# Crossmatching Technique Facilitating Kidney Transplantation

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**Abstract**

Accelerated acute cellular rejection (AR) continues to be a serious problem in kidney transplantation (KT), suggesting that undetected presensitization may be encountered. The purpose of this study was to determine the most sensitive crossmatching (XM) technique to detect the preformed antibody (Ab) which may cause AR. One hundred and twenty two sera from 98 patients, on the waiting list for KT at Ramathibodi Hospital were XMed with 23 cadaveric splenic lymphocytes including 2 living related KT (LR-KT). The XM was performed by 3 different techniques namely, standard microlymphocytotoxicity test (standard NIH), antihuman globulin microlymphocytotoxicity test (AHG) and flow cytometric XM (FCXM). The XM results revealed that 8 out of 75 (10.7%) tests were negative by standard NIH, i.e., 5 tests were positive by AHG only and 1 test was positive by FCXM only and 2 tests were positive by both AHG and FCXM. In addition, the patients who had the AHG technique were not done, 5 out of 47 (10.7%) tests were also negative by standard NIH but were positive by FCXM. The sensitivity of the techniques was done by titrations of anti HLA-A2. It was found that FCXM was the most sensitive technique, followed by AHG and standard NIH, consecutively. In the retrospective study of LR-KT, case #1, the standard NIH for XM using pre-KT blood sample was negative while AHG and FCXM were strongly positive. The patient had AR at day 2 post-KT which confirmed by needle biopsy. The serum at day 11 and day 116 post-KT were tested again and were positive by the 3 techniques. Case #2, pre-KT blood sample showed negative T -XM by the 3 techniques while auto-B and B-XM were positive by standard NIH and AHG but negative by FCXM. This patient had rejection at day 16 after KT. The post-KT blood sample at day 30 showed positive auto T/B

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and T/B-XM by standard NIH and AHG whereas it was still negative by FCXM. It was also noted that Ab to donor B cell was better detected by standard NIH and AHG than FCXM.

In conclusion, FCXM is more sensitive than standard NIH and AHG, however this technique is limtted in detecting IgM T and B cell Ab. AHG technique can detect both IgG and IgM antidonor T and B cell Abs. In addition, AHG technique is more sensitive than standard NIH and does not require sophisticated equipment. AHG technique should be appropriate for routine XM, especially, in LR-KT and sensitized patients.

Kidney transplantation (KT) is the prefered treatment for most patients with end-stage renal failure. Despite the ever increasing success of renal KT, rejection of the graft remains a major problem after KT, especially in cadaveric KT suggesting that undetected presensitization may be encountered[1-4]. Incompatibility between donor and host for antigens of MHC as well as the endothelial cell antigen system or multiple minor histocompatibilities may lead to graft rejection[1,2]. Current pre-KT testing of donor-recipient compatibility using standard microlymphocytotoxicity test (standard NIH), the conventional serological technique has shown a somewhat limited capability to predict the prognosis for graft rejection after KT. Early graft loss (3-6 months) continues to be a major problem. With standard NIH crossmatch (XM), six-month graft survival was 20 per cent in recipients with positive pre-KT antibody (Ab)-dependent cellular cytotoxicity (ADCC) and 75 per cent in ADCC-negative recipient ($P<0.01$)[3]. Early efforts to improve graft survival led to the discovery that circulating antidonor Ab detected by the practical XM method[3]. In this study, two more sensitive techniques, antihuman globulin microlymphocytotoxicity test (AHG) and flow cytometric XM (FCXM), were compared with the conventional serological technique and their relation to the clinical outcome was evaluated.

