MATERIALS AND METHODS
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Specimen Repository

During a period beginning from June 1st 2014 to April 30th 2015, all newly diagnosed patients with UC in, MGM Hospital, Navi Mumbai were included in this study. The experimental protocol of this project was accepted and permitted by the Ethical Review Committee and Institutional Research Review Committee of MGM Institute of Health Sciences, Navi-Mumbai. All the study subjects were enrolled after obtaining their duly signed informed consent reports. Whole blood (5 mL) from patients was collected in sterile vacutainers. It was allowed to clot at 18-24°C. After the clotting, Serum was separated from the rest of the components by centrifugation. Samples were processed within 48 hours of collection or stored in 200µL aliquots at -20°C. Thus a specimen repository of 45 UC positive specimens was created. Along with UC positive specimens, serum samples from 45 healthy individuals were also collected as healthy controls. One serum specimen of a related disease, Autoimmune Vasculitis patient specimen from MGM Hospital, Navi Mumbai, was collected and maintained as related disease control. c-ANCA, p-ANCA and atypical p-ANCA serum specimens were acquired from Plasma Services Group Inc., Southampton, PA, USA and maintained as controls.

Materials

Reagents

The materials used for this project comprised of reagents required for development of the in-house ANCA IIF, UC ELISA and modified UC ANCA IIF assays. IIF slides were sourced from Avishkar International Private Ltd. Mumbai, India. Magnesium chloride, Sodium chloride, Potassium chloride, EDTA, Tween 20, Ethanol and Formalin were purchased from Rankem, India. Human Serum Albumin and Bovine Serum Albumin were purchased from Reliance Lifesciences, Mumbai, India. Sodium phosphate dibasic, Monobasic Sodium Phosphate, Potassium phosphate monobasic, Sucrose and n-propyl gallate were procured from SRL, India. Goat Anti-human FITC (Fluorescein isothiocyanate) Conjugated IgG, Evan’s Blue dye and TMB-H2O2 were purchased from GeNei, India. Glycerol, HEPES (4-(2- hydroxyethyl)-1-
piperazineethanesulfonic acid), and Tris were purchased from Sigma Aldrich, USA. Molecular Grade Ethanol was sourced from Merck, USA. Polymorph prep was procured from Fresenius Kabi, Norway. ELISA plates (MaxiSorp) were obtained from Nunc, Roskilde, Denmark.

**Solutions and Buffers**

1) HBSS Buffer  
   1.36M NaCl  
   50mM KCl  
   25mM NaH$_2$PO$_4$  
   25mM HEPES  
   1mM EDTA  
   pH 7.1 – 7.4

2) RBC Lysis Buffer  
   0.82% NH$_4$Cl  
   pH 7.1 – 7.4

3) Mounting Medium  
   Glycerol  
   0.2M Tris  
   pH 9.3

4) Diluting Buffer  
   25mM Phosphate Buffer  
   0.9% NaCl  
   pH 7.1 – 7.4

5) Coating Buffer  
   50mM Phosphate Buffer  
   0.9% NaCl  
   pH 7.1 – 7.4

6) Blocking Buffer  
   20mM Tris Buffer  
   0.9% NaCl  
   3.0% Tween 20  
   pH 7.1 – 7.4
7) Wash Buffer
   20mM Tris Buffer
   0.9% NaCl
   0.25% Tween 20
   pH 7.1 – 7.4
8) Nuclear Extraction Buffer A
   250 mM Sucrose
   5 mM MgCl₂
   10 mM Tris-HCl
   pH 7.1 – 7.4
9) Nuclear Extraction Buffer B
   1.8 M Sucrose
   1 mM MgCl₂
   10 mM Tris-HCl
   pH 7.1 – 7.4

Samples and Controls
- Serum samples of UC positive patients were collected from MGM Hospital, Vashi, Navi Mumbai and stored at -20°C.
- Normal serum samples of healthy controls were collected from Yashraj Biotechnology Ltd., Navi Mumbai and stored at -20°C.
- Positive control serum samples were obtained from Plasma Services Group Inc., Southampton, PA, USA and stored at -20°C.

Commercial Assay Kits
- ANCA IIF assay was sourced from BioRad, USA.
- ANA IIF assay was sourced from BioRad, USA.
- MPO ELISA assay was sourced from BioRad, USA.
- PR3 ELISA assay was sourced from BioRad, USA.
Methods

Specimen Database

The UC specimens obtained from MGM hospital were coded and stored at -20°C for future experimentation. The specimens collected as whole blood in plain vacutainer tubes. The blood samples were allowed to stand still and coagulate. After coagulation the serum was separated by centrifugation. This serum was further aliquoted in eppendorfs (200 microlitres each). The aliquots were stored at -20°C in with coding done as shown in Table 6.

Table 6: Specimen Database

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Healthy Controls Database

The specimens obtained from healthy individuals were coded and stored at -20°C for future experimentation. The specimens collected as whole blood in plain vacutainer tubes. The blood samples were allowed to stand still and coagulate. After
coagulation the serum was separated by centrifugation. This serum was further aliquoted in eppendorfs (200 microlitres each). The aliquots were stored at -20°C with coding done as shown in Table 7.

**Table 7: Healthy Controls Database**

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</table>

**Standard Controls Database**

The specimens obtained from Plasma Services Group Inc., Southampton, PA, USA were coded and stored at -20°C for further experimentation. The serum was stored in aliquots of 200 microliters each with coding done as shown in Table 8.

**Table 8: Standard Controls Database**

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<th>Sr. No.</th>
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<th>Sr. No.</th>
<th>Specimen Code</th>
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<td>3</td>
<td>Atypical p-ANCA Control</td>
</tr>
<tr>
<td>2</td>
<td>c-ANCA Control</td>
<td>4</td>
<td>Vasculitis Control</td>
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**Localisation of UC Antigens**

It has been postulated in earlier documented research that UC antigens are localised in the nucleus of the neutrophils, unlike the MPO and PR3 antigens which are present in their cytoplasm (Mallolas J, et. al, 2000). In order to establish the fact that UC antigens are localised in the inner periphery of the nucleus, the following experiments were designed and performed.

