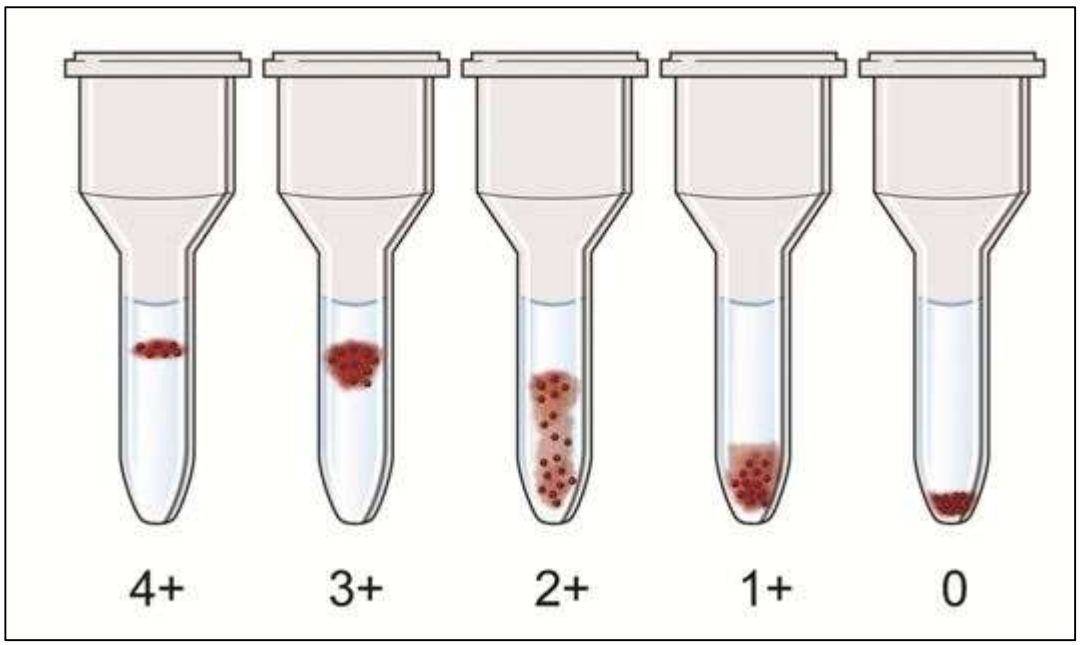


Figure 14 An example of reactions in a typical gel or glass bead column card

2.10.3.2 BioVue technique

Using the OrthoBioVue glass bead column technique, 50µl 0.8% test red cell suspension was added to 40µl of plasma (with or without DTT treatment) in a glass bead cassette (BioVue® -Anti-Human Globulin anti-IgG) and incubated 15 minutes.

The cassettes were spun using OrthoBioVue centrifuge using a two-stage centrifugation first at 793rpm for 2min followed by 1509rpm for 3min in one cycle.

The cards were then manually read on a light box, the results were graded, and the titre recorded as shown in Fig 14.

2.11 Data Analysis

For the method comparison aspect of the study, because 6 methods were being compared for agreement without a reference standard, methods like Correlation or Bland-Altman plot were not suitable as they are suitable mainly for 2 method comparison designs. Repeated Measures ANOVA (using IBM SPSS 25) was therefore used to determine agreement between the antibody titration methods and the following assumptions were made:

- Independent and identically distributed variables observations
- Test variables follow a multivariate normal distribution in the population (Assumption not necessary if sample size ≥ 25)
- Sphericity-The population variances of all possible difference scores are equal. This was tested with Mauchly's test.

The ABO antibody titration results obtained following analysis of the ABOiRTx patient samples using the methodologies used in this study were also compared with the associated results that were obtained by the reference laboratory at the time of transplantation. This was done in order to determine whether different clinical decisions would have been made if any of the methodologies considered in this study were used.

One assumption for this model is that the measured quantity *i.e*. "antibody titre per sample" remains unchanged across the measurements made by the same method(122). Therefore, throughout the study, sample integrity was maintained as samples were kept frozen in aliquots and only thawed when required.

Further:

- If acceptable agreement between methods was noted, then any of the titration methods could be used interchangeably.
- If disagreement between methods was noted, then a proxy reference standard would be used to determine the method that best predicts the clinical outcome *i.e.* transplant survival.

Transplant outcome data was retrieved from patients' historical records and the diagnosis was made through routine biopsies of the renal allografts performed at transplantation and before the first discharge, and episode biopsies were performed in case of graft dysfunction. The biopsy samples were examined using light microscopy and also using immunofluorescent staining in order to check for deposit of C4d in the peritubular capillaries and graft rejection was histologically diagnosed using these graft biopsy specimens using the Banff 2007-2013 criteria (Outcome diagnosis was made prior to this study). Survival analysis was carried out using the Kaplan-Meier over a period of 3 years.

2.11.1 Unique problem with handling titration data

Titres are discrete values following a geometric progression due to serial dilution *i.e.* the results can only take any value as follows: 2, 4, 8, 16 …. $(2)^n$. Therefore, the difference between 2 readings *e.g.* 2 & 4 and 4 & 8 is not the same because the progression follows an exponential function.

In terms of dilution the difference between titres is uniform (*i.e.* 1 dilution between each titre) but for calculation purposes the exponential nature of the titration values distorts the reality and creates a problem when analysing data.

Therefore, to obtain linearity, all titration data was converted to logarithms to base 2 to enable easy analysis. Thus, in all calculations involving titres in this study, titre values were presented as Log (base 2) where a titre of 2 represented $Log_2 2 = 1$, titre of 4 represented $Log_2 4 = 2$ *etc*. as shown in Table 3.

Table 3 Titres expressed as logarithm base 2

Titre	0	2	4	8	16	32	64
Log (base 2)	0	1	2	3	4	5	6

2.12. Pilot Study Results

The 6 methods used in the pilot (and then main) study are shown in Table 4 below. Each titration was tested in duplicate using each method for each of the samples and the duplicate results were designated A1 and A2; B1 and B2 *etc.* However, for subsequent statistical analysis, the duplicate measures were averaged out to allow for comparisons across the different methodologies once good reproducibility was demonstrated.

Table 4 Methods used in the pilot and main studies and their designated codes

Titration Methodology	**Designated code** (Representing duplicate results by each method)
Tube IAT	A1, A2
Tube DRT	B1, B2
Bio-Rad IAT	C1, C2
Bio-Rad DRT	D1, D2
BioVue IAT	E1, E2
BioVue DRT	F1, F2

As stated previously, a pilot study using 10 samples (these were also included in the 50 samples used in the study) from routine ESRD patients on haemodialysis was conducted initially in order to ensure protocol and device familiarisation, to determine reproducibility and also to obtain data for a sample size calculation for the main method comparison study. The pilot study was also used to determine repeatability and hence reliability of each titration method.

Table 5 and Fig 15 shows the results (in duplicates) obtained for all pilot samples which were randomly chosen from ESRD patients on dialysis.

Table 5 Pilot study titration results measured in duplicates.

Sample number	**Tube IAT** (Method A)	**Tube DRT** (Method B)	**Bio-Rad IAT** (Method C)	**Bio-Rad DRT** (Method D)	**BioVue IAT** (Method E)	**BioVue DRT** (Method F)
1	32 32	64 128	256 256	256 256	512 512	256 256
2	64 128	128 128	256 512	512 512	512 1024	512 512
3	32 16	32 32	32 64	64 64	128 128	128 128
4	256 512	256 256	256 512	512 512	512 512	512 512
5	128 128	256 128	512 512	1024 1024	1024 1024	2048 2048
6	512 512	256 256	512 512	512 512	1024 2048	2048 2048
7	32 64	32 64	64 64	64 64	64 64	64 64
8	256 256	256 512	512 512	512 1024	512 1024	1024 1024
9	32 16	16 16	16 32	128 128	128 128	128 128
10	64 32	64 64	32 64	64 64	128 256	128 128

2.12.1 Using pilot study data to calculate sample size

Based on a sample of 10 subjects, pairwise coefficients of individual agreement were calculated. The standard errors (SEs) of the coefficients ranged from 0.03 to 0.22. To make the sample size calculation conservative, the largest standard error of 0.22 was used.

The aim was to use a sample size large enough so that the width of the 95% confidence interval (CI) would not exceed 0.4. Therefore, the CI will be (0.40,0.80). Since the width of the CI is approximately 4 times the SE, the required SE was set to 0.1.

As SE is known to be inversely proportional to the square root of the sample size, therefore in order to decrease the SE from 0.22 to 0.1, the sample size will have to be increased by a factor of $(0.22/0.1)^2$ *i.e.* by 4.84. Since the sample size used in this pilot was 10, at least 49 subjects will be needed to achieve the CI goal. The sample size for titration method comparison in this study was set at 50.

2.12.2 Comparison of titre distributions for pilot samples as measured by different titration methodologies.

Figure 15 shows box and whisker plots of duplicate titre readings measured by the 6 methods chosen in the study as displayed in Table 5.

Method designations *i.e*. Method A1 and Method A2 denote duplicate titres on study samples as measured by each method (For method descriptions, see Table 4)

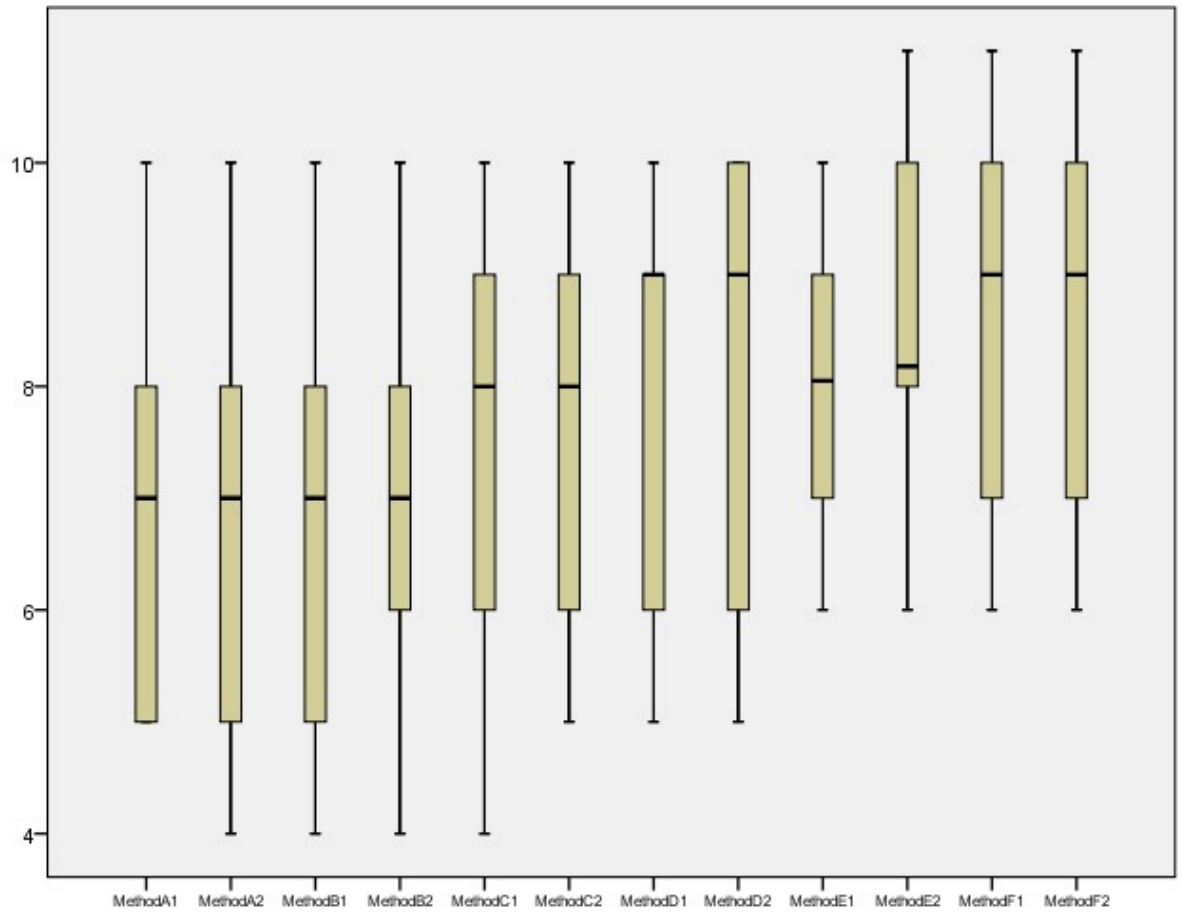


Figure 15 Box and Whisker plots showing duplicate titre readings as measured by the 6 methods in the pilot study

Overall, methods D (Bio-Rad DRT) and F (BioVue DRT) gave higher mean titres (titres expressed to $\log_2$) compared to the others. Methods A (Tube IAT), B (Tube DRT) and C (Bio-Rad IAT) gave the highest range while methods A and B gave the lowest mean titres.

These results demonstrate a wide variation of distributions of results obtained by each method on the same samples. Methods D and F tend to give higher titres than the rest of the methods. In general, this means that choosing to use method D and F over A, B or C may result in overpreparation or underpreparation of patients for ABOiRTx unless one out of the 2 groups of methods is proven to be associated with graft survival compared to the other.

Since the titrations were carried out on different days using the different methodologies, inter-day and intra-test reproducibility needed to be established.

2.12.3 Intra-test and inter-day reproducibility of haemagglutination assay for IgG and IgM ABO antibodies on pilot samples

The Cronbach's coefficient was used to determine the level of reproducibility as shown in Table 6.

Table 6 Determination of Intra-and inter-test reproducibility in the pilot study

Titration Methodology	Intra-test reproducibility Cronbach's Coefficient	Inter-day reproducibility Cronbach's Coefficient	Mean for test	Standard Deviation	Number of samples tested
Tube IAT	0.94	0.95	12.8	3.16	10
Tube DRT	0.95	0.94	13.2	2.79	10
Bio-Rad IAT	0.98	0.97	14.7	3.35	10
Bio-Rad DRT	0.99	0.97	15.9	2.98	10
BioVue IAT	0.97	0.99	17	2.86	10
BioVue DRT	1.0	0.98	17	3.38	10

The intra-test and inter-day repeatability showed good 'test-retest' reliability for all 6 methods as measured by the Cronbach's Alpha reliability coefficient (α) which calculates the inter- and intra-test Correlation coefficient. Effectively, Cronbach's alpha measures internal consistency of a method or how closely related the antibody titres are as a group. As the average inter-method correlation increases, Cronbach's alpha also increases. One of the underlying assumptions for the Cronbach's alpha is that all of the titre measurements were entirely independent from one another. Cronbach's alpha for both inter-day and intra-test was >0.90 thus demonstrating good reproducibility.

This level of consistency as demonstrated in the pilot study provided the confidence needed to proceed with the testing of the rest of the study samples. This high level of reproducibility also justified the use of average titres of each of the pairs of measurements performed on each sample by each individual method. Titration reproducibility was monitored throughout the study especially as different batches of pooled red blood cells were obtained from NHSBT reagents laboratory in Liverpool to ensure consistency of results.

2.12.4 Statistical analysis for pilot study Direct Room Temperature Agglutination (DRT) titration methods

For statistical analysis, methods that used the Direct Room Temperature agglutination (DRT) methodology were analysed separately from those that used Indirect Agglutination (IAT). The BioVue DRT method had the highest mean titre compared to Tube and Bio-Rad DRT methods as shown in Table 7. This might be an early indication of the possibility of overestimation by the BioVue DRT method.

Table 7 Estimated Marginal Means and Standard Error of Titres against their respective Methodologies used.