## MATERIAL AND METHOD

The patient sera and cadaveric splenic lymphocytes from negative standard NIH pairs were selected for study. A total of 122 sera from 98 KT patients (1-4 sera/patient) and 23 cadaveric splenic lymphocytes XMes and 2 living-related KT (LR-KT) were studied for XM techniques by FCXM and/or AHG test[4,5,7,8]. The reaction was compared with standard NIH. Seventy-five samples were tested by the three techniques whereras the remaining samples were tested by standard NIH and FCXM. The XM and autologous control were performed in triplicate for standard NIH and AHG techniques. The positive and negative controls were run parallel with the tested samples. The results were compared among 3 techniques related to the clinical symptom.

The negative control sera were obtained from six healthy pooled AB sera previously tested for lymphocytotoxic Abs. The panel reactive Abs (PRA) less than 20 per cent aginst T and B lymphocytes were accepted. The serum previously tested with PRA>90 per cent was used for positive control.

Lymphoprep$^{TM}$ (Nycomed Pharma, U.S.A.) was used for lymphocyte preparation by gradient centrifugation before further testing by standard NIH, AHG and FCXM. Rabbit antihuman immunoglobulin kappa light chain for AHG technique was obtained from DAKO, Denmark. Fluorescien (FITC)-AffiniPure $F(ab')_2$ fragment goat antihuman IgG, Fc gamma fragment specific was obtained from Jackson ImmunoResarch Labs, U.S.A. whereas Phycoerythrin-conjugated monoclonal anti-CD3 (PE-anti-CD3) and anti-CD20 (PE-anti-CD20) for FCXM technique were purchased from Becton Dickinson Inc, U.S.A.

### Preparation of splenic lymphocyte

The splenic material was kept in McCoy's 5A (GIBCO, U.S.A.) containing 5 per cent HIFCS, 1.25 per cent (v/v) ACD and 50 mg/100 ml DNase at 4°C until processing usually not more than 24 hours. The splenic cells containing media was

agitated in 37°C waterbath for 5-10 min before gradient centrifugation[9]. The total lymphocytes were harvested from the interface and adjusted to the concentration of $5.0 \times 10^5/100$ µl using for FCXM. The T and B lymphocytes were isolated from total lymphocytes by nylon-wool column method followed by AET-rosette formation[9]. Purified T and B lymphocytes were used for standard NIH and AHG techniques.

**AHG**[10]

The antihuman globulin reagent was pretested for the optimal dilution. The 1:50 dilution was used for AHG in this study. The T and B lymphocytes were separately tested against the KT patient serum, then washed with phosphate buffer saline (PBS) before antihuman globulin reagent was added. The strength of reaction was graded from the percentage of killed cells[9,10]. The scores

**Table 1. Crossmatching results of the three methods.**

| Total tested | No of disagreeable (%) | N | Patterns of disagreeable reaction | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | NIH | | AHG | | FCXM | |
| | | | T | B | T | B | T | B |
| 75 | 8/75 (10.7) | 5 | − | − | − | + | − | − |
| | | 1 | − | − | − | − | − | + |
| | | 1 | − | − | + | − | + | − |
| | | 1 | − | − | − | + | + | − |
| 47 | 5/47 (10.7) | 4 | − | − | ND | ND | + | − |
| | | 1 | − | − | ND | ND | − | + |

ND= Not done

NIH = standard microlymphocytotoxicity test

AHG = antihuman globulin microlymphocytotoxicity test

FCXM = flow cytometry crossmatch

**Table 2. Sensitivity of FCXM, AHG and standard NIH techniques.**

| Anti-HLA-A2 Dilution | HLA-A2 cells crossmatching result | | |
|---|---|---|---|
| | FCXM | AHG | NIH |
| 1:1 | P | P | P |
| 1:2 | P | P | P* |
| 1:4 | P | P* | N |
| 1:8 | P* | N | N |
| 1:16 | N | N | N |
| 1:32 | N | N | N |