**Neutrophil Separation from Whole Blood**

The Buffy Coat containing neutrophils was separated using Ficoll-Polymorph Prep Gradient Centrifugation Technique. The pellet thus obtained contained human neutrophils which was further subjected to fractionation and sucrose gradient extraction technique. The protocol developed for neutrophil separation is as follows:

- 5ml blood sample was collect. It was treated with 0.5M EDTA (50µl) (SRL, India) and layered on 5ml Polymorph Prep. (Fresenius Kabi, Norway). The blood was centrifuged at 1500rpm for 30 minutes at 15 °C. The neutrophil layer (buffy coat) was separated by pipetting and washed with 5ml Hanks Basal Salt Solution (HBSS). It was centrifuged once again at 1500 rpm for 15 minutes at 15°C. The supernatant containing HBSS was decanted and cell pellet was treated with RBC Lysis Buffer (0.82% Ammonium chloride solution) to remove RBCs. RBC Lysis: The pellet was dissolved in 5 ml chilled RBC lysis buffer and incubated at 4°C for 10 minutes. Then it was centrifuged once more at 1500 rpm for 15 minutes at 15°C. The above process was repeated 3–4 times till the pellet turned white. This cell pellet was washed with 5 ml HBSS and centrifuged at 1500 rpm for 15 minutes at 15°C. Supernatant containing HBSS was decanted and the pellet was redissolved in 10 ml HBSS.

**Neutrophil Fractionation and Nuclear Extraction**

The Neutrophil Lysis Protocol was designed to separate the nuclear and cytoplasmic fractions of the neutrophils using change in osmotic pressure of the medium in which the neutrophils were suspended. A sucrose gradient was employed to separate the nuclear extract from the cytoplasmic extract of the neutrophils. The protocol developed for neutrophil fractionation and nuclear extraction is as follows:
The HBSS solution containing intact neutrophils was centrifuged at 1500 rpm for 15 min at 15°C. Supernatant containing HBSS was decanted and the cell pellet was redissolved in 1 ml of ice cold Buffer A. This solution was centrifuged at 600 g for 10 minutes. The supernatant was separated as the cytoplasmic extract and previous step was repeated for the pellet. After this step the pellet was redissolved in 1 ml of ice cold Buffer B and centrifuged at 16,000 g for 30 minutes. The supernatant containing Buffer B was discarded and pellet containing the intact nuclei was redissolved in 1 ml Buffer A.

Figure 11: Neutrophil Separation from Whole Blood using Polymorph Prep.

Spectrophotometric Analysis

The cytoplasmic and nuclear extracts obtained from the above mentioned process were analysed for their protein concentration using a spectrophotometer. The spectrophotometric OD of the nuclear and cytoplasmic fractions was recorded at 280nm wavelength, which is used to determine the protein concentration.
**UC-ELISA Analysis**

The cytoplasmic and nuclear extracts were coated onto ELISA plates (MaxiSorp); (Nunc, Roskilde, Denmark) as crude UC antigen source and UC positive specimens were used as source of primary ANCA antibodies. Goat antihuman IgG (Genei, India) was used as HRP conjugated secondary antibody and TMB-H$_2$O$_2$ as the substrate. Absorbance was recorded at 450nm. The protocol develop for this ELISA technique is as follows:

The cytoplasmic and nuclear extracts were diluted 1:20 using the coating buffer (50 mM Phosphate Buffered Saline) and coated onto the ELISA plate. The plate was incubated at room temperature for 120 minutes. After discarding the solution from the wells, the plate was washed with wash buffer (20 mM Tris Buffered Saline with 0.25% Tween 20) 3 times. The wells were blocked with blocking buffer (20 mM Tris Buffered Saline with 3% Tween 20) and incubate at room temperature for 120 minutes After discarding the solution from the wells, the plate was washed with wash buffer 1 time. UC positive serum samples diluted 1:50 with coating buffer were added into the wells (100µl/well). The plate was incubated at 18-24 °C for 60 minutes. After discarding the solution from the wells, the plate was washed with wash buffer 3 times. Secondary Anti-Human IgG-HRP Conjugated Antibody solution (1:50000 diluted with wash buffer) was added to the wells (100µl/well). The plate was incubated at 18-24 °C for 60 minutes. After discarding the solution from the wells, the plate was washed with wash buffer 3 times. 1x TMB-H$_2$O$_2$ solution was added to the wells (100µl/well) and incubated in dark room for 15 minutes. Finally, stop solution was added (100µl/well) and absorbance read in an ELISA plate reader within 15 minutes of adding the stop solution.

**Dot-Blot Analysis**

Purified PR3 and MPO antigen controls, Native nuclear and cytoplasmic extracts and heat denatured nuclear and cytoplasmic extracts were blotted onto a nitrocellulose membrane. The specimens were blotted onto a 3cm x 7cm Nitrocellulose membrane pad using pipette. Droplets of 1 microliter each were blotted onto the Nitrocellulose membrane five times with a gap of 10 minutes between each round of blotting. UC positive serum was used as source of primary ANCA antibody along with
goat anti human IgG as secondary antibody. Goat antihuman IgG (Genei, India) was used as HRP conjugated secondary antibody and TMB-H$_2$O$_2$ as the substrate. The Dot Blot assay of these specimens was performed.