Estimated Marginal Means				
Measure: Antibody titre				
			95% Confidence Interval	
Antibody Titration Method	Mean	Std. Error	Lower Bound	Upper Bound
Tube DRT	6.600	0.464	5.550	7.650
Bio-Rad DRT	7.950	0.497	6.826	9.074
BioVue DRT	8.500	0.563	7.227	9.773

In order to assess for overall comparability across these methods, ANOVA with repeated measures was used as shown in Table 8.

Table 8 ANOVA between all Antibody DRT antibody Titration Methodologies in the pilot

Tests of Within-Subjects Effects

Measure: AntibodyTitre

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
AntibodyTitrationMethod	Sphericity Assumed	19.117	2	9.558	24.995	.000	.735
	Greenhouse-Geisser	19.117	1.721	11.111	24.995	.000	.735
	Huynh-Feldt	19.117	2.000	9.558	24.995	.000	.735
	Lower-bound	19.117	1.000	19.117	24.995	.001	.735
Error (AntibodyTitrationMethod)	Sphericity Assumed	6.883	18	.382			
	Greenhouse-Geisser	6.883	15.485	.445			
	Huynh-Feldt	6.883	18.000	.382			
	Lower-bound	6.883	9.000	.765			

The results show that there was overall significant difference between the ABO antibody titre means as measured by the different titration methods. From these results, the mean scores for the antibody titres were statistically different: F (2, 18) =24.995, p=0.000.

The results demonstrate that there is an overall significant difference in the means obtained by the 3 titration methods. As a result of this statistically significant difference, pairwise comparisons were carried out in order to determine which specific means differed.

Significance testing was done using the Bonferroni *post hoc* test (adjustment for multiple comparisons) and this is shown in Table 9 which shows the significance level for differences between individual antibody titration methods.

Table 9 Pairwise comparison of ABO antibody titres against the DRT Antibody Titration methodology in the pilot

Pairwise Comparisons						
Measure: ABO Antibody Titre						
(I) Antibody Titration Method		Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
Tube DRT	Bio-Rad DRT	-1.350*	0.289	0.004	-2.198	-0.502
	BioVue DRT	-1.900*	0.314	0.001	-2.822	-0.978
Bio-Rad DRT	Tube DRT	1.350*	0.289	0.004	0.502	2.198
	BioVue DRT	-0.550	0.217	0.095	-1.186	0.086
BioVue DRT	Tube DRT	1.900*	0.314	0.001	0.978	2.822
	Bio-Rad DRT	0.550	0.217	0.095	-0.086	1.186
Based on estimated marginal means						
*. The mean difference is significant at the .05 level.						
b. Adjustment for multiple comparisons: Bonferroni.						

There was a significant difference in antibody titre between Tube DRT and Bio-Rad DRT (p=0.004) and between Tube DRT and BioVue DRT (p=0.001). However, there was no significant difference between Bio-Rad DRT and BioVue DRT (p=0.095).

2.13.3 Statistical analysis for pilot study IAT Titration Methods

Statistical analysis was carried for the IAT methods in a similar way as for the DRT methods in Section 2.12 above. Table 10 shows the means and standard errors pilot sample of titres against the IAT methods.

Table 10 Estimated Marginal Means and Standard Error of titres against their respective methodologies

Measure: ABO antibody titre				
AntibodyTitrationMethod	Mean	Std. Error	Interval	
			Lower Bound	Upper Bound
Tube IAT	6.600	0.482	5.510	7.690
BioRad IAT	7.350	0.558	6.088	8.612
BioVue IAT	8.550	0.491	7.439	9.661

BioVue IAT method had the highest mean titre and range compared to Tube IAT and Bio-Rad IAT.

This pattern was also observed above with the DRT methods which seems to suggest that higher titration values are likely to be associated with both the IAT and DRT BioVue methods.

Overall significance testing was performed for IAT methods using ANOVA with repeated measures and the results are shown in Table 11.

Table 11 ANOVA between all IAT antibody titration methodologies in the pilot

Tests of Within-Subjects Effects						
Measure: IAT methods						
Source		Type III Sum of Squares	df	Mean Square	F	Sig.
AntibodyTitration Method	Sphericity Assumed	19.350	2	9.675	16.100	0.000
	Greenhouse-Geisser	19.350	1.467	13.189	16.100	0.001
	Huynh-Feldt	19.350	1.682	11.504	16.100	0.000
	Lower-bound	19.350	1.000	19.350	16.100	0.003
Error (AntibodyTitration Method)	Sphericity Assumed	10.817	18	0.601		
	Greenhouse-Geisser	10.817	13.204	0.819		
	Huynh-Feldt	10.817	15.139	0.715		
	Lower-bound	10.817	9.000	1.202		

There was a statistically significant difference ($p<0.005$) between the IAT methods in this pilot study therefore pairwise comparisons were performed to identify where the specific differences lay (Table 12).

Table 12 Pairwise comparisons of IgG titres against antibody titration methodology

Pairwise Comparisons						
Measure: Antibody titre						
(I) Antibody Titration Method		Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
Tube IAT	Bio-Rad IAT	-0.750	0.417	0.316	-1.972	0.472
	BioVue IAT	-1.950*	0.369	0.001	-3.031	-0.869
Bio-Rad IAT	Tube IAT	0.750	0.417	0.316	-0.472	1.972
	BioVue IAT	-1.200*	0.226	0.001	-1.863	-0.537
BioVue IAT	Tube IAT	1.950*	0.369	0.001	0.869	3.031
	Bio-Rad IAT	1.200*	0.226	0.001	0.537	1.863
Based on estimated marginal means						
*. The mean difference is significant at the .05 level.						
b. Adjustment for multiple comparisons: Bonferroni.						

Table 13 shows overall significant difference between all the methodologies in the pilot study.

Table 13 Multivariate analysis showing overall significance between all IAT antibody titration methodologies in the pilot study

Multivariate Tests					
	Value	F	Hypothesis df	Error df	Sig.
Pillai's trace	0.852	23.114[a]	2.000	8.000	0.000
Wilks' lambda	0.148	23.114[a]	2.000	8.000	0.000
Hotelling's trace	5.779	23.114[a]	2.000	8.000	0.000
Roy's largest root	5.779	23.114[a]	2.000	8.000	0.000
Each F tests the multivariate effect of AntibodyTitrationMethod. These					
a. Exact statistic					

Pairwise investigations showed that the only agreement was found between Tube IAT and Bio-Rad IAT (p=0.316).

2.13.4 Statistical analysis for pilot study IAT and DRT Titration Methods

Multivariate analysis of both IAT and DRT methods was carried out to determine significance. The results are shown in Table 14.

Table 14 Multivariate analysis showing overall significance of both IgG and IgM titres against all methodologies

Multivariate Tests

	Value	F	Hypothesis df	Error df	Sig.	Partial Eta Squared
Pillai's trace	0.875	6.983[a]	5.000	5.000	0.026	0.875
Wilks' lambda	0.125	6.983[a]	5.000	5.000	0.026	0.875
Hotelling's trace	6.983	6.983[a]	5.000	5.000	0.026	0.875
Roy's largest root	6.983	6.983[a]	5.000	5.000	0.026	0.875

Each F tests the multivariate effect of AntibodyTitrationMethod. These tests are

a. Exact statistic

The investigation shows that there was a statistically significant difference between both IAT and DRT methods F (6.983), p=0.026. Since this was a pilot study, pairwise comparisons were performed to obtain a clearer picture of what to expect in the larger sample size i.e. n= 50 in the substantive study as calculated in Section 2.12.1.

Table 15 shows pairwise comparisons of titres performed by the DRT and IAT methods on pilot samples.

Table 15 Pairwise comparisons of titres against DRT and IAT Antibody titration methodologies on pilot samples

Pairwise Comparisons						
Measure: ABO Antibody titre						
(I) Antibody Titration Method		Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
Tube IAT	Tube DRT	-0.750	0.417	1.000	-2.398	0.898
	Bio-Rad IAT	-1.950*	0.369	0.007	-3.407	-0.493
	Bio-Rad DRT	0.000	0.279	1.000	-1.103	1.103
	BioVue IAT	-1.350	0.388	0.104	-2.883	0.183
	BioVue DRT	-1.900*	0.371	0.009	-3.368	-0.432
Tube DRT	Tube IAT	0.750	0.417	1.000	-0.898	2.398
	Bio-Rad IAT	-1.200*	0.226	0.007	-2.094	-0.306
	Bio-Rad DRT	0.750	0.201	0.070	-0.044	1.544
	BioVue IAT	-0.600	0.233	0.452	-1.523	0.323
	BioVue DRT	-1.150*	0.279	0.039	-2.255	-0.045
Bio-Rad IAT	Tube IAT	1.950*	0.369	0.007	0.493	3.407
	Tube DRT	1.200*	0.226	0.007	0.306	2.094
	Bio-Rad DRT	1.950*	0.252	0.000	0.953	2.947
	BioVue IAT	0.600	0.194	0.195	-0.169	1.369
	BioVue DRT	0.050	0.174	1.000	-0.638	0.738
Bio-Rad DRT	Tube IAT	0.000	0.279	1.000	-1.103	1.103
	Tube DRT	-0.750	0.201	0.070	-1.544	0.044
	Bio-Rad IAT	-1.950*	0.252	0.000	-2.947	-0.953
	BioVue IAT	-1.350*	0.289	0.018	-2.493	-0.207
	BioVue DRT	-1.900*	0.314	0.003	-3.143	-0.657
BioVue IAT	Tube IAT	1.350	0.388	0.104	-0.183	2.883
	Tube DRT	0.600	0.233	0.452	-0.323	1.523
	Bio-Rad IAT	-0.600	0.194	0.195	-1.369	0.169
	Bio-Rad DRT	1.350*	0.289	0.018	0.207	2.493
	BioVue DRT	-0.550	0.217	0.477	-1.407	0.307
BioVue DRT	Tube IAT	1.900*	0.371	0.009	0.432	3.368
	Tube DRT	1.150*	0.279	0.039	0.045	2.255
	Bio-Rad IAT	-0.050	0.174	1.000	-0.738	0.638
	Bio-Rad DRT	1.900*	0.314	0.003	0.657	3.143
	BioVue IAT	0.550	0.217	0.477	-0.307	1.407
Based on estimated marginal means						
*. The mean difference is significant at the .05 level.						
b. Adjustment for multiple comparisons: Bonferroni.						

Out of all the pairwise comparisons, the following pairs of methods: Tube IAT/Tube DRT, Tube IAT/Bio-Rad DRT, Tube IAT/BioVue IAT, Tube DRT/BioVue IAT, Bio-Rad IAT/BioVue IAT, Bio-Rad IAT/BioVue DRT, BioVue DRT/BioVue IAT showed no statistically significant difference ($p>0.05$) hence demonstrated agreement.

Following the completion of the pilot study, 40 more samples (30 from ABOiRTx patients and 10 from ESRD patients awaiting transplantation) were processed in a similar manner as the calculated sample size for 80% statistical power was n=50 (Section 2.12.1). The results are shown in the next section.

2.14 Analysis of all study samples against all titration methods

Following the same format as used for the pilot study in Section 2.13 above, the same analyses were carried out on the full complement of study samples (n=50) which also included the pilot samples (n=10). Table 16 shows ANOVA results between DRT methods on all study samples.

Table 16 ANOVA between DRT titres against Titration methodology

Tests of Within-Subjects Effects							
Measure: **Antibody Titres (Both IgG and IgM)**							
Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
AntibodyTitrationMethod	Sphericity Assumed	161.090	5	32.218	51.425	0.000	0.512
	Greenhouse-Geisser	161.090	2.877	55.997	51.425	0.000	0.512
	Huynh-Feldt	161.090	3.075	52.384	51.425	0.000	0.512
	Lower-bound	161.090	1.000	161.090	51.425	0.000	0.512
Error(AntibodyTitrationMethod)	Sphericity Assumed	153.493	245	0.627			
	Greenhouse-Geisser	153.493	140.960	1.089			
	Huynh-Feldt	153.493	150.684	1.019			
	Lower-bound	153.493	49.000	3.133			

Sphericity assumption for ANOVA was not met hence the Greenhouse-Geisser correction was used. Repeated measures ANOVA determined that the mean antibody titres differed statistically significantly between the different titration methodologies $F(2.877, 14,96) = 51.42$, $p<0.005$.

Table 17 Pairwise comparisons between antibody titres and Titration methodologies on all study samples

Pairwise Comparisons						
Measure: Antibody Titres						
(I) AntibodyTitrationMethod		Mean Difference (I-J)	Std. Error	Sig.[b]	Interval for Difference[b]	
					Lower Bound	Upper Bound
Tube IAT	Tube IgM	-0.170	0.094	1.000	-0.461	0.121
	BioRad IAT	-.520*	0.149	0.016	-0.980	-0.060
	BioRadIgM	-1.140*	0.196	0.000	-1.746	-0.534
	BioVue IAT	-1.550*	0.205	0.000	-2.183	-0.917
	BioVueIgM	-2.020*	0.176	0.000	-2.563	-1.477
Tube IgM	Tube IAT	0.170	0.094	1.000	-0.121	0.461
	BioRad IAT	-0.350	0.115	0.054	-0.703	0.003
	BioRad IgM	-.970*	0.178	0.000	-1.518	-0.422
	BioVue IAT	-1.380*	0.188	0.000	-1.961	-0.799
	BioVue IgM	-1.850*	0.166	0.000	-2.363	-1.337
BioRadIAT	Tube IAT	.520*	0.149	0.016	0.060	0.980
	Tube IgM	0.350	0.115	0.054	-0.003	0.703
	BioRad IAT	-.620*	0.174	0.013	-1.157	-0.083
	BioRad IgM	-1.030*	0.128	0.000	-1.425	-0.635
	BioVue IgM	-1.500*	0.153	0.000	-1.973	-1.027
BioRad IgM	Tube IAT	1.140*	0.196	0.000	0.534	1.746
	Tube IgM	.970*	0.178	0.000	0.422	1.518
	BioRad IAT	.620*	0.174	0.013	0.083	1.157
	BioVue IAT	-0.410	0.154	0.159	-0.886	0.066
	BioVue IgM	-.880*	0.124	0.000	-1.263	-0.497
BioVue IAT	Tube IAT	1.550*	0.205	0.000	0.917	2.183
	Tube IgM	1.380*	0.188	0.000	0.799	1.961
	BioRad IAT	1.030*	0.128	0.000	0.635	1.425
	BioRad IgM	0.410	0.154	0.159	-0.066	0.886
	BioVue IgM	-.470*	0.126	0.008	-0.860	-0.080
BioVue IgM	Tube IAT	2.020*	0.176	0.000	1.477	2.563
	Tube IgM	1.850*	0.166	0.000	1.337	2.363
	BioRad IAT	1.500*	0.153	0.000	1.027	1.973
	Biorad IgM	.880*	0.124	0.000	0.497	1.263
	BioVue IAT	.470*	0.126	0.008	0.080	0.860
Based on estimated marginal means						
*. The mean difference is significant at the .05 level.						
b. Adjustment for multiple comparisons: Bonferroni.						

The results of pairwise comparisons in Table 17 show that the only methods that demonstrated agreement were Tube IAT and Tube IgM, Tube IgM and Bio-Rad IAT, Bio-Rad IgM and Bio-Vue IAT i.e. (p=1.00) (CI: -0.12, 0.461), p=0.054 (CI -0.703-0.003) and p=0.159 (CI -0.886-0.066) respectively.

2.15 Risk factors affecting transplant outcome

Several factors may affect graft outcome and thus acting as confounders when assessing the effect of antibody titres on ABOiRTx outcome. These factors include HLA incompatibility, comorbidities like diabetes mellitus, age at transplant and whether the transplant was pre-emptive or followed a period of dialysis and are listed below.

- Number of previous transplants
- Immunosuppressive drugs
- Age/Gender of donor or recipient
- Weight
- Dialysis vs pre-emptive transplantation
- HLA antibody sensitisation

Table 17 below summarises the most common risk factors associated with ABOiRTx outcome as identified by the British Transplantation Society in the Guidelines for Antibody incompatible transplantation (123).