* = the highest titer of each method

P = positive N = negative

NIH = standard microlymphocytotoxicity test

AHG = antihuman globulin microlymphocytotoxicity test

FCXM = flow cytometry crossmatch

**Table 3. The results of three crossmatching techniques and autoantibodies in living related kidney transplantation.**

**Case #1**

| Period of study | | Crossmatching Result | | | | | |
|---|---|---|---|---|---|---|---|
| | | NIH | | AHG | | FCXM | |
| | | T | B | T | B | T | B |
| 28 days-Pre KT | Test | N | N | SP | SP | SP | N |
| | Autologous | N | N | N | MP | ND | ND |
| 11 days-Post KT (after rejection) | Test | SP | N | SP | MP | WP | N |
| | Autologous | ND | ND | SP | MP | ND | ND |
| 116 days-Post KT (after rejection) | Test | SP | SP | MP | SP | WP | N |
| | Autologous | N | SP | WP | SP | ND | ND |

**Case #2**

| Period of study | | Crossmatching Result | | | | | |
|---|---|---|---|---|---|---|---|
| | | NIH | | AHG | | FCXM | |
| | | T | B | T | B | T | B |
| 225 days-Pre KT | Test | N | WP | N | WP | N | N |
| | Autologous | WP | SP | N | MP | N | N |
| 28 days-Pre KT | Test | N | WP | N | WP | N | N |
| | Autologous | MP | MP | MP | MP | ND | ND |
| 30 days-Post KT (after rejection) | Test | SP | SP | SP | SP | N | N |
| | Autologous | WP | SP | WP | SP | ND | ND |

N= Negative WP= Weakly Positive MP= Moderately Positive SP= Strongly Positive ND=Not done

NIH = standard microlymphocytotoxicity test

AHG = antihuman globulin microlymphocytotoxicity test

FCXM = flow cytometry crossmatch

KT = kidney transplantation

T = T lymphocyte B = B lymphocyte

of 4, 6 and 8 were interpreted as positive reaction whereas 1 and 2 were negative.

## FCXM[7]

One hundred microliter of sensitized donor splenic lymphocytes reacted with 50 µl of FITC and 10 µl of PE-anti-CD3 for T lymphocytes or PE-anti-CD20 for B lymphocytes. The reaction was read and dual-color flow cytometric analysis was performed by FACscan (Becton Dickinson, U.S.A.) [7]. The positive FCXM should be greater than 2 standard deviations (SD)[4,7]. From the experiments of 23 cadaveric splenic lymphocytes, 2SD of T and B lymphocytes were 21.79 and 41.97 mean channel fluorecence (MCF), respectively.

## RESULTS

The comparison of 3 different XM techniques, standard NIH, AHG and FCXM, is shown in Table 1. It was found that the result of 67 out of 75 (89.3%) XMes were agreeable by 3 different XM techniques whereas 8 out of 75 (10.7%) were

disagreeable. There were 4 patterns of disagreeable reactions, i.e., positive reactions with both AHG and FCXM, AHG only, and FCXM only. One positive AHG and FCXM showed a different pattern of reaction, i.e., it was positive with B cells by AHG but positive with T cells by FCXM. In addition, 47 XMes were tested by standard NIH and FCXM. The XM result in this group was similar to the first group that 5 out of 47 (10.7%) XMes were disagreeable, i.e., they were positive by FCXM but negative by standard NIH. Four XMes were positive with T cells and one was positive with B cells by FCXM.

The sensitivities of FCXM, AHG and standard NIH were tested by using two-fold dilution of anti-HLA-A2 tested against A2 cells (Table 2). The titers of anti-HLA-A2 were 1:8, 1:4 and 1:2 by FCXM, AHG and NIH, respectively.