**Autoimmune Profile Survey of UC Cases**

**Prospective Registration**

During a period beginning from June 1$^{st}$ 2014 to April 30$^{th}$ 2015, 45 patients diagnosed with UC at MGM Hospital, Navi Mumbai along with 45 healthy controls with similar gender, age and geographical location characteristics were included in the case-control survey. All UC positive subjects included in the study had to satisfy the criteria for diagnosis of UC employed by the hospital. The physical symptoms, endoscopy data and clinical observations were taken into consideration to determine the presence of UC and such UC positive patients were requested to volunteer for the study with their informed consent. (Appendix I)

**Data Collection**

Based on the information collected from the subjects, following criteria were examined: time of diagnosis, severity of disease, extra-intestinal indicators, smoking, occupation, eating habits, family history of UC, etc. This information was collected from the subjects at the time of hospital visit. The patients answered a questionnaire based on the survey proposed by IOIBD containing questions pertaining to various environmental risk factors. (Appendix II) The questionnaire included details of ethnicity, infections occurred during childhood, vaccines administered, smoking, eating habits, health and hygiene, physical exercise and occurrence of stressful events. The data collected from the subjects was grouped under 10 variables as follows: infections occurred during childhood, vaccines administered, consumption of sugar, intake of fibres, consumption of fast food, intake of caffeine (tea and coffee), physical exercise, smoking, health and hygiene and major stressful events. These criteria were compared with the severity of disease. Also the impact of these indicators on extent of UC and the quality of living of the patients was analysed. This data was further processed using statistical software to interpret it with respect to the variables.
**Ethical Considerations**

All studies were executed by the consent and permission of the Ethics Committee of MGM Institute of Health Sciences. The subjects involved in the study gave their consent in writing. The data was analysed statistically using SPSS (10.1) statistical software.

**Review of Currently Available Diagnostic Assays**

A comprehensive review of the serological testing carried out by two of world’s leading diagnostic laboratories, namely Quest Diagnostics and Mayo Clinic, was performed to determine the types of serological tests undertaken for the diagnosis of UC along with other clinical findings and screening tests. The tests offered by these laboratories for the diagnosis of UC in adjunct with the clinical findings and symptoms were documented. The major platforms of testing were found to be IIF and ELISA assays. Both the laboratories performed ANCA detection using ANCA IIF assays. In order to rule out interference by ANA antibodies, ANA IIF assay was performed. Finally, in order to rule out other related autoimmune diseases Wegener’s Granulomatosis and Microscopic Polyaengitis, the PR3 and MPO ELISA assays were performed. Thus the specimens positive on the ANCA screen and negative on the other tests were considered to be most likely cases of UC. The findings are tabulated in Table 9 and Table 10.

**Table 9: Diagnostic Panel for UC Serological Testing employed by Quest Diagnostics (Source: Quest Diagnostics Test Catalogue for IBD)**

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<td>Negative</td>
<td>Equivocal</td>
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<tr>
<td>ANCA Screen</td>
<td>Not detected</td>
<td>—</td>
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<tr>
<td>ANA Screen</td>
<td>Not detected</td>
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<td>ASCA IgG</td>
<td>≤20 U</td>
<td>20.1-29.9 U</td>
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<td>MPO Antibody</td>
<td>&lt;1 AI</td>
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<tr>
<td>PR-3 Antibody</td>
<td>&lt;1 AI</td>
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Table 10: Diagnostic Panel for UC Serological Testing employed by Mayo Clinic (Source: Mayo Clinic Test Catalogue for IBD)

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Immunofluorescence Assays

Indirect Immunofluorescence (IIF) is a diagnostic technique which employs fluorescence based antibodies to visualize results of the test under ultraviolet light. In this method, the test serum is spotted onto a slide which contains specialized cells impregnated onto it. The autoantibodies present in test serum interact with the cells affixed on the slides and attach to those cells. The slide is further coated with a fluorescent labelled secondary antibody against the specific autoantibodies which are under consideration. The observations are studied based upon the presence or absence of fluorescent patterns under UV light. IIF technique helps to detect and quantitate autoantibodies which in turn is useful for diagnosis of autoimmune diseases (Renato et.al, 2013). During the IIF procedure, a fluorescent secondary antibody serves as a tagging agent for the antigen- autoantibody reaction that occurs in the cells affixed onto the glass slide. human epithelial cell line (HEp-2) and neutrophils are amongst the most frequently used cells for diagnosis of autoimmune diseases using IIF assays. The end result is interpreted based upon observation of a specific fluorescent pattern on the IIF slides under UV light, indicating the role of autoantibodies.

Based upon the literature review, it was suggested that ANCA antibodies are generally found amongst UC patients. ANCA are autoantibodies, majorly of the IgG class which are specific to antigens in the cytoplasm of neutrophils. ANCA play a major
role in pathogenesis of many autoimmune diseases including UC. ANCA are detected using IIF technique on neutrophil slides fixed with ethanol. Formalin-fixation is used to differentiate between the different ANCA patterns. They are majorly divided into three different patterns based on the localization of the fluorescence inside the neutrophils; cytoplasmic (c-ANCA), perinuclear (p-ANCA) and atypical. c-ANCA shows granular fluorescence in the cytoplasm with central interlobular prominence. p-ANCA shows staining in the perinuclear region and atypical p-ANCA often shows a thick perinuclear staining along with infrequent nuclear extensions. Antigen present in the cytoplasm of the neutrophils is responsible for the c-ANCA type staining pattern. Classical p-ANCA occurs with antibodies directed to myeloperoxidase (MPO) antigen and lysozyme. Atypical p-ANCA pattern is created by antigens similar to that of the p-ANCAs namely, BPI, cathepsin G, elastase, lactoferrin and other uncharacterized antigens.