Table 17 Main risks associated with ABOiRTx

Factor	**High risk**	**Low risk**
Haemagglutination titre	>1/256	Any level
If donor is group A	Group A1	Group A2
Donor type	Deceased donor	Living Donor
HLA antibody incompatibility	Yes	No

(Adapted from British Transplant Society Guidelines 2015)(107)

Unfortunately, these potential confounders could not effectively be factored into graft outcome statistical inferences as the information was not consistently recorded.

Therefore, the only risk factor which could be investigated was 'baseline ABO antibody levels' against transplant outcome.

2.15 Comparison of titration methodologies used in the study against methodologies used by other centres in the NEQAS program

In order to determine comparability of the study results against other centres, the study was registered to participate in a NEQAS ABO antibody titration pilot exercise from 2012 to 2015 and a total of 12 exercises were performed. A maximum of 441 submissions from all participants were sent in this exercise, most of them from the United Kingdom and some from other parts of the world.

In the current study only the proposed standard DRT and IAT methods were registered and submitted to the NEQAS survey with and also without DTT treatment. The survey looked at 14 different titration methods. Figure 16 summarises the methodologies used and the respective submissions made under each category. Reproducibility was also assessed in one of the survey exercise distributions through blind-testing of 2 identical samples containing anti-A antibody *i.e.* Sample 2 and sample 3 as shown in the Table 18.

Table 18 Intra-laboratory reproducibility of testing 2 identical samples against different methodologies

Technique (Number of results)	Results Patient 2 vs. Patient 3		
	Number (%) Identical	Number (%) 1 dilution apart	Number (%) >1 dilution apart
DRT Standard (48)	43 (90%)	5 (10%)	0 (0%)
IAT Standard (54)	41 (76%)	13 (24%)	0 (0%)
DRT In-house (46)	38 (83%)	8 (17%)	0 (0%)
IAT In-house untreated (29)	16 (55%)	12 (41%)	1 (4%)
IAT In-house DTT treated (8)	4 (50%)	4 (50%)	0 (0%)

'DRT standard' and 'DRT In-house' techniques showed good reproducibility *i.e.* both showing the highest number of identical results (90% and 83% respectively) and 0% of results showed a difference of greater than 1 dilution on the identical samples tested. The proposed IAT Standard technique also showed good reproducibility with 0% of the results >1 dilution apart and 76% of identical titres obtained. The IAT in-house untreated technique was the only one that had 4% of results showing >1 dilution difference with 41% of the results 1 dilution apart. The IAT in house DTT treated technique was the least used technique with equal proportions (50%) of titres identical and also within 1 dilution difference, however, the proportion may have been affected by the small number of samples tested using this methodology.

Figure 16 shows the number of submitted results against respective methodologies as submitted to NEQAS. This shows that DRT and IAT standard methods were the most frequently used methodologies.

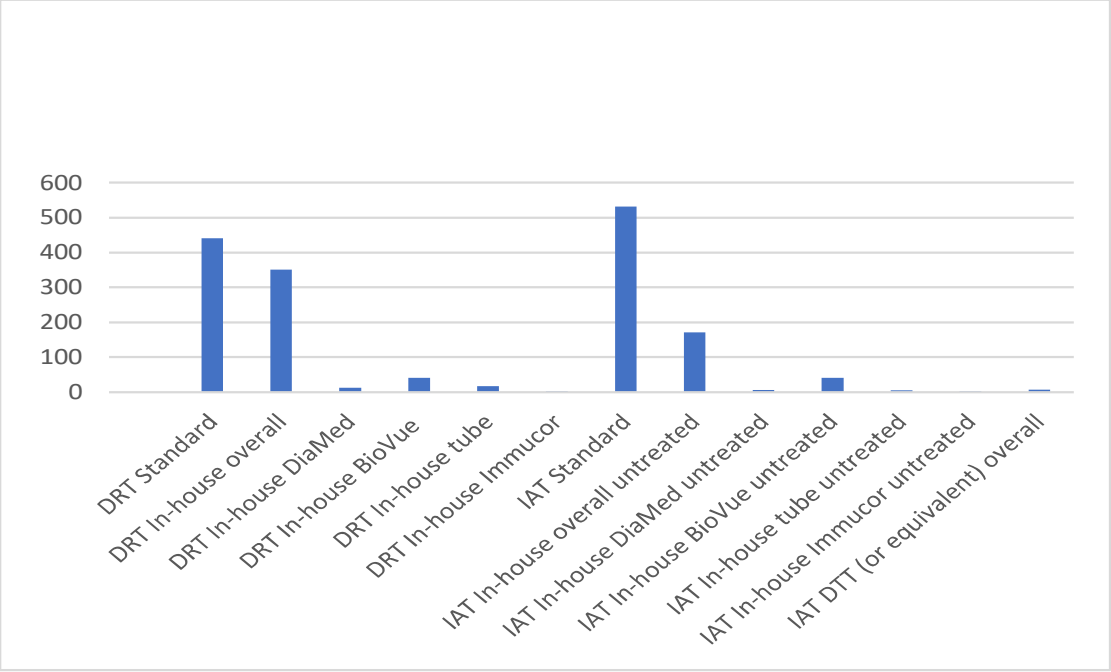


Figure 16 Number of submitted results against titration methodology in a UK NEQAS exercise

Immucor and Tube methodologies were the least used methodologies in the survey while DTT treatment was only used by a small number of centres.

Overall, the results obtained through participation of our study in this scheme were either identical to or within 1 dilution of the method median. This overall comparability demonstrated that the methodologies used in this study produced results that were in consensus with the majority of centres using similar or different methodologies.

The titrations in this study were run by one individual to mitigate the effect of inter-operator variability thus maintaining a level of objectivity throughout the study.

2.11.5 Transplant survival against antibody titration results for the ABOiRTx patients

A Kaplan Meier method was used for survival analysis for determining if there was any difference in survival between ABOiRTx patients with low and those with high baseline ABO antibody titres.

High or Low titre levels were defined as ≤64 and >64 respectively. The titre levels were determined for each patient based on this criterion as measured by each of the methods considered in this study. For graft outcome, records were retrospectively reviewed over a 5-year period and any cases that went over this period were “censored” (*i.e*. patients that did not experience graft rejection or died with a functioning graft in the defined period).

Table 19 below shows that a high percentage 66.7 % (n=14) of cases were censored in the low ABO antibody group compared to the high antibody group *i.e*. 33.3% (n=3). The event in this study was defined as “Graft rejection”. "High” antibody titre was defined as >64 while “Low” titre was defined as ≤64. From Table 19, it appears that high antibody level is associated with poor graft survival.

Table 19 Antibody titre against censored cases over a 5- year period

Case Processing Summary				
		N of Events	Censored	
Antibody Level	Total N		N	Percent
High	9	6	3	33.3%
Low	21	7	14	66.7%
Overall	30	13	17	56.7%

Table 20 demonstrates that poor prognosis is associated with high ABO antibody group *i.e*. mean survival time for the Low antibody group was higher at 3.789 (SE 0.473) years (CI=0.466-3.872) compared to the High antibody group which was 2.169 years (SE=0.869) (CI=0.466-3.872).

Table 20 Measures of Central tendency for Survival time between High and Low antibody levels

Means and Medians for Survival Time									
	Mean[a]				Median				
			Interval				Interval		
Antibody Level	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound	
High	2.169	0.869	0.466	3.872	1.100	0.481	0.156	2.044	
Low	3.789	0.473	2.862	4.717					
Overall	3.313	0.431	2.469	4.157	5.000				
a. Estimation is limited to the largest survival time if it is censored.									

Overall, there was no statistically significant difference in graft survival (p>0.05) between the High and Low ABO antibody titre groups over a 5-year period as shown in Table 21 as tested by the Log Rank (Mantel-Cox) Chi-Square.

Table 21 Overall comparison of graft survival between high and low ABO antibody titre groups

Overall Comparisons			
	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	3.185	1	0.074
Breslow (Generalized Wilcoxon)	2.878	1	0.090
Tarone-Ware	3.093	1	0.079
Test of equality of survival distributions for the different levels of AbLevel.			

The Kaplan–Meier survival curves for the 2 ABO antibody groups (High and Low) as measured by the Reference Laboratory IAT method, Fig17, show different survival patterns with the Low antibody titre group showing longer survival as demonstrated by the high cumulative survival.

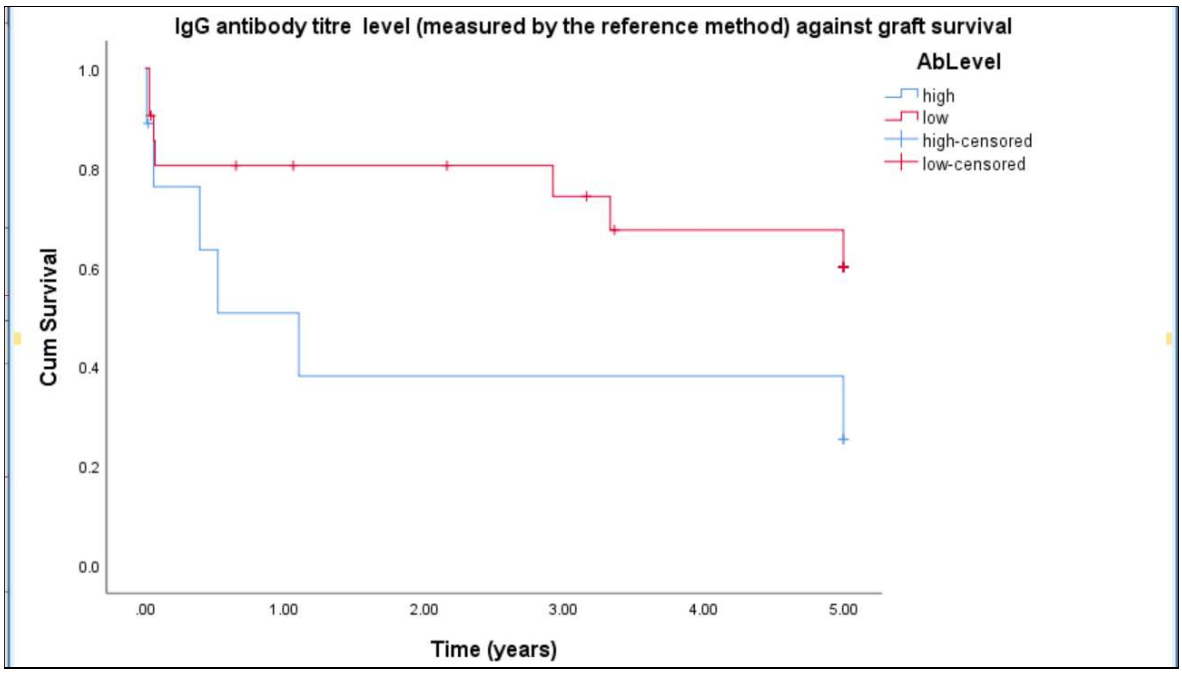


Figure 17 ABO IgG antibody titre level (measured by the reference method) against graft survival

However, there was no statistically significant difference in graft survival between the 2 antibody level groups (p-value=0.074) as calculated by the Log Rank Mantel-Cox test.

Fig 18 shows the survival curves for High and Low antibody levels on the same samples but measured by the Tube IAT method used in the study.

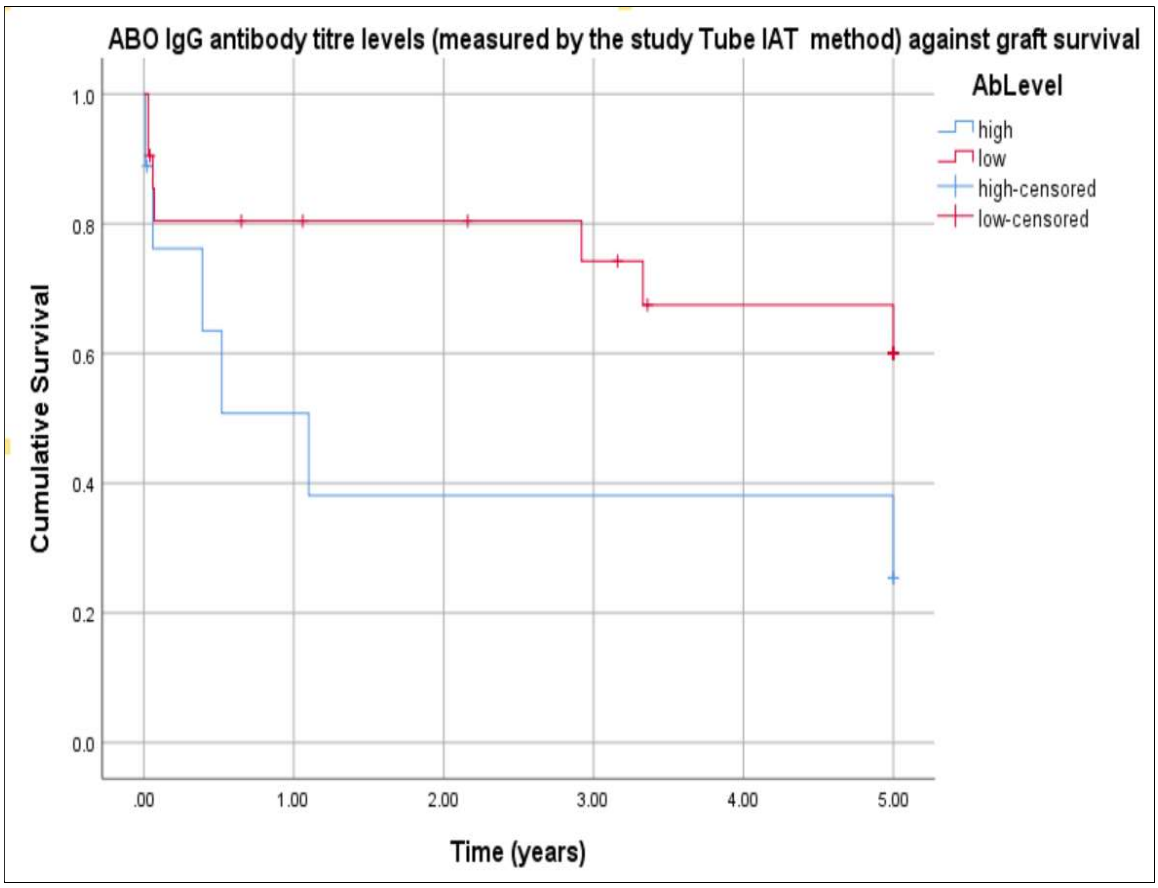


Figure 18 ABO IgG antibody titre levels (measured by the study Tube IAT method) against graft survival.

There was also no statistically significant difference in graft survival between the 2 antibody level groups with significance level of p-value=0.074, similar to that calculated for the Reference method in Fig 17.

When the study Bio-Rad IAT method was used to determine the antibody levels, the survival curves in Fig 19 were obtained for the High and Low antibody categories.

