DR RAJAN DUGGAL
MD;DNB PATHOLOGY
PDCC RENAL AND TRANSPLANT PATHOLOGY
ASSOCIATE CONSULTANT
DEPARTMENT OF PATHOLOGY AND LABORATORY MEDICINE
MEDANTA, THE MEDICITY HOSPITAL
GURGAON (HARYANA)

C4d in Transplant biopsies - *Immunofluorescence*

PRINCIPLE

The immunohistological evaluation can be done by immunofluorescence (IF). It is based on the use of antisera conjugated with fluorescein directed against human antigens C4d

SPECIMEN REQUIREMENTS

Frozen Tissue :- Tissue, Store at -20°C and ship on Normal Saline or dry ice. Avoid Freeze thraw or Frozen – Fresh Tissue in Michel transport media, shipped at ambient temperature.

REAGENT

1) Normal Saline or Michel transport media

2) Antibodies
   a) Rabbit anti human C4d (Polyclonal)– AbD SEROTEC – catalogue No – 0300-0230
   b) Mouse anti human C4d (monoclonal)- AbD SEROTEC catalogue no - 2222 -8004

3) FITC Labelled Secondary antibody
   a) Donkey anti-rabbit antibody (Polyclonal)– AbD SEROTEC-catalogue no- 644002
   b) Rabbit antimouse antibody (Monoclonal)- AbD SEROTEC- catalogue no- star 9b
4) Diluent – DAKO – Code No – 50809
5) Phosphate buffer (Coon's buffer) pH 7.4
6) Buffered Glycerol (Mounting Media)

These antibodies are used in our laboratory but any alternative with similar specifications can be used

CHEMICALS

1) Citric Acid free acid monhydrous, Cat. No – C7129 – Sigma

2) N – Ethylmaleimide Cat. No. – E3876 – Sigma
3) Magnesium Sulfate Anhydrous. Cat. No M7500 – Sigma
4) Propidium Iodide 95 – 98% Cat. No. – P4170 – Sigma
5) P-phenylenediamine Free base Cat. No. P6001 – Sigma
6) Sodium Citrate Dihydrate ACSR Cat. No. 54641 – Sigma
7) Glycerol - Merk – Art 4094.
8) Ammonium Sulphate – Merk – 17507
9) Sodium Hydrogen phosphate – Merk – 17845
10) Disodium Hydrogen phosphate – Merk – 17549
11) Sodium Chloride – Rankem – Product No 50160.

EQUIPMENTS / MATERIALS

1) Light Microscope – Olympus – BX-51'
2) Fluorescence Microscope – Olympus BX-51.
3) Graduated glass cylinder 1000 mL – Borosil.
4) Graduated glass cylinder 100 mL – Borosil.
5) Hand gloves, Disposable (Sergun)
6) Poly – prep slides – Sigma diagnostic
7) PAP Pen – Binding site Cat. No. AD 1005
8) Micropipettes 0.5 µL to 10 µL – Finnpipette India
   10 µL to 100 µL – socorex.
9) Pipette tips – Tarson Micro tip.
10) Slide Staining tray (Humidity Chamber)
    Scientific Products Cat. No M6304
11) Frosted Micro Slides – Blue ribbon.
12) Cryostat – Cryocut – 1800 – Reichert – Jung.
13) Weighing Balance – Dhona 200 D.
14) Refrigerator – Quality
15) Incubator 37°C.

REAGENT PREPARATION AND STORAGE

All reagents must be labelled with date of preparation, expiration date, storage requirements, Lot number and the initials of the technologist preparing the reagent. Solutions are prepared individually at room temperature (RT)

1) Michel Transport Media / Normal Saline.

Solution A (buffer)

a) 1M Sodium / pot. Citrate buffer (pH 7.4) = 10 mL
b) 0.1 M Magnesium sulphate (sigma) = 1.2 gms.
c) 0.1 M n-Ethylmetemide (sigma) = 0.02 gms
d) Ammonium Sulphate = 250 gms

e) Dist. Water = 490 mL.

Citrate buffer

Solution – A
Sodium Citrate 14.2 gms in 100 mL of distilled water

Solution – B
Citric Acid 21.0 gms in 100 mL of distilled water.

To 80 mL of solution A add Solution B until the pH of solution is 7.4.
Make up to 100 mL with distilled water.

This media can be stored at room temperature and the shelf life is 1 year.

The biopsies can be left in this medium at room temperature for 6 Months without any deterioration of antigen / antibody complexes.

2) Phosphate buffered saline pH 7.4 (coon's buffer)
A) Sodium chloride = 20.25 gms
B) Disodium Hydrogen phosphate = 3.20 gms
C) Sodium dihydrogen phosphate = 0.39 gms
D) Distilled water = 2.5 lits.
(Check pH 7.4 Adjust if necessary)

3) Buffered Glycol (Mounting Media)
a) Sodium Hydrogen phosphate = 0.072 gms
b) Disodium Hydrogen phosphate = 0.016 gms.
c) P – phenylenediamine (Sigma) = 10 mgms.
d) Distilled Water = 10 mL.
e) Glycerol = 90 mL.
(Adjust = pH between 8.9)

METHOD

1. Cut 4µ thick Sections of Snap Frozen biopsies in a cryostat Set at - 25°C (Two slides are needed for each biopsy).

2. Frozen sections are incubated with rabbit anti human C4d (polyclonal antibody catalogue no - 0300 – 0230) or mouse anti human C4d (monoclonal antibody catalogue no - 2222 -8004) in a dilution of 1:20 in phosphate buffered saline (PBS) for 30 minutes at room temperature. For 1:20 dilution add 19 microlitre of PBS and 1 microlitre of antibody.

3. Wash 4 times with PBS at an interval of 15 minutes.
4. After washing with PBS, incubate with an FITC-labeled secondary donkey anti-rabbit antibody for polyclonal (catalogue no- 644002) or FITC-labeled secondary rabbit antimouse antibody for monoclonal (catalogue no- star 9b) in a dilution of 1:10 in PBS for 30 min at RT. For 1:10 dilution add 9 microlitre of PBS and 1 microlitre of antibody.
5. Wash 4 times with PBS at an interval of 15 minutes.
6. Coverslip sections using an aqueous mounting media.

QUALITY ASSURANCE

This procedure is in compliance with the general guidelines. (Refer to Quality Assurance statement)

RESULTS

Reporting Results

Glomerular mesangial staining is taken as interna; control and positivity in peritubular capillary is taken as positive