The international consensus statement recommends IIF to be used as the ideal test for ANCA screening (Savige et.al, 2003). IIF results help to distinguish among c-ANCA, p-ANCA and atypical p-ANCA. The autoantibodies found in UC are called atypical p-ANCA because they are significantly different from the conventional c-ANCA and p-ANCA with respect to their antigens (Maria et.al, 2009). Various research groups have detected atypical p-ANCA amongst UC cases in varying proportions ranging from 50% up to 90% of the sample population (Ali et.al, 2013). p-ANCA are generally seen in patients with MPA and are reactive to myeloperoxidase antigen (MPO) whereas c-ANCA are observed in patients with WG and are reactive to proteinase 3 antigen (PR3) (Joode et.al, 2013). Both MPO and PR3 are localized in the azurophilic granules present in the cytoplasm of the neutrophils. p-ANCA pattern is considered as an artifact of ethanol fixation, which is observed due to the positively charged cytoplasmic proteins migrating of from the azurophilic granules in the cytoplasm onto negatively charged nuclear surface. This is the reason why typical p-ANCA shows perinuclear pattern on ethanol fixation whereas on formalin fixation, it shows diffused cytoplasmic pattern where in the migration of cytoplasmic proteins is restricted. The target antigens which are specific to atypical p-ANCA are yet not elucidated. They may include lysozyme, elastase, lactoferrin, catalase, cathepsin G, BPI, α-enolase, and lamin B1 amongst many others which still remain to be
characterized. (Barahona et al., 2009). Various studies have indicated that these are not cytoplasmic antigens like MPO, PR3, but they are present inside the nucleus of human neutrophils (Mallolas J, et al., 2000). It has been reported that the High Mobility Group Proteins (HMG1 and HMG2) and few granular and non-histone chromosomal proteins may be the potential target antigens of atypical p-ANCA (Takaishi et al., 2012). Hence as a unique characteristic, atypical p-ANCA does not show any change in the ANCA IIF pattern when fixed with either ethanol or formalin. This is the differentiating criteria employed to differentiate between atypical p-ANCA, c-ANCA and typical p-ANCA. The ANCA pattern images captured during the experimentation process using BioRad ANCA IIF assay are shown in Figure 12.

Figure 12: Human Neutrophils coated on BioRad ANCA IIF Slides and fluoresced under UV light showing ANCA patterns using FITC. (40X)

Anti Nuclear Antibodies (ANA) are autoantibodies generated by the host immune cells when it falts to differentiate the "self-antigens’ from the "nonself-antigens." They generally target nuclear antigens causing tissue and organ damage. Also, ANA antibodies interfere in the detection of ANCA since both these autoimmune antibodies have similar target antigens present within the human cells. Hence it is necessary to screen every ANCA positive specimen using ANA screen so as to ensure that the ANCA positivity is not due to the coexistence of ANA antibodies. Positive ANA results on IIF are indicated by the presence of a particular type of fluorescence pattern. Generally, three major types of ANA patterns can be observed: Homogenenous (diffuse), Speckled and Nucleolar pattern. The ANA pattern images captured during the experimentation process using BioRad ANA IIF assay are shown in Figure 13. We
tested the UC specimens on BioRad ANCA IIF Assay and BioRad ANA IIF assay to develop an algorithm for the diagnosis of UC in Indian Population.

![Image of ANA Staining patterns on Hep-2 IIF slides](image)

**Figure 13:** ANA Staining patterns observed on Hep-2 IIF slides (from top to bottom): Homogenous, Speckled and Nucleolar pattern. (40X)

**Enzyme Immunoassays**

Anti Neutrophil Cytoplasmic Antibodies are autoantibodies generated by the host immune cells when it falters to differentiate the "self-antigens’ from the "nonself-antigens" and attack certain proteins present within the neutrophils. Two of the most
commonly found ANCA are the autoantibodies that target the neutrophil proteins proteinase 3 (PR3) and myeloperoxidase (MPO). They help detect and diagnose autoimmune diseases like Wegener granulomatosis (WG), Microscopic Polyangiitis (MPA). MPO and PR3 antibodies are tested using Enzyme Immunoassay technique. A positive test for PR3 antibodies is observed in more than 80% of the WG patients. A positive test for MPO antibodies is consistent with MPA. The negation of presence of MPO or PR3 antibodies is crucial for diagnosis of UC since their fluorescence pattern is very much similar to that of atypical pANCA antibodies and it requires experience as well as technical expertise for the analyst to differentiate between these three patterns of fluorescence.

Anti-Saccharomyces cerevisiae antibodies (ASCA) are autoantibodies that are often found in people suffering from CD. Patients suffering from CD often possess significant amount of IgG autoantibodies that react with cell wall mannan of Saccharomyces cerevisiae. These two classes of autoantibodies are detected by ELISA testing. The ASCA antibodies are more prevalent in CD whereas atypical pANCA is more prevalent in UC. If ASCA is positive, there is a high possibility that the person is suffering from CD. If ANCA is positive, there is a high possibility that the person is suffering from UC. Approximately 55 to 75% of patients with CD show elevated levels of IgA or IgG ASCA antibodies. Approximately 60 to 85% of patients show presence of atypical pANCA.

**Development of Diagnostic Algorithm for UC**

Based upon the available literature, the following algorithm was tested for its validity amongst the Indian population by testing the UC specimens with the commercial assays available in the market. The assays were sourced from BioRad and specimens were tested for the respective biomarkers to check for the diagnosis of UC. ANCA and ANA testing was performed by IIF whereas PR3 and MPO analysis was performed using ELISA. There was a need felt for a systematic algorithm for UC diagnosis in India due to the large amount of expenses incurred by the patients for performing various diagnostic tests, many of which could be avoided if a systematic algorithm was adopted. Also, the algorithm was necessary to save upon the precious
time wasted during the diagnosis phase due to which treatment gets delayed. This diagnostic algorithm has been validated in the western countries but is not established in the Indian setting.

Table 11: Diagnostic Algorithm for diagnosis of UC and other related autoimmune diseases (To be interpreted along with clinical evidence)

<table>
<thead>
<tr>
<th>Auto immune Biomarkers/Tests</th>
<th>ANCA IIF</th>
<th>ANA IIF</th>
<th>ASCA IgG ELISA</th>
<th>PR3 ELISA</th>
<th>MPO ELISA</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Atypical p-ANCA)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>UC positive</td>
</tr>
<tr>
<td>(ASCRA)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>CD positive</td>
</tr>
<tr>
<td>(c-ANCA)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>WG positive</td>
</tr>
<tr>
<td>(p-ANCA)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>MPA positive</td>
</tr>
</tbody>
</table>

Testing of UC Specimens with BioRad ANCA IIF Assay

All the 45 UC specimens along with standard controls were tested using BioRad ANCA IIF Assay. The protocol was as follows. UC specimens were diluted 1:20 using sample diluent. IIF slides were coated with the diluted specimen solution (approx. 50µl/well). They were incubated in a humid chamber at 18-24°C for 30 minutes. Edges of the slides were blotted to remove excess liquid. 1 drop (approx. 50µl/well) of FITC Conjugate was added per well and the slides were incubated in a humid chamber at room temperature for 30 more minutes. After discarding excess liquid, the slides were washed gently with PBS. They were further immersed in PBS with 2-3 drops of Evan’s Blue counter stain for 10 minutes. Slides were blotted on tissue paper to remove excess liquid. 1 drop of Mounting Medium was placed on each well and the slides were observed under UV microscope at 40X magnification.