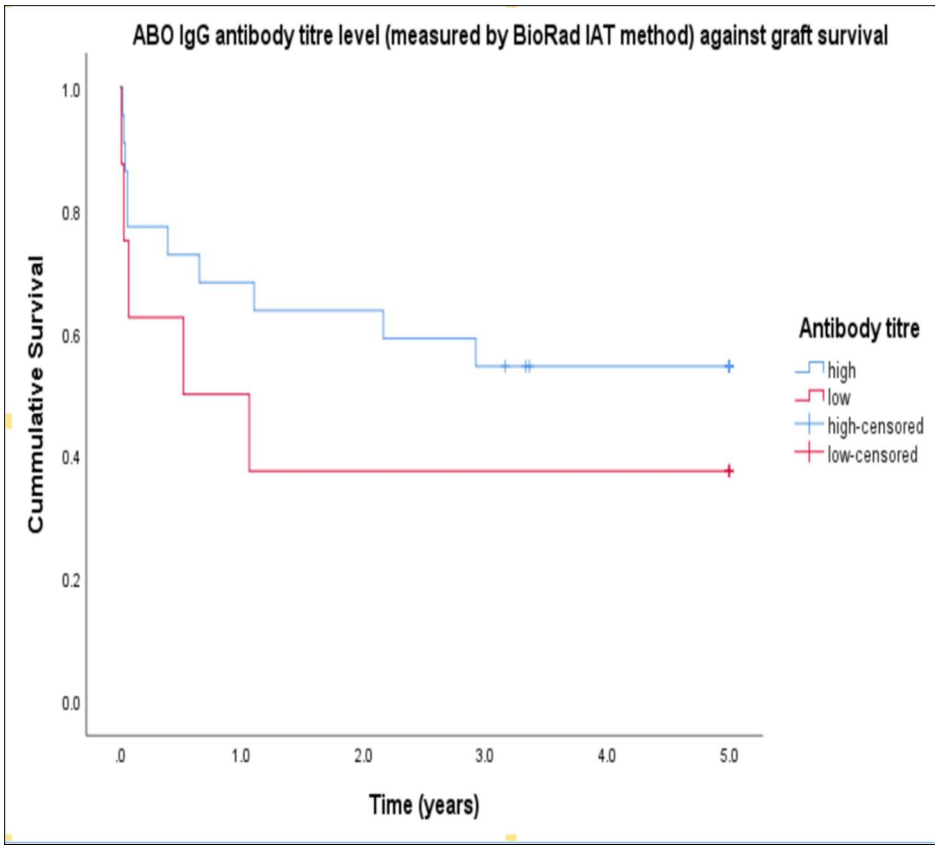


Figure 19 ABO IgG antibody level (measured by Bio-Rad IAT method) against graft survival

There was no significant difference between graft survival in the 2 antibody groups, p-value=0.315. However, the High antibody level group had longer cumulative survival compared to Fig 17 and 18.

Survival curves were also prepared for High and Low antibody groups determined by the study BioVue IAT method and the results are shown in Fig 20.

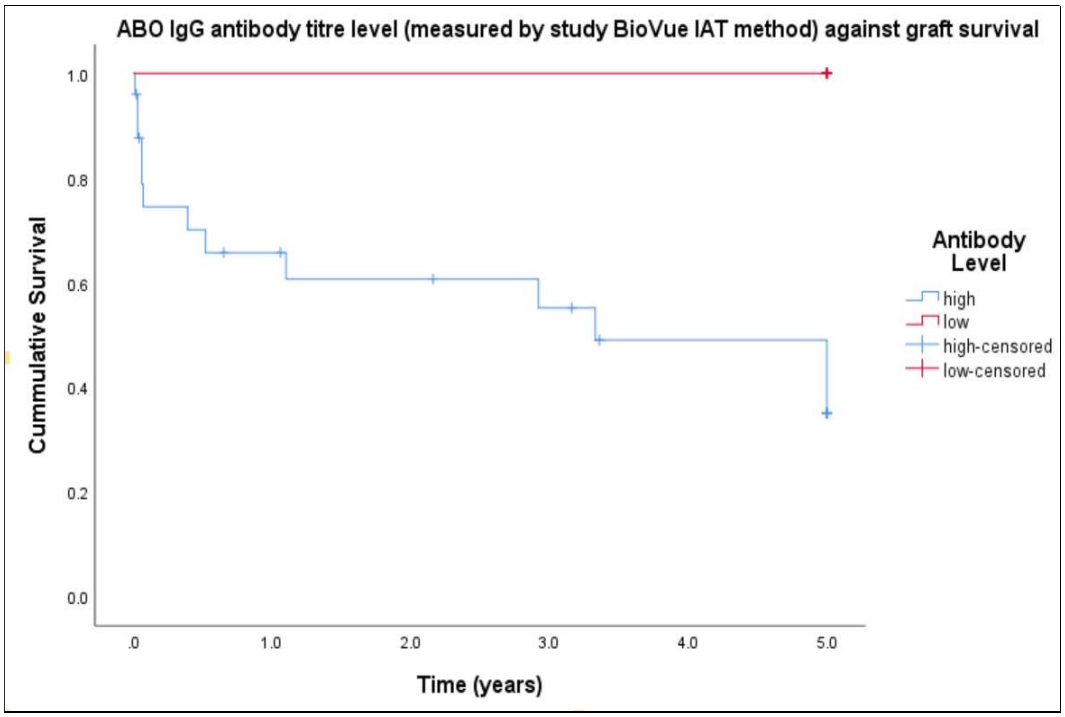


Figure 20 ABO IgG antibody titre level (measured by study BioVue IAT method) against graft survival

Study BioVue IAT was the only method that demonstrated statistically significant difference between the 2 antibody groups, p=0.025 and also, more patients were censored in the high antibody group than in Figs 17-19. This was also the only method that demonstrated no graft rejection nor censoring in the 5-year period.

2.11.7 Managing ABO incompatible transplants with respect to each titration methodology investigated in 2.116 above.

Use of any antibody titration method in ABOiRTx has accompanying cost implications. A method which underestimates the antibody titre would mean that patients will be transplanted before they are ready *i.e.* when their antibody levels are still high thus resulting in unnecessary cost and time wastage in trying to deal with transplant rejection. Conversely, the titration method that overestimates titres will result in pre-transplant over preparation of patients or total exclusion from transplantation for patients that could either proceed to transplantation with minimal preparation.

The titration methods were therefore assessed for cost-effectiveness by reviewing the decisions made based on each method and the transplant outcome. This would give an indication of patients that were possibly transplanted before they were ready (and *vice versa)*. An ideal antibody titration method will give an accurate titre thus allowing the correct decision to be made and appropriate allocation of resources.

In the results obtained in 2.11.6, there was no significant difference in graft survival between high and low antibody levels as measured by Reference IAT, study Tube IAT and study Bio-Rad IAT although the low antibody group had the lowest cumulative graft survival and also had the most censored patients who did not experience graft rejection. The study BioVue IAT method was the only method that categorised the antibody levels to cause a significant difference in the graft survival of the patients investigated.

2.12 Maximum pre-transplant/Baseline ABO antibody titres and plasma exchange in the 30 study patients

Table 22 shows the baseline (maximum ABO antibody measured before plasma exchange) and pre-operative (maximum ABO antibody measured after effective plasma exchange cycles) antibody titres of the study transplant recipients. The titration shown in the table was performed by the Reference Laboratory (Red Cell Immunology Laboratory at Colindale) therefore, information presented is retrospective. The same samples used pre-transplantation were tested using the 6 antibody titration methodologies chosen in this study to determine agreement or disagreement of results. The results are shown in Table 22.

Table 22 Baseline antibody titres (measured by different titration methodologies) against number of pre-transplant plasmapheresis.

Case number	Baseline antibody levels (Measured by Reference Laboratory)		Pre-operative ABO antibody levels		
	IgG + IgM Blood group antibody titres	IgG Blood group antibody titres	IgG + IgM Blood group antibody titres	IgG Blood group antibody titres	Number of pretransplant plasmapheresis
1	256 (8)	64(6)	16(4)	4(2)	10
2	128(7)	64(6)	4(2	Neat (0)	11
3	128(7)	128(7)	2(1)	Neat (0)	9
4	16(4)	4(2)	Neat (0)	Neat (0)	7
5	16(4)	128(7)	Neat (0)	Neat (0)	6
6	128(7)	4(2)	4(2)	2(1)	8
7	16(4)	4(2)	Neat (0)	Neat (0)	2
8	128(7)	2(1)	Neat (0)	Neat (0)	8
9	64(6)	16(4)	2(1)	Neat (0)	7
10	128(7)	128(7)	2(1)	Neat (0)	11
11	512(9)	256(8)	8(3)	8(3)	15
12	128(7)	64(6)	4(16)	Neat (0)	8
13	16(4)	16(4)	2(1)	Neat (0)	5
14	64(6)	64(6)	8(256)	2(1)	4
15	64(6)	64(6)	4(2)	Neat (0)	4
16	128(7)	32(5)	8(3)	2(1)	8
17	128(7)	64(6)	4(2)	Neat (0)	9
18	64(6)	2(1)	8(3)	Neat (0)	4
19	16(4)	4(2)	Neat (0)	Neat (0)	2
20	2(1)	32(5)	8(3)	2(1)	3
21	8(3)	32(5)	8(3)	Neat (0)	3
22	8(3)	64(6)	4(2)	Neat (0)	4
23	512(9)	128(7)	16(4)	4(2)	12
24	2(1)	64(6)	Neat (0)	Neat (0)	2
25	2(1)	128(7)	Neat (0)	Neat (0)	8
26	128(7)	128(7)	4(2)	Neat (0)	10
27	128(7)	128(7)	2(1)	2(1)	11
28	8(3)	32(5)	Neat (0)	Neat (0)	3
29	16(4)	4(2)	Neat (0)	Neat (0)	2
30	64(6)	64(6)	2(1)	Neat (0)	4
Figures in parenthesis represent the respective titres expressed as logarithm_2					

Plasma exchanges were carried out for each recipient until the ABO antibody titres were ≤64. However, for 2 of the recipients (1 and 23), transplant was carried out at a combined IgG + IgM titre of 16 but IgG only titre of 4. The Reference laboratory used Bio-Rad DRT with untreated plasma to measure Combined IgM+IgG titre and DTT treated plasma for the titration of IgG only ABO antibodies.

Pearson's correlation was carried out to investigate for relationships between the variables "Combined baseline IgG and IgM antibody titre", "baseline IgG only antibody titre" and "number of Plasma exchanges" carried out to reduce the titres of the measured antibodies. Data is presented in Table 23.

Table 23 Investigation of Correlation between antibody titre and Number of Plasma Exchanges

Correlations

		IgG and IgM ABO Antibody Titre	IgG only Antibody Titre	Number of Plasma Exchanges
Combined IgG and IgM ABO antibody Titre	Pearson Correlation		0.189	.740**
	Sig. (2-tailed)		0.318	0.000
	N	30	30	30
IgG only Antibody Titre	Pearson Correlation	0.189	1	.495**
	Sig. (2-tailed)	0.318		0.005
	N	30	30	30
Number of Plasma Exchanges	Pearson Correlation	.740**	.495**	1
	Sig. (2-tailed)	0.000	0.005	
	N	30	30	30
**. Correlation is significant at the 0.01 level (2-tailed).				

There was significant evidence of a relationship between Combined baseline IgG+IgM antibody titre and Number of Plasma Exchanges ($r=0.740$, $p<0.05$) and also between baseline "IgG only" antibody titre and Number of plasma exchanges ($r=0.495$, $p=0.005$) although the latter had a weaker correlation coefficient.

Figure 19 demonstrates that, in general, as the baseline IgG and IgM ABO antibody titre increases, the required number of Plasma Exchanges also increases proportionally.

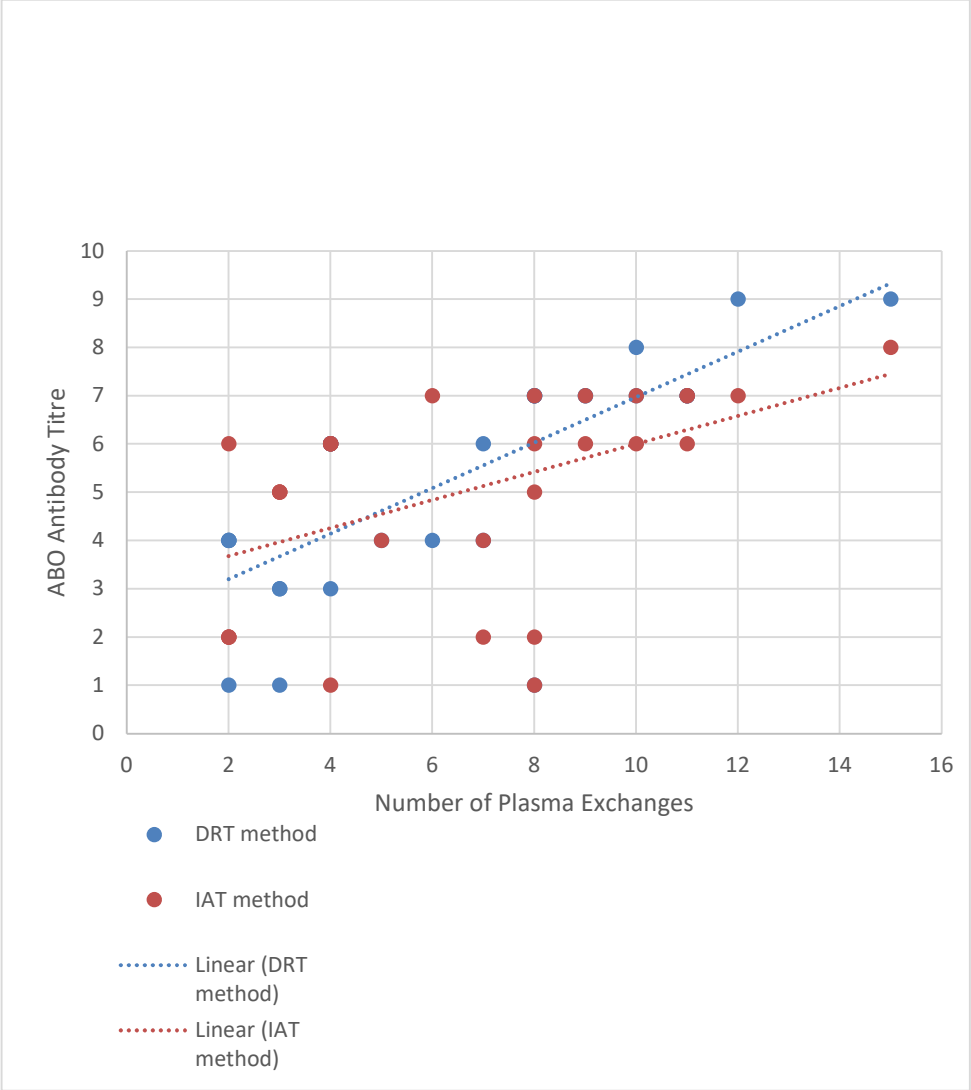


Figure 21 Pre-transplant Baseline ABO antibody titres (by the Reference Laboratory DRT and IAT methods) against number of plasma exchanges

DRT in Fig 21 represents combined IgM+IgG titration while IAT represents IgG only measured.

2.13 A comparison of the titration methods investigated in this study against the titration methods used by the Reference Laboratory at the time of transplantation

Table 24 shows combined IgM+IgG titres and IgG only titres measured by the different methods, IAT methodologies used in the study (and the method used by the Reference Laboratory at the time of transplantation), against the number of respective plasma exchanges.