The kinetic variations of Ab (pre-KT, recently rejection and post KT) were studied in 2 patients with rejection by standard NIH, AHG and FCXM (Table 3). In case #1, the positive reaction was not detectable by standard NIH at day 28 before KT whereas strongly positive reactions against T and B cells were detected by AHG and against T by FCXM in the same serum. The patient developed AR at day 2 post-KT which was confirmed by needle biopsy. After rejection, the strong reaction was observed with T cells at day 11 and T and B cells by standard NIH technique at days 116 post-KT. In case #2, auto-B cell Ab and B lymphocyte XM were detected in serum at day 225 and day 28 pre-KT by standard NIH and AHG whereas FCXM was negative. The patient developed rejection at day 16 post-KT which was documented by $^{99m}$Tc DTPA and $^{131}$I Hippuran Renal Scintigraphy. The serum at day 30 post-KT was strongly positive with T and B cells auto-Abs and XMes by both standard NIH and AHG whereas it was still negative by FCXM.

## DISCUSSION

AHG and FCXM are more sensitive techniques in both qualitative and quantitative measures than standard NIH which was agreeable to the previous studies[4,7,8,10]. The positive AHG-B cell/FCXM-T cell XM may be due to more HLA class I antigen presented on B than T lymphocytes rendering positive B cell XM with a sensitive AHG technique. FCXM is more sensitive than AHG, this technique can detect a very low Ab on T lymphocyte. However, FCXM technique is limited to the detection of IgG antibody[7,11].

Previous studies showed that FCXM is useful for kidney retransplantation and LR-KT [9,10]. The IgM Ab, mostly from auto-Ab, that detected by AHG and standard NIH except FCXM seemed to have no effect on primary KT. The sensitivity of FCXM in this study was not as high as other studies. This may be due to the presence of aggregated immunoglobulin that causes high background of MCF in autologous and test sera rendering low sensitivity of FCXM[7,12]. However, the sensitivity of FCXM can be improved by ultraspeed centrifugation to remove aggregated immunoglobulin[6,7]. This study suggests that either FCXM or AHG should be used for pre-KT XM in both kidney retransplantation and LR-KT.

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# วิธีทดสอบการเข้ากันได้ในผู้ป่วยเปลี่ยนไต