Testing of UC Specimens with BioRad ANA IIF Assay

All the 45 UC specimens along with standard controls were tested using BioRad ANA IIF Assay. The protocol was as follows. UC specimens were diluted 1:40 using sample diluent. IIF Hep-2 slides were coated with the diluted specimen solution
They were incubated in a humid chamber at 18-24°C for 30 minutes. Edges of the slides were blotted to remove excess liquid. 1 drop (approx. 50µl/well) of FITC Conjugate was added per well and incubated at room temperature for 30 minutes in a humid chamber. After discarding excess liquid, the slides were washed gently with PBS. They were further immersed in PBS with 2-3 drops of Evan’s Blue counter stain for 10 minutes. Slides were blotted on tissue paper to remove excess liquid. 1 drop of Mounting Medium was placed on each well and the slides were observed under UV microscope at 40X magnification.

**Testing of UC Specimens with BioRad ASCA IgG ELISA Assay**

All the 45 UC specimens along with standard controls were tested using BioRad ASCA IgG ELISA Assay. The protocol was as follows. The specimens were diluted 1:100 in sample diluent buffer and coated onto the ELISA plate. The plate was incubated 18-24°C for 30 minutes. After discarding the solution from the wells, the plate was washed with wash buffer 3 times. EIA Conjugate was added to the wells (100µl/well). The plate was incubated at 18-24°C for 30 minutes. After discarding the solution from the wells, the plate was washed with wash buffer 3 times. 1x TMB substrate was added to the plates (100µl/well) and incubated in dark for 30 minutes. Finally, the stop solution was added (100µl/well) and absorbance read in an ELISA plate reader within 15 minutes of adding the stop solution.

**Testing of UC Specimens with BioRad MPO ELISA Assay**

All the 45 UC specimens along with standard controls were tested using BioRad MPO ELISA Assay. The protocol was as follows. The specimens were diluted 1:100 in sample diluent buffer and coated onto the ELISA plate. The plate was incubated 18-24°C for 30 minutes. After discarding the solution from the wells, the plate was washed with wash buffer 3 times. EIA Conjugate was added to the wells (100µl/well). The plate was incubated at 18-24°C for 30 minutes. After discarding the solution from the wells, the plate was washed with wash buffer 3 times. 1x TMB substrate was added to the plates (100µl/well) and incubated in dark for 15 minutes. Finally, the stop solution was
added (100µl/well) and absorbance read in an ELISA plate reader within 15 minutes of adding the stop solution.

**Testing of UC Specimens with BioRad PR3 ELISA Assay**

All the 45 UC specimens along with standard controls were tested using BioRad PR3 ELISA Assay. The protocol was as follows. The specimens were diluted 1:100 in sample diluent buffer and coated onto the ELISA plate. The plate was incubated at 18-24°C for 30 minutes. After discarding the solution from the wells, the plate was washed with wash buffer 3 times. EIA Conjugate was added to the wells (100µl/well). The plate was incubated at 18-24°C for 30 minutes. After discarding the solution from the wells, the plate was washed with wash buffer 3 times. 1x TMB substrate was added to the plate (100µl/well) and incubated in dark for 30 minutes. Finally, the stop solution was added (100µl/well) and absorbance read in an ELISA plate reader within 15 minutes of adding the stop solution.

**ANCA IIF Assay Development**

An In-house ANCA IIF assay was developed in order to create a cheaper alternative to the available expensive assays in the market. The Buffy Coat containing neutrophils was separated using Ficoll-Polymorph Prep Gradient Centrifugation Technique. The neutrophils thus obtained from the buffy coat were used for neutrophil slide preparation. The neutrophils mounted on IIF slides using the in-house technique were bigger in size as compared to the ones on BioRad slides, which made the nucleus very prominent for differentiating between typical and atypical p-ANCA staining patterns (Figure 14, 15). The specimen results were interpreted based upon the pattern showed by them on the neutrophil slides fixed using ethanol and formalin. The ANCA patterns seen on ethanol and formalin fixed slides were: c-ANCA pattern was presented as coarse speckled granular cytoplasmic staining; p-ANCA pattern was seen as fine rimmed perinuclear staining on ethanol fixation and diffused granular staining on formalin fixation. Atypical p-ANCA pattern was observed as thick perinuclear staining along with infrequent coarse granular cytoplasmic staining on ethanol fixation and faint or no staining on formalin fixation. The absence of staining of atypical pANCA on
formalin fixed slides may be due to the conformational change occurring to the native structure of atypical pANCA antigens located on the inner periphery of the nucleus rendering them incapable of binding to the autoantibodies.