Table 24 ABO antibody titres (as measured by different titration methodologies) against number of Plasma Exchanges

Sample number	Number of Plasma Exchanges	IgM+IgG titre (Log2) by Reference laboratory	IgG titre (Log2) by Reference Laboratory (DTT treatment)	IgM+IgG titre (Log2) by Tube IAT	IgM+IgG titre (Log2) BioRad IAT	IgM+IgG titre (Log2) BioVue IAT	IgG titre Biorad (Log2) (DTT treatment)
1	10	8	9	5	8	9	9
2	11	7	6	6.5	8.5	9.5	5
3	9	7	7	4.5	5.5	7	7
4	7	4	2	8.5	8.5	9	1
5	6	4	7	7	9	10	6
6	8	7	2	9	9	10.5	4
7	2	4	2	5.5	6	6	2
8	8	7	1	8	9	9.5	2
9	7	6	4	4.5	4.5	7	4
10	11	7	7	5.5	5.5	7.5	7
11	15	9	8	7	7	9	7
12	8	7	6	5.5	7	8	4
13	5	4	4	8	8	8	4
14	4	6	6	10	8	8	Not done*
15	4	6	6	10	9	8	4
16	9	7	6	7	9	10	6
17	3	3	5	4.5	4.5	7	5
18	4	3	6	5.5	5.5	7.5	6
19	12	9	7	7	7	9	7
20	2	1	6	5.5	7	8	Not done*
21	10	7	7	6.5	8.5	9.5	7
22	11	7	7	4.5	5.5	7	5
23	4	6	6	9	9	10.5	4
24	4	6	1	4.5	4.5	7	1
25	2	4	2	5.5	5.5	7	Not done*
26	2	2	4	2	6	6	3
27	7	4	2	4.5	4.5	7	4
28	6	4	7	5.5	5.5	7.5	6
* Test not performed due to sample insufficiency							

*3 samples could not be processed using the DTT treatment method for IgG due to sample insufficiency. These samples were therefore excluded from the comparison between Plasma exchanges and IgG titre by Bio-Rad method using the IgG Bio-Rad with DTT treatment method chosen for this study. Data shown in shaded columns was obtained from the current study while the rest was performed by the reference laboratory at the time of transplantation.

The results in Table 24 are displayed in a scatter plot in Fig17 in order to demonstrate the relationship between the number of plasma exchanges and the titration methodology used.

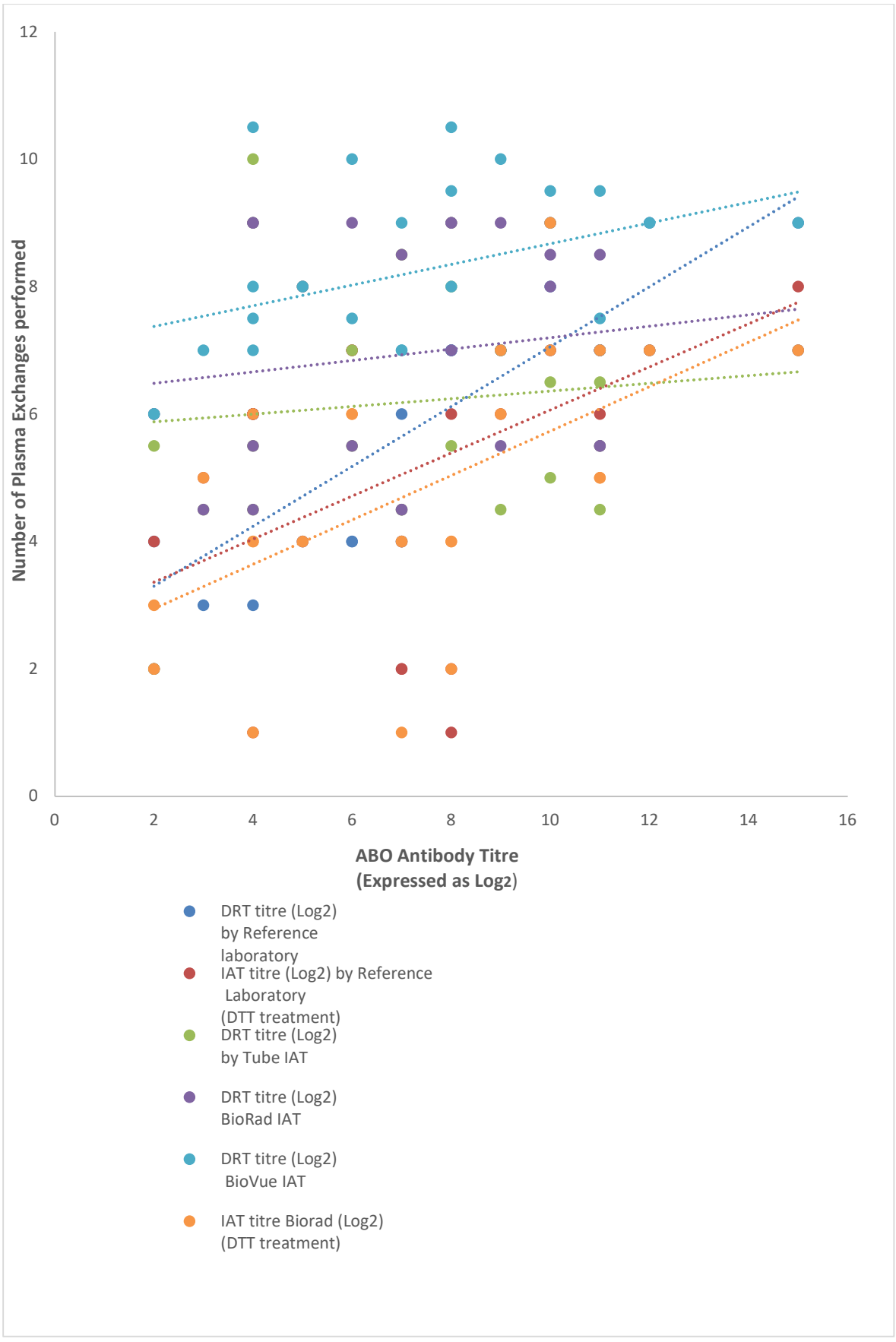


Figure 22 A comparison of the number of plasma exchanges against ABO antibody titres measured by different methodologies. A line of best fit was drawn for each methodology to demonstrate the correlation between Log_2 of ABO antibody titres and number of plasma exchanges. DRT titre showed the highest correlation. Table 25 summarises the levels of correlation.

By inspection, the IAT titre by the reference laboratory method and that by the Bio-Rad method with DTT treatment showed comparable correlation with the associated number of plasma exchanges. DRT titre by tube IAT and DRT by Bio-Rad IAT also showed a similar trend of weak but positive correlation with the number of plasma exchanges.

DRT titre by BioVue IAT method had the highest overall number of plasma exchanges while the Bio-Rad IAT (with DTT treatment) had the lowest. The correlation and significance values for each methodology are shown in Table 25 below.

Table 25 Correlation between Number of Plasma Exchanges and the antibody titre measured by each titration method

Titre obtained by each titration methodology against the Number of Plasma Exchanges	Correlation Coefficient (r)	Approximate significance P value
DRT titre by Reference laboratory	0.826	0.001
IAT titre by Reference Laboratory (DTT treatment)	0.489	0.013
DRT titre by Tube IAT	0.109	0.604
DRT titre Bio-Rad IAT	0.175	0.402
DRT titre BioVue IAT	0.401	0.047
IAT titre Bio-Rad (DTT treatment)	0.574	0.003

There was strong positive correlation between the number of plasma exchanges and the DRT titre measured by the reference laboratory (r=0.826, p=0.001) and moderate correlation when Bio-Rad IAT with DTT treatment was used (r=0.574, p=0.003).

2.14 Implications of the differences in titration methodologies and Plasma Exchange in this study

As the only meaningful linear correlation with number of plasma exchanges was found between DRT titres measured by the Reference laboratory method and Bio-Rad IAT method with DTT treatment, predictions using regression equations could only be performed for these 2 methods.

The table below shows a comparison between the number of plasma exchanges that were performed as informed by titration results from the reference laboratory method (DRT titres) and the predicted plasma exchanges if the IAT method by Bio-Rad (with DTT treatment) method was used.

Table 26 Number of Plasma exchanges as determined by antibody titre according to each titration methodology

DRT titre (Log2) by Reference laboratory	Number of Plasma Exchanges as determined by the Reference laboratory method	IgG titre (Log2) (DTT treatment) by Bio-Rad	Number of Plasma Exchanges as determined by the Bio-Rad IAT method with DTT treatment
8	10	9	6
7	11	5	4
7	9	7	5
4	7	1	3
4	6	6	4
7	8	4	4
4	2	2	3
7	8	2	3
6	7	4	4
7	11	7	5
9	15	7	5
7	8	4	4
4	5	4	4
6	4	4	4
7	9	6	4
3	3	5	4
3	4	6	4
9	12	7	5
7	10	7	5
7	11	5	4
6	4	4	4
6	4	1	3
2	2	3	3
4	7	4	4
4	6	6	4

The mean number of plasma exchanges by DRT titre Reference laboratory method and by Bio-Rad IAT with DTT treatment was 7.320 and 4.080 respectively.

A paired t-test showed that there was a significant difference between the plasma exchanges measured by the 2 methods ($p<0.005$).

These results demonstrate that if the Bio-Rad IAT method had been used to guide pre-transplantation decision-making then significantly less plasma exchange cycles would have been required compared to the Reference laboratory DRT method.

2.16 Discussion

Comparison across IAT and DAT methods: The pilot study demonstrated good reproducibility and hence reliability of each of the selected titration methods through the pilot study. A major advantage of this study was that all titrations were carried out by one operator and hence no inter-operator variation considerations were necessary. This is one of the main issues which currently makes inter-laboratory comparison of titres unideal. However, despite one operator using all 6 methods, there was a wide variation in the results obtained on a set of 10 samples tested during the pilot study which suggested that there were other factors inherent in each method that resulted in the observed variation. This further confirmed this difference in results as discussed in Section 1.15.1. The variation in titres in the 10-sample pilot study informed the calculation of an ideal sample size of 50. This proved to be complex as there had not been any prior studies that looked at 6 titration methodologies with no agreed gold standard for comparison. Therefore, the calculation involved pairwise comparison of the 6 methods to determine level of variation within and across tests. This study may have set precedence for other similar subsequent studies in the area of antibody titration.

Participation of this study in the UK NEQAS ABO antibody titration pilot exercise further validated the reliability of the methodologies used as the results were either identical or within 1 dilution (or titre step) of the method median.

The pilot study showed that there was a significant difference between the DRT methods except between Bio-Rad DRT and BioVue DRT.

There was also an overall statistically significant difference between the IAT methods used in the pilot study except between Tube IAT and Bio-Rad IAT (p=0.316). An overall assessment across all methodologies (both IAT and DRT) in the pilot revealed a significant difference (p=0.026) with the following agreements between methods upon pairwise investigation: Tube IAT/Tube DRT, Tube IAT/Bio-Rad DRT, Tube IAT/BioVue IAT, Tube DRT/BioVue IAT, Bio-Rad IAT/BioVue IAT, Bio-Rad IAT/BioVue DRT, BioVue DRT/BioVue IAT.

However, when all study samples were considered, using multivariate analysis overall significant difference across all methods (IAT and DRT) was observed (p=0.000) as seen in the pilot. However, upon pairwise comparisons, the only pairs of methods that showed agreement were Tube IAT/Tube DRT, Tube DRT/Bio-Rad IAT and Bio-Rad DRT/BioVue IAT. The differences in the results between pilot and full study was most likely due to the differences in the sample sizes, however, agreement between these methods out of all the possible pairs of methods shows that only methods within each pair can be used interchangeably and not across pairs.

The pairs of methods in agreement seem to be IAT against DRT each. Ideally, the agreement should have been between techniques that use the same methodology as in general IAT techniques detect IgG and very little IgM antibodies compared to DRT techniques which seem to detect more of the IgM than IgG.

A study at John Hopkins hospital(124) reported that tube DRT ABO antibody titres were not necessary and do not offer any more data pertinent to clinical decision-making than IAT titres and when they compared DRT titres to gel card IAT they found that 86% of the results were identical. They concluded that the DRT method and IAT by gel card method were comparable, but the gel card method reduced turnaround times through reduced incubation times and performing the titrations in batches(124). However, in this study, there was no overall comparability across all titration methodologies although there were individual pairs of methodologies that demonstrated a level of agreement.

Other studies that looked at only 2 methodologies *e.g.* Cho *et al*(125) reported that column agglutination technology was more sensitive compared to the tube technique. However, they used the immediate spin technique for their tube method which does not have an incubation step instead of the more sensitive 30 min room temperature incubation version, so this may have affected the sensitivity of their tube method.

It is also important to note that with card agglutination techniques that utilise glass beads *e.g.* in the case of the BioVue method, there is a tendency to obtain higher ABO antibody titres (101). This was noted initially in the pilot study results whereby the average titre obtained by the BioVue IAT method was higher. Tanabe(104) observed that bead column agglutination method demonstrated worse reproducibility with high maximum titre than gel column agglutination method and also that serum to cell ratio is slightly higher in column agglutination methods using glass beads. Serum -cell ratios are determined and proposed by manufacturers and are different depending on column ingredient, this information is essential so that a distinction is made between methods that use different specifications when comparing target levels of ABO antibody titres.

It may be argued that in cases where a standard method has been found, a method that reasonably overestimates titres is preferable as plasma exchange can then be carried out accordingly, unlike when a method underestimates and patients are possibly underprepared for transplant.

Lack of consensus on the definition of the reaction grade for the titration endpoint has been another source of variation in ABO antibody titration surveys. In this study a 1+ agglutination reaction was used as the endpoint because of its unambiguity compared to a weaker (w+) reaction which is less discernible and subjective between different operators.

Use of different definitions of endpoints makes comparisons across studies difficult e.g. Cho *et al* (125) used a 'w+' endpoint instead of the '1+ grade used by Kang's study (101) and this clearly causes disparity in results obtained.

Some previous studies have reported that the titres obtained with gel column IAT are comparable to those obtained with tube IAT(124). In Kang's study (101), they looked at 3 methods without DTT treatment which showed different detection capabilities of the IgG type of ABO antibody according to the ABO group.

Card agglutination technique has been proven to reduce the inter-laboratory variations in ABO antibody titres as well as the turnaround times. However, given the requirement of expensive reagents and the financial limitations, its use as a routine method is limited in some countries.

It is also important to note that the monospecific antihuman (AHG) used in the gel card technique could cross-react with IgA and IgM isotypes as well as with complement components.

Hence the tube or gel techniques used with the AHG can potentially overestimate the IgG type of ABO antibody compared to flow cytometry that uses an isotype specific antibody.

Lack of overall agreement between methods suggests that, for standardisation purposes, the proposed method has to be chosen from the identified pairs in agreement.

The study showed significant differences in the results obtained by different titration methodologies on the same set of samples. These methodologies are in current use across the world and being utilised to inform patient management regarding pre- and post-ABOiRTx treatment.

DTT treatment: Shirey et *al* (124) reported that ABO antibody titres measured using AHG are critical for clinical management.

However, the IgM isotype of ABO antibody can interfere in the detection of the IgG type of ABO antibody when DTT treatment of plasma is not used. This mainly affects blood groups A and B which mostly consist of the IgM type of ABO antibody. DTT treatment is needed to measure the exact IgG titration. However, the process of DTT treatment is cumbersome, time-consuming, and may even destroy the IgG. According to the ‘uniform procedure’ for ABO antibody titration suggested by the College of American Pathologists, the DRT tube test can be converted to the AHG phase after 30 mins of RT incubation without DTT treatment. Therefore, if DTT has not been used for titration, of the IgG type of ABO antibody, results need to be reported as a measure of ‘total antibody’ and not IgG. Alternatively, the gel card test is better in detecting the IgG type of ABO antibody and may be used instead.

In Kang *et al's* study(101) however, three methods using sera without DTT treatment showed different detection capabilities of the IgG type of ABO antibody according to the ABO blood group. Therefore, caution should be exercised when interpreting the detection of the ABO antibodies.

Standardisation of ABO antibody titration methodologies: ABO antibody titration by haemagglutination is a very simple, convenient and accessible method which, if all variables affecting this technique are appropriately controlled, it would be easier to compare, transfer patients across institutions and also perform metanalyses. Due to significant inter-laboratory variations in widely used tests for ABO antibody titre measurement, the use of card agglutination techniques has increased in recent years. However, some laboratories still use the tube haemagglutination technique and a few have begun to use flow cytometry.

From Fig16, the 2 most popular titration methods "DRT standard" and "IAT standard", would be most ideal for standardisation.