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Accelerated acute cellular rejection (AR) ยังเป็นปัญหาที่ยุ่งยากอย่างยิ่งในการเปลี่ยนไต ทำให้ตระหนักว่ายังมีการตรวจไม่พบภูมิต้านทานในผู้ป่วยที่ได้รับการกระตุ้น การศึกษานี้มุ่งเน้นที่จะศึกษาวิธีการทดสอบที่มีประสิทธิภาพเพื่อให้สามารถที่จะตรวจสอบภูมิต้านทานที่มีอยู่แล้วซึ่งอาจเป็นสาเหตุของ AR น้ำเหลืองจำนวน 122 ตัวอย่าง จากผู้ป่วยจำนวน 98 ราย ที่เข้ารายชื่อรอรับการเปลี่ยนไตที่ รพ.รามาธิบดี ได้รับการทดสอบกับเซลเม็ดเลือดขาวชนิดลิมโฟซัยจากผู้บริจาคที่เสียชีวิตจำนวน 23 ราย รวมถึงได้ศึกษาย้อนหลังของการทดสอบการเข้ากันได้ ในผู้ป่วยที่ได้รับการเปลี่ยนไตจากผู้บริจาคที่มีชีวิต (living related kidney transplantation, LR-KT) วิธีการทดสอบต่างๆ ที่นำมาศึกษามีดังนี้ วิธีการตรวจมาตรฐาน (Standard microlymphocytotoxicity test, Standard NIH) วิธีการตรวจโดยสารเร่งปฏิกริยา (Antihuman globulin microlymphocytotoxicity test, AHG) และวิธีการตรวจนับเซลที่ไหลเป็นกระแส (Flow cytometric crossmatch, FCXM) ผลการทดสอบแสดงให้เห็นว่า 8 ราย (10.7%) จากทั้งหมด 75 ราย ที่ให้ผลลบโดย Standard NIH มี 5 ราย ให้ผลบวกด้วยวิธี AHG และ 1 รายให้ผลบวกด้วยวิธี FCXM เพียงอย่างเดียว ส่วน 2 รายที่เหลือ ให้ผลบวกทั้ง AHG และ FCXM และในกลุ่มที่ไม่ได้ทำการทดสอบด้วยวิธี AHG พบว่า 5 ราย (10.7%) จากทั้งหมด 47 รายที่ให้ผลลบโดย Standard NIH ให้ผลบวกด้วยวิธี FCXM และเมื่อทำการทดสอบความไวของแต่ละวิธีด้วยการเจือจางน้ำเหลืองชนิด Anti HLA-A2 ปรากฏว่า FCXM ให้ผลไวที่สุด รองลงมาคือ AHG และ Standard NIH ตามลำดับ ผลจากการศึกษาย้อนหลังใน LR-KT ในรายที่ 1 ในน้ำเหลืองก่อนการเปลี่ยนไต Standard NIH ไม่สามารถตรวจพบภูมิต้านทานต่อผู้บริจาคในขณะที่ AHG และ FCXM สามารถตรวจพบภูมิต้านทานต่อผู้บริจาคได้ ผู้ป่วยปฏิเสธไตอย่างเฉียบพลันในวันที่ 2 หลังเปลี่ยนไตซึ่งมีการยืนยันผลด้วย needle biopsy น้ำเหลืองในวันที่ 11 และ 116 หลังเปลี่ยนไต สามารถตรวจพบภูมิต้านทานต่อผู้บริจาคได้จากการตรวจทั้ง 3 วิธี ในรายที่ 2 น้ำเหลืองก่อนการเปลี่ยนไตให้ผลลบต่อเม็ดเลือดขาวชนิด ที-ลิมโฟซัย โดยการทดสอบทั้ง 3 วิธี ในขณะที่พบภูมิต้านทานต่อตัวเองของเม็ดเลือดขาวชนิด บี-ลิมโฟซัย และภูมิต้านทานต่อเม็ดเลือดขาวชนิด บี-ลิมโฟซัย โดยการทดสอบด้วยวิธี Standard NIH และ AHG ผู้ป่วยปฏิเสธไตอย่างเฉียบพลันในวันที่ 16 หลังเปลี่ยนไต น้ำเหลืองในวันที่ 30 หลังเปลี่ยนไต ตรวจพบภูมิต้านทานต่อตัวเองของเม็ดเลือดขาวชนิด ทีและบี-ลิมโฟซัย และภูมิต้านทานต่อเม็ดเลือดขาวชนิด ทีและบี-ลิมโฟซัย โดยการทดสอบด้วยวิธี Standard NIH และ AHG ขณะที่วิธี FCXM ยังคงให้ผลลบอยู่เช่นเดิม จึงมักพบรายงานอยู่เสมอว่าภูมิต้านทานต่อเม็ดเลือดขาวชนิด บี-ลิมโฟซัย สามารถตรวจพบโดยวิธี Standard NIH และ AHG ได้ดีกว่าการตรวจด้วยวิธี FCXM

สรุปวิธี FCXM มีความไวมากกว่าวิธี Standard NIH และ AHG แต่ถึงอย่างไรวิธีนี้ก็มีขีดจำกัดในการตรวจภูมิต้านทานชนิด IgM ต่อเม็ดเลือดขาวชนิด ทีและบี-ลิมโฟซัย AHG เป็นวิธีที่สามารถตรวจภูมิต้านทานได้ทั้งชนิด IgG และ IgM ทั้งต่อเม็ดเลือดขาวชนิด ทีและบี-ลิมโฟซัย อีกทั้ง AHG มีความไวมากกว่า วิธี Standard NIH และไม่มีความต้องการเครื่องมือที่สลับซับซ้อนแต่อย่างใด จึงเป็นวิธีที่เหมาะสมในการนำมาใช้ในการตรวจในงานประจำวัน โดยเฉพาะอย่างยิ่งใน LR-KT หรือผู้ป่วยเคยได้รับการกระตุ้น

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