Figure 14: Neutrophils fixed onto IIF slides using an in-house developed protocol

Figure 15: Neutrophils mounted on BioRad ANCA IIF assay slide (Ref. slide) and in-house ANCA IIF assay slide (Slide 3) at 40X

IIF Slide Preparation

5ml blood sample was collected from a healthy individual as source of neutrophils. It was treated with 0.5M EDTA (50µl) and layer it on 5ml Polymorph Prep. The solution was centrifuged at 1500rpm for 30 minutes at 15 °C. The neutrophil layer (buffy coat) was separated by pipetting and washed with 5ml Hanks Basal Salt Solution. It was re-centrifuged at 1500 rpm for 15 minutes at 15°C. The supernatant containing HBSS was decanted and cell pellet was treated with RBC Lysis Buffer (0.82%
Ammonium chloride solution) to remove RBCs. RBC Lysis: The pellet was dissolved in 5 ml chilled RBC lysis buffer and incubated at 4°C for 10 minutes. Then it was centrifuged once more at 1500 rpm for 15 minutes at 15°C. The above process was repeated 3–4 times till the pellet turned white. This cell pellet was washed with 5 ml HBSS and centrifuged at 1500 rpm for 15 minutes at 15°C. Supernatant containing HBSS was decanted and the pellet was redissolved in 10 ml HBSS. A cell count of the solution was taken using cell counter and the solution was diluted with (HBSS+HSA) solution to obtain 1x10⁶ cells/ml concentration. This cell suspension (50µl/well) was coated on IIF slide and the slide was incubated at 18-24 °C for 45 minutes. The slide was blotted on tissue paper to remove excess liquid. Further, ¼th Saline solution (50µl/well) was added to the slide and it was incubated at 18-24 °C for exactly 1 minute. The ¼th saline created a hypotonic environment for the neutrophils enabling the water molecules to enter into them leading to the swelling of neutrophils. The time of treatment was crucial because excessive treatment with the hypotonic saline would have led to bursting of the neutrophils. The slide was blotted on tissue paper to remove excess liquid and immersed in molecular grade ethanol (100%) or molecular grade formalin (10%) for 2 hours. The slide was dried with dryer and used for ANCA IIF or stored at 4 °C in dry condition for further use. The process is outlined in Figure 16.

**ANCA IIF Protocol**

The UC specimens were diluted 1:40 using phosphate buffered saline. The IIF slides were coated with the diluted specimen solution (30µl/well). They were incubated in a humid chamber at 18-24 °C for 30 minutes. Excess liquid was blotted from edges. 1 drop of FITC Conjugated Goat Anti-human IgG antibody (1:50000) was added in every well and the slide was incubated at 18-24 °C for 30 minutes in a humid chamber. Excess liquid was discarded and the slide was washed gently with PBS. The slide was immersed in PBS with 2-3 drops of Evan’s Blue counter stain for 10 minutes. After blotting the excess liquid on tissue paper, 1 drop of Mounting Medium (glycerol) was placed on each well. The slide was observed under UV microscope for fluorescence. The process is outlined in Figure 17. All the 45 UC specimens along with standard controls were tested using In-house ANCA IIF Assay.
An In-house UC ELISA assay was developed in order to create a cheaper alternative to the available expensive assays in the market. It had been established in the earlier part of the experiments that UC antigens are located in the nucleus of the
neutrophils. Hence human neutrophils were lysed to extract intact nuclei as source of UC antigens using an in-house developed and standardised protocol. The Neutrophil Lysis Protocol was designed to separate the nuclear and cytoplasmic fractions of the neutrophils using change in osmotic pressure of the medium in which the neutrophils were suspended. A sucrose gradient was employed to separate the intact nuclei from the cytoplasmic extract of the neutrophils. The intact nuclei were coated onto ELISA plates (MaxiSorp); (Nunc, Roskilde, Denmark) and UC positive specimens were used as source of primary ANCA antibodies. Goat antihuman IgG (Genei, India) was used as HRP conjugated secondary antibody and TMB-H₂O₂ as the substrate. Absorbance was recorded at 450nm. The protocol developed for neutrophil fractionation and nuclear extraction and UC ELISA technique was as follows:

**Extraction of Intact Nuclei from Human Neutrophils**

Human neutrophils were obtained from whole blood of a healthy individual using the Neutrophil separation protocol as described in the first chapter. The HBSS solution containing intact neutrophils was centrifuged at 1500 rpm for 15 minutes at 15°C. Supernatant containing HBSS was decanted and the cell pellet was redissolved in 1 ml of ice cold Buffer A. This solution was centrifuged at 600 g for 10 minutes. The supernatant was separated as the cytoplasmic extract and previous step was repeated for the pellet. After this step the pellet was redissolved in 1 ml of ice cold Buffer B and centrifuged at 16,000 g for 30 minutes. The supernatant containing Buffer B was discarded and pellet containing the intact nuclei was redissolved in 1 ml Buffer A (Figure 18).

**UC ELISA Assay Protocol**

The solution of intact nuclei was diluted 1:20 in coating buffer (50 mM Phosphate Buffered Saline) and coated onto the ELISA plate. The plate was incubated at 18-24 °C for 120 minutes. After discarding the solution from the wells, the pate was washed with wash buffer (20 mM Tris Buffered Saline with 0.25% Tween 20) 3 times. The wells were blocked with blocking buffer (20 mM Tris Buffered Saline with 3% Tween 20) and incubate at 18-24 °C for 120 minutes After discarding the solution from the wells, the pate was washed with wash buffer 1 time. UC positive serum samples
diluted 1:50 with coating buffer were added into the wells (100µl/well). The plate was incubated at 18-24 °C for 30 minutes. After discarding the solution from the wells, the plate was washed with wash buffer 3 times. Secondary Anti-Human IgG-HRP Conjugated Antibody solution (1:50000 diluted with wash buffer) was added to the wells (100µl/well). The plate was incubated at 18-24 °C for 30 minutes. After discarding the solution from the wells, the plate was washed with wash buffer 3 times. 1x TMB-H2O2 solution was added to the wells (100µl/well) and incubated in a dark room for 15 minutes. Finally, the stop solution was added (100µl/well) and absorbance read in an ELISA plate reader within 15 minutes of adding the stop solution. (Figure 19)