Unfortunately, standardisation of antibody titres has continued to be elusive with proficiency testing still demonstrating large variations in titration results. By using a detailed procedure and a weak positive endpoint (+w), the College of American Pathologists was able to reduce the interlaboratory variation (106). They also concluded that using a detailed and consistent laboratory method for antibody titres may render the current practice of repeating titres from a previous sample with the current sample unnecessary, because the variation of titres decreases over time. Furthermore, UKNEQAS and NIBSC are currently working together to develop lyopholised ABO antibody standards which can be used for proficiency testing and as references when setting up titrations.

The implications of not having a standardised assay include poor allograft, listing for paired exchange programmes unnecessarily and excessive desensitisation. Using a non-standardised protocol causes considerable variability in the ABO titre targets used for clinical decision-making and may play a role in poorer allograft outcomes. There is no evidence yet to demonstrate effect of titre variation on clinical outcomes(105). Kumlien *et al*(112) conducted a three-centre study whereby a standard method was compared to local results but no clinical outcomes were correlated with differences in titre values. This finding was also confirmed in the current study whereby no overall correlation was observed between graft outcome and the different types of antibody titration methodologies. This correlation may be difficult to objectively investigate unless all other variables, including immunosuppression regimen, number of dialysis sessions pretransplant etc. are reasonably controlled.

The issue of A or B antigen densities on titration cells has not been investigated as a factor that might affect the antibody titration results. However, no difference in results was observed between different batches of titration cells when the same samples were tested.

This is most likely due to the use of different donors pooled together in the manufacturing of titration cells which reduces the effect of biological variation.

Baseline antibody titres: Some studies have indicated that high baseline anti-A/B IgG titre is a predictor for AMR (126) and poor long term allograft survival in ABOiRTx(78).

However, most clinicians remain focused only on the target antibody titre (*i.e.* pre-transplant titre following antibody removal) and do not take into account the differences that may be as a result of the choice of the method used. This has constituted part of the problem in ABOiRTx because antibody titre has been considered as an arbitrary term assumed to have a definitive methodology for measurement and therefore thought to be standard across all institutions.

This has resulted in differences in outcome results thus making comparisons across studies difficult and making it hard to define an upper limit of antibody level at which transplantation is high risk.

Shimmura *et al* (127) investigated if an immunosuppressive regimen involving the administration of tacrolimus, MMF and steroid, prior to transplantation would negate the influence of preoperative anti-A/B titres on graft survival. Their study indicated that the use of this regimen may result in a much better survival and eliminate the influence of preoperative antibody titres on graft outcomes. This was an important finding because the effect of baseline antibody titre will be rendered unnecessary if this finding was to be replicated in other studies. Nakagawa *et al* (128, 129) investigated the necessity of ABO antibody removal in ABOiRTx in 2 groups of patients, one did and the other did not undergo pre-transplant removal of ABO antibodies. They found no significant difference in graft outcome between the 2 groups and concluded that the most important consideration was the inhibition of *de novo* formulation of ABO antibodies rather than removal of preformed ones.

However, due to different immunosuppressive regimens across institutions, it is difficult to make comparisons and confirm this finding without carrying taking into consideration all the immunosuppressive regimens used by different groups or rather without standardising treatment modalities across all ABOiKTx groups.

In the current study, the results showed that the number of plasma exchanges was strongly correlated to the baseline (maximum pre-transplant titre).
Therefore, accurate measurement of the baseline titre is critical to ensure the correct number of plasma exchange cycles are carried out.

The results in Section 2.14 showed that if the Bio-Rad method with DTT treatment used in this study had been used to guide plasma exchange, then significantly less plasma cycles would have been carried out compared to when the Reference laboratory DRT method was used at the time of transplant. This comparison between these 2 methods was made because they were the only methods that showed strong linear correlation to plasma exchange. Therefore, if any of the titration methods, currently in use in different institutions, will result in significantly different decisions with regards to PE then it is difficult to identify which one is ideal in the absence of a gold standard method.

AMR and antibody titre: AMR may occur even if the pre-treatment level of ABO antibody is low. Although it can occur with any pre-transplant level, AMR is more likely when the ABO titre is greater than 1/256(107-109). In this study, no statistically significant difference was observed between 'High'/ 'Low' ABO antibody levels and graft survival when Reference laboratory IgG, study tube IAT and study Bio-Rad IAT were used ($p>0.05$). The only statistically significant difference in survival was noted between the 2 antibody categories and graft survival when study BioVue IAT method was used ($p=0.025$).

Overall, this might suggest that baseline ABO antibody titre is not associated with graft outcome or that, even if there may be an association, it is confounded by the antibody depletion interventions used pre- and post-transplant. Other groups like that of Montgomery (67, 70) at Jones Hopkins, used the pre-transplant baseline antibody level to decide on initiation of post rather than pre-transplant plasma pheresis. This was in contrast to other studies(130) who hold the view that an on-demand strategy for plasma pheresis guided by post antibody titres is ideal.

2.17 **Conclusion**

Overall, the 6 ABO antibody titration methods in this study showed an overall statistically significant difference using ANOVA multivariate analysis although upon pairwise analysis association between the following pairs of methods were observed: Tube IAT/Tube DRT, Tube DRT/Bio-Rad IAT and Bio-Rad DRT/BioVue IAT. The absence of overall agreement between the titration methodologies meant that the methods cannot be used interchangeably. However, since in general, the pairs that showed agreement upon pairwise investigation seemed to be all combinations of IAT and DRT methodologies, a proposal to select column agglutination can be made. In this case a reasonable choice would be Bio-Rad IAT which is reproducible and reasonably accessible to most laboratories even as a manual technique. Of the 2 column IAT techniques BioVue can be discounted because of its reported tendency to overestimate titres.

DTT treatment of plasma is rather cumbersome and is more skill intensive and can easily be replaced by column agglutination which utilises anti-IgG for the measurement of IgG isotype.

With regard to ABOiRTx, the number of plasma exchanges was closely linked to baseline ABO antibody titres, therefore objective measurement is required and can be achieved through standardisation of the proposed Bio-Rad IAT method that can be refined overtime and proficiency testing implemented across all centres for comparison and performance monitoring.

No overall association was found between baseline ABO antibody titres and graft outcome. This was most likely due to the possible confounding use of plasma exchange and immunosuppression.

Chapter Three: Measurement of IgG Subclasses

3.0 Measurement of ABO antibody IgG subclasses

Antibody titration described in Section 2 using haemagglutination methodologies does not discriminate subtype specific ABO antibodies and provides limited information on antibody isotypes or subclasses with respect to ABOiRTx.

This part of the study is aimed at developing an ELISA (Enzyme Linked Immunosorbent Assay) method for the determination of IgG subclass profiles in order to correlate them to their respective clinical outcomes *i.e*. graft rejection or survival. In order to enhance the detection capability in the current study as frozen sample volumes were a limiting factor, the 'indirect' as opposed to 'direct' ELISA technique was used. The following sections describe how this Indirect ELISA technique was developed for the measurement of ABO antibody IgG subclasses.

3.1 Developing the ELISA technique for measurement of ABO subclasses

An Indirect ELISA was developed in this study for the measurement of ABO IgG antibody subclasses. In this technique, micro-titre plates are incubated with antigens of interest, washed and blocked with Human Serum Albumin (HSA) to reduce non-specific binding of subsequent antibodies to the micro-titre plate. Samples suspected of having the antibody of interest (primary antibody) are added, washed and then followed by the addition of secondary antibody conjugated with enzyme. A substrate is then added which is catalysed by the enzyme on the secondary conjugate to produce a chromogenic signal which can then be detected either quantitatively or qualitatively.

This principle was used in developing the Indirect ELISA technique in this study and this involved coating polystyrene microtitre plates with blood group A or B neoglycoproteins (NGPs) conjugated to HSA *i.e.* A-HSA and B-HSA and optimising the concentrations of patients' plasma, primary antibody and secondary conjugate. The method was modified from a similar procedure used at the Leslie Brent Laboratory in a separate study which used a direct ELISA technique with no secondary conjugate.

The basic principle is illustrated in Figure 23.

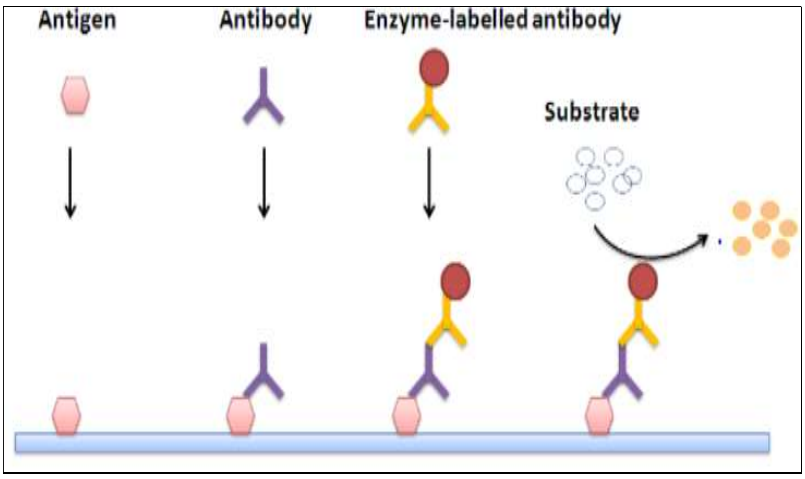


Figure 23 Illustration of Indirect ELISA technique

Antigen is immobilised onto a microtiter plate and samples suspected of having antibody of interest are added. Plate is then washed and a secondary antibody conjugated to an enzyme added followed by the substrate which is then catalysed by the enzyme to produce a chromogenic signal. The signal is then detected and measured.

Section 3.1 below shows details of the procedure and reagents used for the indirect ELISA developed for this study.

3.2 Indirect ELISA protocol

The ELISA technique for each subclass as optimised in Section 3.3 above was then used for measuring ABO IgG subclass levels. All samples were assayed in duplicate and the results obtained for each pair of microtiter plate were averaged.

A-HSA and B-HSA (Dextra Laboratories *Ltd*) were diluted in 0.1 M bicarbonate buffer, PH 9.6 (4.24g Na_2CO_3, 5.04g $NaHCO_3$) [“Coating Buffer”] to a concentration of 10µg/ml. (Human serum albumin) HSA was also diluted in 0.1M bicarbonate buffer, PH 9.6, to a concentration of 10µg/ml.

50µl of A-HSA, B-HSA and HSA were added to appropriate wells in the ELISA plates (Polysorb, Nunc). The ELISA plates were incubated overnight at 4°C. Following incubation, the ELISA plates were washed 4 times with phosphate buffered saline containing 0.05% v/v Tween 20 (PBS-$T_{0.05\%}$: 0.012M Na_2HPO_4, 0.003M NaH_2PO_4, 0.15M NaCl made up to 1 litre with dH_2O and adjusted to PH 7).

50µl of each sample was added to appropriate wells and the ELISA plates incubated at room temperature for 45 min-1 h. The ELISA plates were then washed 4 times with PBS-$T_{0.05\%}$. Rabbit antihuman globulin (RAH)-IgG1, RAH -IgG2, RAH -IgG3 and RAH-IgG4 (Sigma-Aldrich) were diluted (as optimised in Section 3.3) with PBS-$T_{0.05\%}$ and 50µl of each subclass specific antibody was added to appropriate wells. The ELISA plates were then incubated at RT for a further 1h, after which the plates were washed 4 times with PBS-$T_{0.05\%}$. 50µl of mouse antirabbit globulin conjugated to horse radish peroxidase (HRP) (Sigma-Aldrich) diluted with PBS working buffer, PH 7.4 (16.7g Na_2HPO_4, 5.70g NaH_2PO_4) (as optimised in Section 3.3) as added to all wells and incubated for a further 30 minutes followed by washing 4 times with PBS-$T_{0.05\%}$.

To develop the assay, Orthophenylene diamine (OPD) substrate (Sigma-Aldrich) was prepared using SigmaFast kit (Sigma-Aldrich) by dissolving 1 tablet of OPD and 1 of H_2O_2 in 20ml of de-ionised water. 50µl of prepared substrate was added to each well and the reaction allowed to develop for 5-10 minutes. The reaction was then stopped by the addition of 50µl of 0.5M H_2SO_4 (BDH) to each well.

Absorbance measurements (Optical density-OD) were determined at 492nm using a (Anthos Labtec Instruments, Anthos 2000) plate reader. (This is procedure is illustrated in Fig 23)

It is important to confidently define a negative value in a chosen assay. A positive optical density (OD) on an ELISA readout includes binding to the specific antigen, binding to HSA, additional non-specific binding and the OD of the ELISA plate. In the NGP ELISA it an essential initial step to subtract the background values obtained from either binding to HSA or the OD obtained from the binding to the neoglycoprotein. In the neoglycoprotein (NGP) ELISA a sample was considered negative when the mean background OD +3 SD (Standard deviation) of HSA subtracted from the OD obtained against the neoglycoprotein was zero or less *i.e.*

(mean NGP OD)- (mean OD HSA +3SD) = “True OD” of antibody binding.

To obtain the OD HSA+3SD ELISA plates were coated with HSA and “normal” background binding established by incubating sera from several individuals in duplicate and establishing the mean background HSA and mean and mean SD. To establish background binding for each antibody isotype (IgG1, IgG2, IgG3 and IgG4), ELISA plates were coated with HSA at 10µg/ml. Sera from a number of individuals was used as shown for one patient below:
Individual 1: serum was applied to 2x24 wells on HSA-coated ELISA plates and incubated as described above with RAH-IgG1/HRP, RAH-IgG2/HRP, RAH-IgG3/HRP and IgG4/HRP. For each plate and for each individual, 12 duplicate pairs of readings for each HSA duplicate were obtained. For each HSA pair, the mean OD was obtained, giving in total 12 mean ODs (A).The mean (X) of these 12 means (A) and the SD of these 12 means (Y) was obtained. This process was performed on each of the study patients used and for each antibody class.

Once all readings were obtained, the mean SD (Z) of the mean SDs (Y) was obtained. This was the value that was then used in the NGP ELISA.

When an NGP ELISA was performed, 4 readings were obtained for each individual – 2 neoglycoprotein ODs and 2 HSA ODs. The mean OD for each duplicate was obtained. If the conjugates were conjugated to HSA and IgG was being investigated, the mean OD obtained for HSA was then multiplied by 0.075 to obtain the HSA SD. The value was multiplied by 3 to give a final value that was then subtracted from the mean neoglycoprotein OD. This was performed for all study subjects.

3.3 Optimisation of the ELISA technique

During the development of the ELISA methodology for the detection of the ABO IgG antibody subclasses, reagent volumes and concentrations had to be optimised to ensure detectability and cost effectiveness of the procedure. For this purpose, the checkerboard technique (CBT) method was used, and the optimisation process involved testing different dilutions of the reagents and plasma used in order to determine the optimal concentrations using the least amount of reagent and plasma but yielding a signal strong enough to be detected.

The need to use the optimal amount of antigen coated wells that will successfully bind to antibodies, which in turn can be detected with an optimal amount of anti-species conjugate. In the CBT method, only 2 variables can be titrated in one assay and the principal considerations are:

- That enough antigen is available for antibody binding so as to prevent wastage through adding concentrations of antigen that are too high, thus causing wells to receive a large excess of antigen compared with the amount needed to fill available plastic sites.

- That the amount of the conjugate is optimised to avoid high non-specific backgrounds and to allow the detection of all bound antibody molecules to give the required analytical sensitivity expected.

Figure 24 shows an illustration of the CBT method whereby dilutions of antigen and serum containing antibodies are tested against a pre-titrated secondary conjugate.

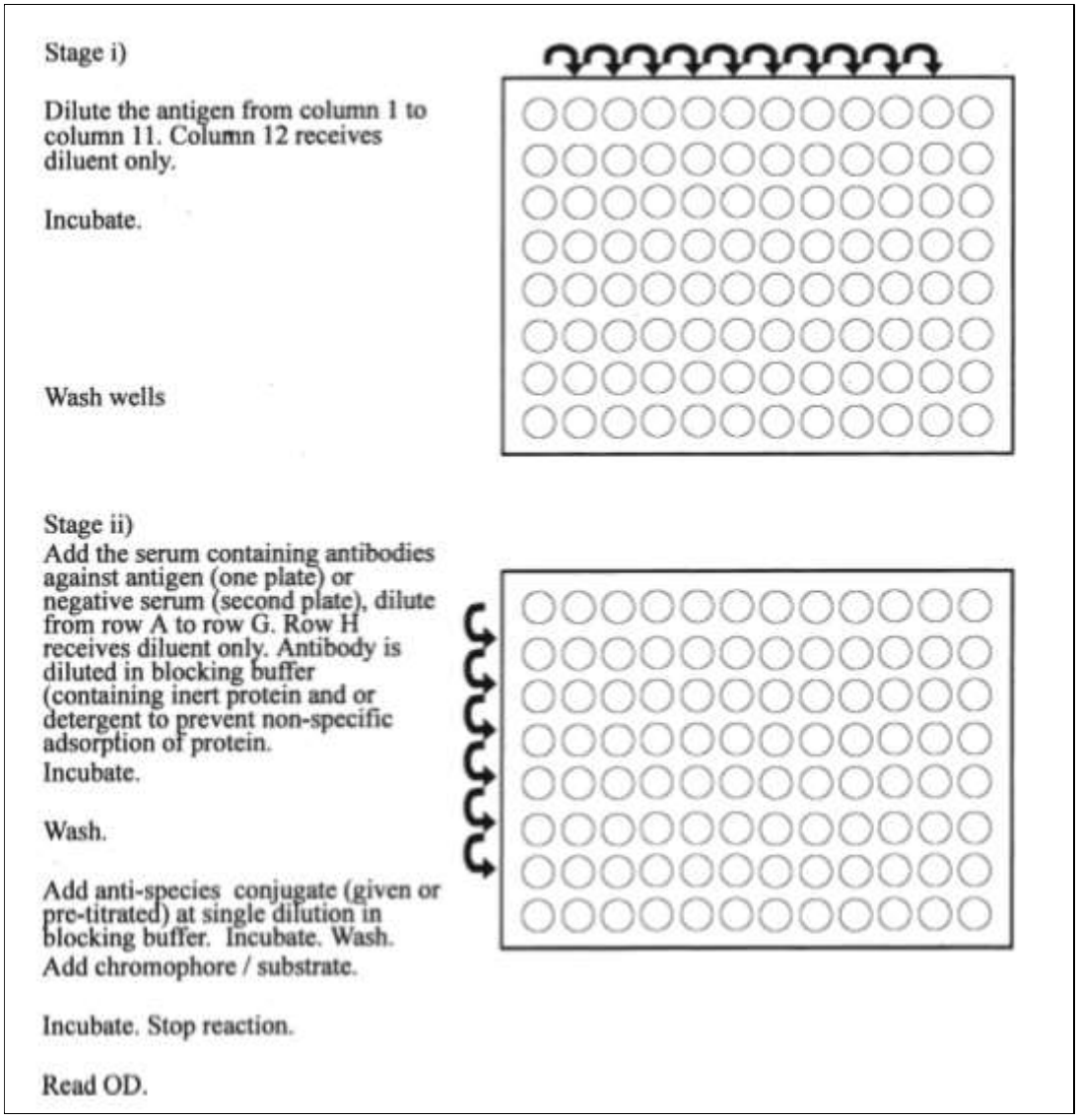


Figure *24* Illustration showing a basic example of the Checkerboard technique for ELISA Optimisation.

Optimisation also assesses the effect of diluting negative sera on an assay to establish the background readings of such antisera at various dilutions.

As part of the optimisation process, different concentrations of plasma were prepared in PBS working buffer and different concentrations of both the primary antibody and secondary conjugate were prepared.

The optimisation process was carried out for all 4 IgG subclasses and the results are shown in Figs 23-26 incl.

3.3.1 ELISA Optimisation for the measurement of IgG1 subclass

Different dilutions of Rabbit antihuman globulin IgG1 (RAH)-IgG1 were set up against dilutions of pooled plasma and a set dilution of the secondary conjugate at 1 in 5000 dilution.

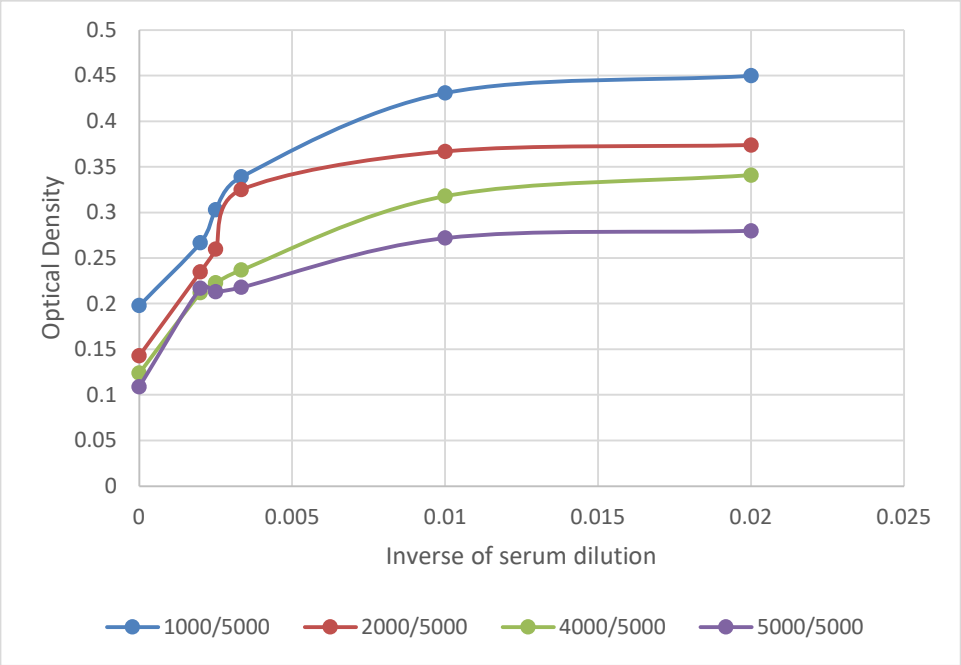


Figure 23 ELISA Optimisation for IgG1 subclass measurement (Different dilutions of anti-IgG1 and serum but secondary conjugate at 1 in 5000)

From these results, the ideal dilutions of reagents for the IgG1 subclass ELISA were as follows: Plasma (0.01=1 in 100), anti-IgG1 (1 in 2000) and secondary conjugate (RAH-IgG/HRP) (1 in 5000)

3.3.2 ELISA Optimisation for the measurement of IgG2 subclass

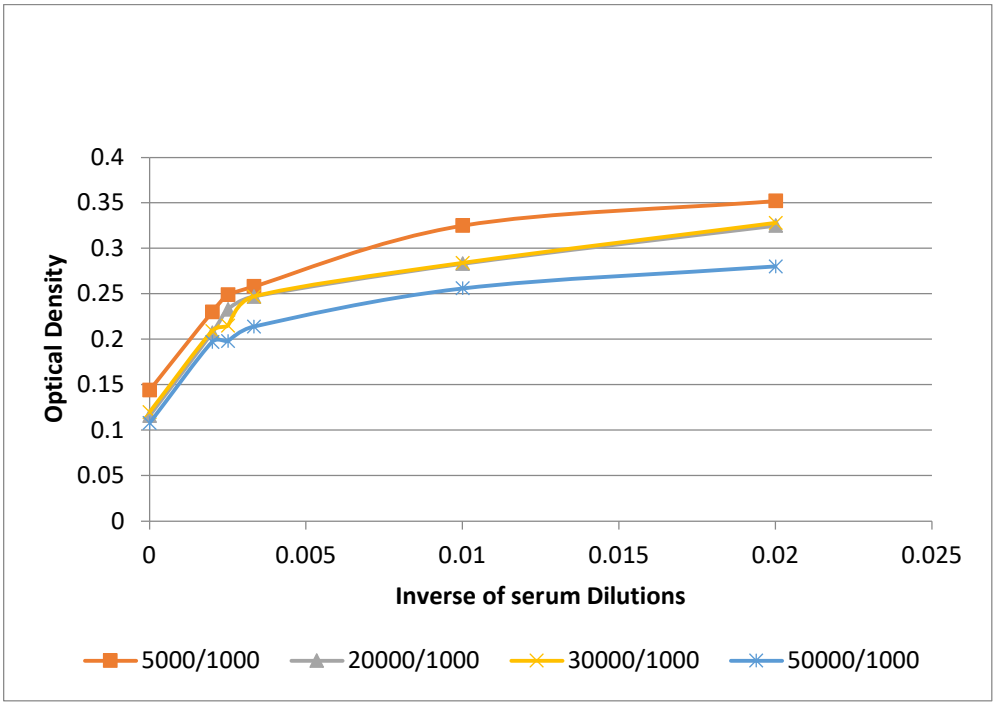


Figure 24 ELISA Optimisation for IgG2 subclass (Different dilutions of anti-IgG2 and serum against secondary anti-IgG at 1 in 1000 dilution)

The selected ideal dilutions of reagents for the IgG2 subclass ELISA were as follows: Plasma (0.01=1 in 100), anti-IgG2 (1 in 20000) and secondary conjugate (RAH-IgG/HRP) (1 in 1000).

3.3.3 ELISA Optimisation for the measurement of IgG3 subclass

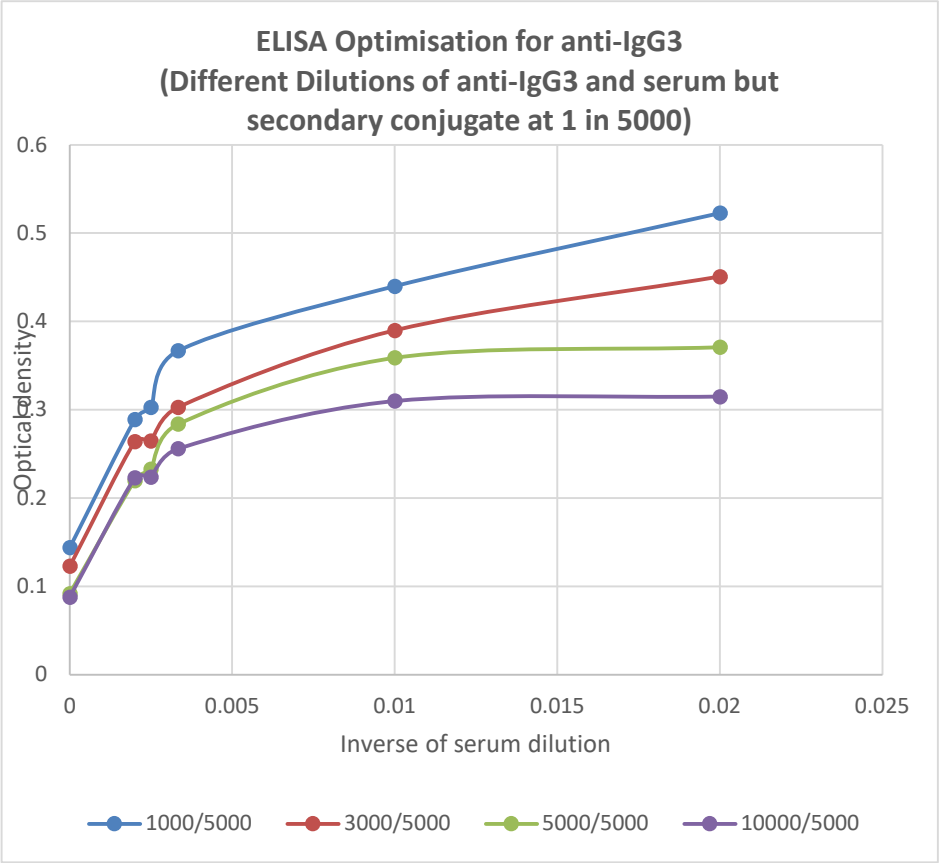


Figure 25 ELISA Optimisation for IgG3 subclass

The selected ideal dilutions of reagents for the IgG3 subclass ELISA were as follows: Plasma (0.01=1 in 100), anti-IgG3 (1 in 5000) and secondary conjugate (RAH-IgG/HRP) (1 in 5000).

3.3.4 ELISA Optimisation for the measurement of IgG4 subclass

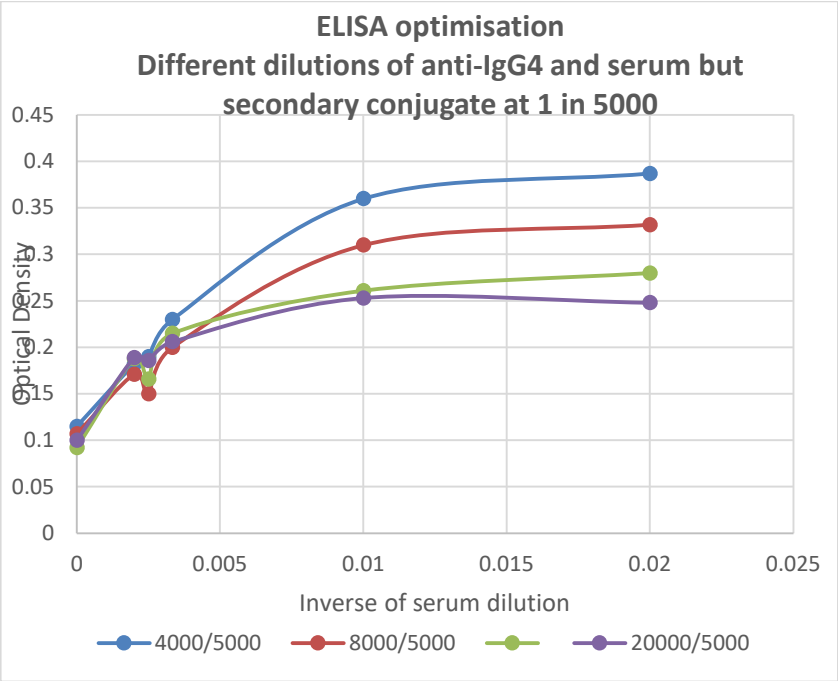


Figure 26 ELISA Optimisation for IgG4 subclass

The selected ideal dilutions of reagents for the IgG4 subclass ELISA were as follows: Plasma (0.01=1 in 100), anti-IgG2 (1 in 8000) and secondary conjugate (RAH-IgG/HRP) (1 in 5000).

Once all the optimisations were completed, the respective dilutions of antibodies were then applied to the method, as detailed previously in Section 3.2.

3.4 Testing patient samples for IgG subclasses

Once the method was established, the ABOiRTx patient samples were then tested for IgG antibody subclasses. Only 9 out of the 30 study samples had sufficient plasma to carry out this second part of the study. The results obtained are presented in Table 27.

Table 27 ABO IgG antibody subclass status against ABO blood group and Transplant outcome on 9 study samples

	IgG antibody Subclass					
Case Number	**IgG1**	**IgG2**	**IgG3**	**IgG4**	**Recipient's Blood group**	**Graft Outcome**
1	Pos	Pos	Neg	Neg	O	Rejected
2	Pos	Pos	Pos	Neg	O	Rejected
3	Pos	Pos	Pos	Neg	B	Rejected
4	Pos	Pos	Neg	Neg	A	Censored
5	Neg	Pos	Pos	Neg	A	Censored
6	Neg	Pos	Neg	Neg	B	Censored
7	Neg	Pos	Neg	Neg	B	Censored
8	Pos	Neg	Pos	Neg	O	Rejected
9	Pos	Pos	Pos	Pos	O	Rejected

The results show that 5/9 patients whose samples were tested demonstrated graft rejection. In all cases of graft rejection, 4/5 of recipients had both IgG1 and IgG 2 subclasses. In fact, IgG1 and IgG2 subclasses were detected in 5/9 of the patients and, of these, 4 experienced graft rejection. The 5th patient to undergo rejection tested for IgG1 and IgG3 only. Only in 1 case of the 4 patients that did not experience rejection (censored) were both IgG1 and IgG2 subclasses detected and these subclasses these were not found in samples from the remaining 3 patients that did not experience rejection.