**Figure 18: Extraction of intact neutrophil nuclei and coating on to ELISA plate**

In order to establish the qualitative reference range for the UC ELISA assay, 45 healthy control specimens were tested and the reference range was established based upon their ELISA OD values. Cut-off = (Mean+3*SD). An OD < Cut-off value was considered as negative for UC and OD ≥ Cut-off value was considered positive for UC. All the 45 UC specimens along with standard controls were tested using In-house UC ANCA Assay.
It has been established in the earlier part of the experiments and through published literature that UC antigens are located in the nucleus of the neutrophils (Mallolas J, et. al, 2000). Hence an attempt was made to develop an assay which would utilise this fact to enhance the performance characteristics of diagnostic test for UC. The intact nuclei extracted from human neutrophils were coated onto IIF slides with an assumption that the UC antigens would be better exposed to the ANCA antibodies in the UC positive patient sera and hence this modified UC ANCA IIF assay will exhibit enhanced performance characteristics as compared to the traditional ANCA IIF assay. Thus human neutrophils were lysed to extract intact nuclei as source of UC antigens using an in-house developed and standardised protocol. The Neutrophil Lysis Protocol was designed to separate the nuclear and cytoplasmic fractions of the neutrophils using change in osmotic pressure of the medium in which the neutrophils were suspended. A sucrose gradient was employed to separate the intact nuclei from the cytoplasmic extract of the neutrophils. The intact nuclei thus obtained were used for UC ANCA IIF slide preparation. The UC ANCA IIF slides were treated with UC specimens followed by FITC conjugated Goat Anti Human IgG antibodies. The slides were visualised under UV microscope. Due to the absence of whole neutrophils, the result interpretation was

Figure 19: Protocol for UC ELISA Assay

Modified UC ANCA IIF Assay Development

It has been established in the earlier part of the experiments and through published literature that UC antigens are located in the nucleus of the neutrophils (Mallolas J, et. al, 2000). Hence an attempt was made to develop an assay which would utilise this fact to enhance the performance characteristics of diagnostic test for UC. The intact nuclei extracted from human neutrophils were coated onto IIF slides with an assumption that the UC antigens would be better exposed to the ANCA antibodies in the UC positive patient sera and hence this modified UC ANCA IIF assay will exhibit enhanced performance characteristics as compared to the traditional ANCA IIF assay. Thus human neutrophils were lysed to extract intact nuclei as source of UC antigens using an in-house developed and standardised protocol. The Neutrophil Lysis Protocol was designed to separate the nuclear and cytoplasmic fractions of the neutrophils using change in osmotic pressure of the medium in which the neutrophils were suspended. A sucrose gradient was employed to separate the intact nuclei from the cytoplasmic extract of the neutrophils. The intact nuclei thus obtained were used for UC ANCA IIF slide preparation. The UC ANCA IIF slides were treated with UC specimens followed by FITC conjugated Goat Anti Human IgG antibodies. The slides were visualised under UV microscope. Due to the absence of whole neutrophils, the result interpretation was
simplified and could be stated as UC positive for fluorescence observed in the nuclei and UC negative for absence of fluorescence. The in-house developed modified UC ANCA IIF assay has a rather simplified interpretation of the results due to only the nuclei, being present on the slides as the source of UC antigens. There was no need to interpret the various patterns of fluorescence, rather only a qualitative estimation of presence or absence of fluorescence. The protocol developed for modified UC ANCA IIF Assay was as follows.

**Modified UC ANCA IIF Slide Preparation**

5ml blood sample was collected from a healthy individual as source of neutrophils. It was treated with 0.5M EDTA (50µl) and layered on 5ml Polymorph Prep. The blood was centrifuged at 1500rpm for 30 minutes at 15°C. The neutrophil layer (buffy coat) was separated by pipetting and washed with 5ml Hanks Basal Salt Solution (HBSS). It was centrifuged once again at 1500 rpm for 15 minutes at 15°C. The supernatant containing HBSS was decanted and cell pellet was treated with RBC Lysis Buffer (0.82% Ammonium chloride solution) to remove RBCs. RBC Lysis: The pellet was dissolved in 5 ml chilled RBC lysis buffer and incubated at 4°C for 10 minutes. Then it was centrifuged once more at 1500 rpm for 15 minutes at 15°C. The above process was repeated 3–4 times till the pellet turned white. This cell pellet was washed with 5 ml HBSS and centrifuged at 1500 rpm for 15 minutes at 15°C. Supernatant containing HBSS was decanted and the pellet was redissolved in 10 ml HBSS.

The HBSS solution containing intact neutrophils was centrifuged at 1500 rpm for 15 min at 15°C. Supernatant containing HBSS was decanted and the cell pellet was redissolved in 1 ml of ice cold Buffer A. This solution was centrifuged at 600 g for 10 minutes. The supernatant was separated as the cytoplasmic extract and previous step was repeated for the pellet. After this step the pellet was redissolved in 1 ml of ice cold Buffer B and centrifuged at 16,000 g for 30 minutes. The supernatant containing Buffer B was discarded and pellet containing the intact nuclei was resuspended in 1 ml Buffer A and centrifuged at 600 g for 10 min. The supernatant was separated as the cytoplasmic extract and previous step was repeated for the pellet. After this step the pellet was resuspended in 1 ml of ice cold Buffer B and centrifuged at 16,000 g for 30 minutes. The supernatant was discarded and pellet containing the intact nuclei was resuspended.
in 1 ml Buffer A. This cell suspension was diluted 1:2 using PBS, was coated (50µl/well) on IIF slide and the slide was incubated at room temperature for 45 minutes. The slide was blotted on tissue paper to remove excess liquid. The slide was then immersed in molecular grade ethanol (100%) for 2 hours. The slide was dried with dryer and used for Modified UC ANCA IIF or stored at 4 ºC in dry condition for further use. The process is outlined in Figure 20.

![Figure 20: Modified UC ANCA IIF Slide Preparation Protocol](image)

**Modified UC ANCA IIF Assay Protocol**

The UC specimens were diluted 1:40 using phosphate buffered saline. The Modified UC ANCA IIF slides were coated with the diluted specimen solution (30µl/well). They were incubated in a humid chamber at 18-24ºC for 30 minutes. Excess liquid was blotted from edges. 1 drop of FITC Conjugated Goat Antihuman IgG antibody (1:50000) was added to the plate and the slide was incubated at 18-24ºC for 30 minutes in a humid chamber. Excess liquid was discarded and the slide was washed gently with PBS. The slide was immersed in PBS with 2-3 drops of Evan’s Blue counter stain for 10 minutes. After blotting the excess liquid on tissue paper, 1 drop of Mounting
Medium (glycerol) was placed on each well. The slide was observed under UV microscope for fluorescence. The process is outlined in Figure 21.