IgG4 subclass was only detected in 1/9 patient samples and this patient also experienced graft rejection. Furthermore, this patient tested positive for all subclasses of IgG (IgG 1,2 and 3).

IgG3 was detected in 4/5 of the graft rejection cases but in only 1/4 of the censored cases.

A Chi-Square test was used to check for any association between graft outcome and ABO IgG subclass. Due to the small sample size used, expected frequencies in the Chi-Square contingency table were less than 5, therefore Fisher's Exact test version of Chi-Square was used. The results are shown in Table 28.

Table 28 Significance levels of ABO antibody IgG Subclass status against transplant outcome of 9 study samples

IgG Subclass against graft outcome	Test statistics and significance
IgG1	χ^2 (1) =5.625, p=0.048
IgG2	χ^2 (1) =0.900, p=1.000
IgG3	χ^2 (1) =2.723, p=0.206
IgG4	χ^2 (1) =2.057, p=0.358

There was no association between IgG2, IgG3 and IgG4 subclasses and graft outcome. The only association with graft outcome was noted with the IgG1 subclass.

No association was found between IgG subclass and ABO blood group of the recipients as shown in Table 29.

Table 29 Significance levels of ABO IgG antibody subclass status against respective ABO blood groups on 9 study samples

IgG Subclass against ABO blood groups (A, B and O)	Test statistics and significance
IgG1	χ^2 (2) =2.625, p=0.269
IgG2	χ^2 (2) =1.406, p=0.495
IgG3	χ^2 (2) =1.238, p=0.539
IgG4	χ^2 (2) =5.143, p=0.273

However, the sample size for this part of the study was small and hence statistical power is too low to draw conclusive deductions on this association. The study investigations were limited to the available volumes of frozen plasma samples.

3.5 Discussion

The results in this study showed that in all cases of graft rejection investigated, 4 out of the 9 recipients had both IgG1 and IgG 2 subclasses. This corresponds well with Lee *et al*'s study(114) where they found that, using flow cytometry, the baseline level IgG1 and 2 were the predominant subclasses in ABOiRTx. However, another study has shown that IgG2 is predominant in cases of ABO incompatibility rather than IgG3 (117).

There was no association between IgG2, IgG3, IgG4 subclasses (as individual entities) and graft outcome. The only association with graft outcome was noted with the IgG1 subclass disregarding presence of the other subclasses.

IgG2 was detected in 4/4 of the patients who did not experience graft rejection. This may support the hypothesis put forward by Kirk *et al*(85) who suggested that accommodation of graft is related to a shift within the IgG isotype to the IgG2 subclass which is considered less efficient at activating complement. This subclass competitively inhibits the binding of the other subclasses known to be more cytotoxic. This concept can only hold true if this antibody is consistently present pre- and post-transplant and must demonstrate some high level of resistance to plasma exchange.

IgG3 was detected in 4 of the graft rejection cases and in 1 of the censored cases. This subclass is thought to be the one mainly responsible for increased risk of HAR as it is highly effective at activating complement. However, the sample size was very small to make a substantive conclusion regarding this subclass and graft survival although this subclass was found in 4 out of 5 patients who experienced graft rejection in this study.

IgG4 is normally present in the least concentration in humans and increases are normally associated with chronic antigenic stimulation (131, 132). This isotype was detected in only 1 out of the 9 cases in this study. This could be because no antigenic stimulation had been introduced yet through graft transplant and therefore the levels were still low pre-transplant. It is also interesting that this was in a patient with rejection which might suggest that the IgG4 subclass level may have increased after transplantation and was possibly involved in graft rejection. However, it is also possible that the patient may have had other comorbid factors that may have resulted in graft rejection.

This brings about the question of the relevance of testing subclasses pre-transplant when the patient will receive immunosuppression to suppress the effects (if any) of these preformed antibodies. It may be more useful to look at the ABO IgG antibody subclass profile post-transplant in order to monitor for any changes that might be suggestive of graft rejection activity.

When considering blood group specificity, no association was found between ABO IgG subclass and ABO group, however, the sample size was too small to make a generalised conclusion due to lack of fair representation across all ABO blood groups.

The sample size for testing with the IgG subclass ELISA was limited by the available sample volumes as the study relied on historical samples that were frozen and had been kept for many years. Therefore, out of the 30 ABOiKTx samples, only 9 had sufficient volume to perform both antibody titration and IgG subclass investigation. A prospective study will therefore be ideal as samples and relevant study information can be collected contemporaneously.

Apart from sample adequacy, sample integrity in terms of IgG subclass activity after a prolonged period in frozen state could not verified as no measurements had been conducted pre-transplant so there was no basis for comparison.

Developing an ELISA is a process is quite cumbersome and requires skill and experience and therefore may not be suitable for a routine Blood Transfusion laboratory. Several runs with different dilutions of reagents and plasma were performed to achieve reasonable optimisation before the final method for each isotype was decided upon.

3.6 Conclusion

The aim of this part of the study was to develop an ELISA method that would determine the presence or absence of IgG subclasses in renal transplant patients and determine if there was any association with graft outcome

The objectives of this section of the study were achieved but the only drawback was the number of samples available for ELISA testing. However, the indirect ELISA technique developed in this study can be used in other prospective studies with larger sample sizes for more statistical power.

Nevertheless, based on this sample size, it was discovered that most of the cases that experienced graft rejection had a combination of IgG1 and IgG2 subclasses present.

No association was found between ABO IgG2, IgG3 and IgG4 as individual entities and graft outcome. IgG1 was the only antibody that demonstrated association with graft outcome.

IgG2 was detected in 4 out of 9 cases who did not experience graft rejection which suggests a confirmation of the protective nature of this subclass through reduced effectiveness in activating complement.

IgG3 was detected in 4/5 out of the graft rejection cases which also confirms one of its features which is efficiency in activating complement.

IgG4 was detected in 1 out of the 9 cases. This subclass is usually found only in low quantities in healthy individuals. However, the sensitivity of the ELISA methodology used in this study may be queried in light of this result.

Indirect ELISA is considered to be a very sensitive method and therefore able to detect the intended analyte once test has been developed and optimised. In this study, the ELISA methodology for detecting IgG4 was tested against a pooled plasma sample and was proven to be able to detect this subclass.

No association was found between the ABO IgG subclasses and patients' blood groups, however, a larger sample size is required to further establish this finding.

3.7 Future Work

Flow cytometry and ELISA

It is evident that although haemagglutination methods for quantifying ABO antibodies are ideal in terms of accessibility, cost and simplicity, they are not easy to standardise. It may take a very long time before a standard method is agreed upon and proficiency surveys conducted to monitor performance across centres.

Techniques like flow cytometry and ELISA, though expensive and therefore out of reach of many laboratories, may provide an ideal opportunity for standardisation. Flow cytometry can be used to measure ABO antibodies through binding to natural ABO antigen on the red cells and offers more specificity and sensitivity. This may be an advantage over ELISA which utilises synthetic immobilised ABO antigens.

Both methods can be used to investigate the binding of different anti-A/B isotypes and subclasses through the use of specific secondary antibody.

The dynamics of ABO IgG antibody subclasses need to be investigated further (using larger sample sizes) in terms of pre- and post-transplantation periods while taking into account other factors like standardised immunosuppression regimens.

Therefore, if upon further studies, these methods prove to be objectively essential in the area of ABOiRTx, they may have to be performed at centralised laboratories for cost effectiveness.

Surface Plasmon Resonance (SPR)

ABO antibodies can be quantified using another uncommon method called SPR used by Kimura *et al* (133). This method measures changes of the physical properties of ABO antibody/antigen complexes of which the antigens are immobilised on a sensor chip. This technique can be instrumental in determining the antibody affinity as opposed to the just the quantity as measured by other methods. This method can therefore be utilised to predict the strength of interaction between ABO antibodies and the graft and because it is rather expensive, can be used pre-transplant to determine level of risk where pre-transplant PE may be indicated by the presence of high affinity antibodies.

ABH-glycan Microarray

Another specialised methodology was proposed by Jeyakanthan *et al* (26) which uses ABH-glycan microarray for the determination of donor-specific ABO antibodies in ABOiRTx.

This method offers the advantage of being able to provide a profile of recipient antibodies directed against ABO antigen subtypes expressed in the donated organ (*e.g.* A types -I-VI, B types I-VI and H types I-VI)(22). This type of study would be instrumental in predicting graft outcome based on the ABO-glycan profile thus enabling assessment of patients against donors. Due to the accuracy offered by this approach to the identification of pre-formed ABO antibodies to ABO antigen subtypes, it is possible to avoid unnecessary interventions through aggressive PE and immunosuppression.

Chapter Four: My journey on the DBMS and the project

The Professional review and development module in the 1st year was quite useful in setting up objectives and also identifying areas of strengths and weaknesses. The PGS development programme helped me to address my weaknesses through various courses throughout the DBMS programme. Through this system I learnt presentation, Advanced MS Word, SPSS, writing your thesis and how to publish articles etc. These are all skills that I have been using in different areas e.g. at work and other activities.

4.0 Presentations

I have had the opportunity to present my findings at different stages of the study and have received useful feedback. As a result, I have improved on my presentation skills and I now apply this experience regularly e.g. in meetings, delivering lectures or training sessions.

Class presentations and group activities during the programme were quite useful as I managed to learn from my colleagues from different disciplines. The opportunity to interact with them and share ideas and skills helped me to improve on my presentation skills particularly in articulation of ideas and dealing with questions. The group activities were instrumental in that they taught me group dynamics and how to function in a group with individuals of varying opinions and backgrounds.

4.1 Networking

Within the DBMS class and within the profession, interaction with organisations like NEQAS, Leslie Brent Laboratory, Red Cell Immunology Reference Laboratory NHSBT Colindale,

Imperial College Healthcare NHS Trust, Bio-Rad and Ortho Clinical Diagnostics, academic staff from other Universities, Renal and Haematology consultants.

This has helped me to establish a network of communication with these individuals and organisations and I can confidently draw upon these relationships in future projects or discussions. Through these networks, I have been able to acquire a vast amount of knowledge ranging from clinical to laboratory and manufacturing processes.

Working with other students within the class gave me an appreciation of the interaction between different professions within health sciences and how evidence-based approach in the disciplines is essential in the development of patient care.

4.2 Designing a method from scratch

Designing the Indirect ELISA method for the measurement of ABO IgG antibody subclasses in ABOiRTx was a challenge as this was somewhat outside my area of expertise *i.e.* Blood Transfusion where ELISAs are not routinely used, let alone designed. However, with guidance and support, I managed to design and optimise the test. In this exercise I learnt how to formulate ideas for new technologies and be able to bring them to life. In this process, I learnt to interact with other scientists from research laboratories and share ideas and resources. I also learnt how to adapt to an unfamiliar scientific field. Designing the ELISA method was a major undertaking as not many groups have looked into this are with respect to ABOiRTx.

4.3 Annual Reviews

I found annual reviews very useful as I always received constructive ideas and comments from assessors and from other students.

Reviews always kept me on track and provided me with new insights into my area of study and gave me a chance to review some of my colleagues' work as well and share ideas. Interacting with other professionals helped in cultivating confidence.

4.4 Constraints

Time has always been a major constraint as there was a great need to effectively balance this resource between family, work, studying and social life. Competing priorities in all these areas helped me to learn time management. I cannot say I managed to master this, but I can confirm that that I did my best given the challenges I encountered.

During one of my annual reviews, it was agreed that the initial plan to investigate antibody titres in ABOiRTx would not produce enough new information given the rate at which this field was moving. Therefore, it was suggested that in addition, IgG antibody subclasses should also be investigated. This meant that additional time and resources were needed. This happened at a time when I was changing jobs, which meant that the original arrangement to use the research facilities at my old workplace had to be reviewed and new resources procured. This took time but once a new arrangement was made, it meant travelling from my new workplace to the old research laboratory which now had limited access.

Starting a job as a manager at one of the largest Red Cell Immunology Laboratories in the country made time management a huge challenge as there was not much time for studying or anything else, so I resorted to using travelling time on public transport to cover various aspect of my project.

Due to all these pressures, I had to request for an extension which went a long way in helping me to put more work into the project until completion. Overall, I learnt a lot in time management, especially the aspect of using every available time to cover different concepts.

4.5 Professional review and development module

The professional Review module covered in this Professional Doctorate programme presented me with opportunity to review my strength and weaknesses in a structured manner and to learn how to enhance the identified strengths and remedy the weaknesses.

This module helped me set goals for the rest of the programme which were reviewed regularly with the supervisor and also annually at the appraisal meetings.

4.6 Advanced Statistics and Research Methodology

Covering Advanced Statistics and Research Methodology enabled me to understand different approaches to statistical analyses ranging from qualitative to quantitative methods. Although my area of study is mainly quantitative in nature but learning qualitative methods made it possible for me to understand studies that make use of these methods. In addition, this made it possible for me to be able to supervise other students in my workplace whose areas of study require qualitative methodologies.

Notably, in my own study, establishing the most appropriate statistical method for comparing the 6-antibody titration method in the absence of a gold standard was quite major undertaking involving a few statisticians.

This also meant that sample size determination was also a challenge as this type of calculation had no precedence in this field of titration where the data was not linear and therefore needed manipulation before any meaningful statistics could be applied.

Knowledge of various statistical processes through the DBMS programme has made it possible for me to engage a local journal club which is also interested in the cultivation of evidence-based practice and continuous professional development.

4.7 Application of experience and results from this study

My project in ABO incompatible renal transplantation made it possible for the Hammersmith Hospital laboratory to gain knowledge and experience in ABO incompatible renal transplantation in terms of ABO antibody titration techniques and therapeutic plasma exchange.

It has made it possible for antibody titration to be available for both ABOiRTx and other purposes like ABOi bone marrow transplants whenever required.

Sharing knowledge on ABOiRTx and ABOcRTx has increased the laboratory's expertise in this field and this has made it worthwhile as Hammersmith Hospital has one of the largest renal units in Europe

The results of this study will be shared with UK NEQAS ABOi antibody titration pilot exercise whose aim is to standardise the titration methodology for ABOi transplantation. The findings in this study will add to the body of information available to inform formulation of guidelines.

4.8 Teaching university students and training laboratory staff

As Imperial College NHS Trust is a major teaching hospital and has one of the largest Renal units in Europe, I had the opportunity to use the knowledge and expertise that I gained through the Professional Doctorate to teach other members of staff or directly apply the knowledge. I had the opportunity to be part of a team of Training Officers who devised and harmonised a functional training system across 4 hospital sites. Through the DBMS programme I managed to successfully supervise 3 MSc students, 4 Specialist portfolio students while carrying out routine training of staff in the laboratory.

I was also involved in teaching practical aspects of Blood Group Serology to Imperial College BSc students. I managed to use the Learning models learnt in my first year DBMS.

The feedback from most students was very encouraging and the teaching made it possible for most of them to understand all other theoretical aspects they were learning in their Transfusion module.

4.9 Publications

I have not had a chance to publish any journal article during the course of this programme although plans were there to do so. However, plans are still there to publish 2 papers from this thesis and the proposed titles are:

- ABO Antibody titration methodologies against renal transplant outcome
- The problem with ABO antibody titration in ABO incompatible renal transplantation

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