**Figure 21: Modified UC ANCA IIF Assay Protocol**

**Comparison of Performance Characteristics of In-house Assays with Commercial Gold Standard**

The performance characteristics of in-house assays developed for diagnosis of UC were compared to those of the commercially available gold standard BioRad assays. The comparison of Specificity, Sensitivity, Accuracy, PPV, NPV and ROC Curve Analysis was performed with the help of statistical tools SPSS 10 software. The Specificity, Sensitivity, NPV and PPV were calculated using a 2x2 contingency table. (Table 12)

- Specificity = Σ True negative / Σ Condition negative
- Sensitivity = Σ True positive / Σ Condition positive
- Negative Predictive Value = Σ True negative / Σ (True Negative + False Negative)
- Positive Predictive Value = Σ True positive / Σ (True Positive + False Positive)
Accuracy: To test the intra and inter assay variability of the In-house Assay, three UC positive and three healthy control specimens were tested at different dilutions (1:20, 1:40 and 1:60) during a period of one month using the three in-house developed assays to check whether the positives were reported as positives and negatives were reported as negatives.

Table 12: 2x2 contingency table to calculate the performance characteristics

<table>
<thead>
<tr>
<th></th>
<th>Total Population</th>
<th>Predicted Condition Positive</th>
<th>Predicted Condition Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>True Condition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Condition Positive</td>
<td>True Positive</td>
<td>False Negative</td>
<td>(Type II Error)</td>
</tr>
<tr>
<td>Condition Negative</td>
<td>False Positive</td>
<td>False Positive</td>
<td>(Type I Error)</td>
</tr>
</tbody>
</table>

Receiver-Operating Characteristic (ROC) Curve Analysis

ROC curve is a statistical tool used to analyze the efficiency of diagnostic assays. A ROC curve, is a presentation of the sensitivity against the specificity of a test in a graphical format. The curve is obtained by plotting the true positive rate (TPR or Sensitivity) against the false positive rate (FPR or (1-Specificity)). The TPR is the number of specimens correctly identified as positive by the assay amongst all the positive specimens. The FPR is the number of specimens incorrectly identified as positive by the assay amongst all the negative specimens. The ROC space is plotted on the graph with X axis as the FPR and Y axis as the TPR. TPR is equivalent to sensitivity and FPR is equivalent to (1 – specificity). Hence the ROC graph is also called the sensitivity vs (1 – specificity) plot. Thus the ROC curve helps to test the ability of the assay to differentiate between the true positive and the false positive. The most desirable assay would result in a point in the upper left corner (0,1) of the ROC space, signifying 100% sensitivity with no false negatives and 100% specificity with no false positives. A bad test which would differentiate between the test results randomly, like flipping a coin, would be place on the diagonal line dividing the ROC space into two halves. An assay which produces grossly erroneous results would be placed in the
bottom right area of the ROC space. In the figure presented below, the ROC space is depicted with a diagonal of random guess dividing the ROC space. The points above the diagonal, moving towards perfect classification of (0,1) represent a better prediction by the assay (Point A). The points on the diagonal represent completely random assays with no ability to distinguish between the true positives and false positives (Point B). The points below the diagonal moving towards bottom right corner represent assays which give erroneous results which are completely unreliable. (Point C). (Figure 22)

**Figure 22: The ROC space and plots**
(Source: http://gim.unmc.edu/dxtests/roc3.htm)

**ROC Area Under Curve:**

The ROC Area Under Curve is a statistical tool to compare two or more assays for their worthiness in application. The ROC-AUC graph in figure 23 depicts 3 curves representing three different assays one of which is excellent, the other is good and the third one is worthless as a diagnostic test. The efficiency of an assay depends upon its ability to correctly distinguish between the positives and negatives in a test population. This efficiency is measured as the function of the ROC-AUC. An AUC value of 1 depicts a perfect classification with 100% sensitivity and 100% specificity. An AUC value of 0.5 represents a worthless assay which can distinguish between positives and negatives with the same probability as flipping a coin or rolling a die. The AUC value between 0.5 to 1 represents the assays which are better than random. Hence as the value of AUC increases from 0.5 up to 1, the ability of the test to correctly diagnose the condition without detecting false positives or negatives enhances and so does the
worthiness of the assay. It is desirable to have a AUC of higher value in order to grade a test as a good diagnostic assay. When comparing the accuracy of two or more assays, their ROC curves are plotted on the ROC graph and the ROC-AUC is calculated. The assay which shows the highest AUC value is considered to be the best and the most accurate amongst the assays being compared.

Area Under Curve:

- 0.90-1 = excellent
- 0.80-0.90 = good
- 0.70-0.80 = fair
- 0.60-0.70 = poor
- 0.50-0.60 = fail

![ROC Curve for Comparison of Diagnostic Tests](http://gim.unmc.edu/dxtests/roc3.htm)

**Figure 23: ROC curve for Comparison of Diagnostic Tests**  
(Source: [http://gim.unmc.edu/dxtests/roc3.htm](http://gim.unmc.edu/dxtests/roc3.htm))

An ROC curve helps to determine the relationship between sensitivity and specificity of an assay. As the ROC curve moves closer to the top left of the ROC space, the accuracy of the test increases. As the curve moves closer to the diagonal dividing the ROC space, the randomness of the test increases; i.e. the accuracy decreases. Thus the AUC becomes the quantifiable indicator of the accuracy of the assay. To compare two or more assays the ROC-AUC of the tests helps to determine which is better overall at distinguishing patients with and without disease.

The three in-house developed assays were compared to the commercial standard BioRad assay. The performance characteristics of each of the assay were determined and their worthiness was analyzed. Finally, the ROC-AUC were plotted and compared for all the four assays and the best assay for diagnosis of UC was determined.