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Development of an indirect immunofluorescence based assay for diagnosis of ulcerative colitis in Indian population

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A B S T R A C T

The prevalence of Ulcerative Colitis (UC), once thought to be negligible, has increases exponentially in the Indian population. The development of novel, cost effective and time efficient Indirect Immunofluorescence (IIF) based assay for detection of anti-neutrophil cytoplasmic antibodies (ANCA) and diagnosis of UC in the Indian population is discussed. A novel IIF based assay was developed using intact nuclei from healthy patients to detect atypical p-ANCA in patients suffering from UC. Sera from 45 patients diagnosed with UC, 45 healthy controls and one related disease control were tested using a novel UC-ANCA assay and validated by commercially available ANCA IIF assay. Prevalence of ANCA amongst UC patients in the Indian population was determined. Atypical p-ANCA was detected in 86.6% of the patients using the UC-ANCA assay as compared to 71.1% using the commercial ANCA assay. The validation of UC-ANCA assay with a commercially available ANCA IIF assay resulted in higher sensitivity. The UC-ANCA assay proved to be not only enhanced in terms of performance but also comparatively economical and rapid. The novel UC-ANCA assay may prove to be very useful in identification and differentiation of UC patients from typical ANCA positive subjects suffering from other autoimmune diseases at one tenth the cost of clinically available ANCA IIF tests which will immensely benefit the cost constrained diagnostic field of developing countries.

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1. Introduction

Inflammatory bowel disease (IBD) is a broad term that describes conditions with chronic or recurring immune response and inflammation of the gastrointestinal tract. The two most common types of inflammatory bowel diseases are UC and CD [1,2]. In CD, inflammation affects the entire digestive tract whereas in UC, only the large intestine is affected. Both the types of IBD are characterized by an abnormal response to the body’s immune system [1–3]. Substantial advances in the understanding of the molecular pathogenesis of IBD have been made in recent times [4,5]. Genetic studies highlight the importance of host–microbe interactions in the pathogenesis of IBD [6–8]. Autoimmune processes may play a role in the pathogenesis of IBD since many independent studies have found several types of auto antibodies involved in the diseases, such as antibodies to neutrophils like ANCA and anti-Saccharomyces cerevisiae antibodies [ASCA] [9–14]. UC has an incidence rate of 1–20 positive cases per 100,000 individuals per year, and a prevalence of 8–246 per 100,000 individuals [15,16]. The incidence rate is the number of new cases per population in a given time period whereas the prevalence rate is the proportion of cases in the population at a given time. According to the studies in rural Indian subcontinent, the proportion of UC is higher as compared to the western countries where CD is seen in a higher proportion [18].

According to the international consensus statement, indirect immunofluorescence should be used for ANCA screening [17]. Indirect immunofluorescence results help to distinguish among c-ANCA, p-ANCA and atypical p-ANCA patterns. ANCA detected in UC are called atypical p-ANCA, since they differ substantially from c-ANCA and p-ANCA [3]. Different groups have identified atypical p-ANCA in UC in varying proportions ranging from 50% up to 90% [10]. p-ANCA are generally seen in patients with microscopic polyangi-
itis and are reactive to myeloperoxidase antigen (MPO) whereas c-ANCA are observed in sera from patients with Wegener’s granulomatosis and are reactive to proteinase 3 antigen (PR3) [19]. Both MPO and PR3 are known to be present in the azurophilic granules observed in the cytoplasm of the neutrophils. p-ANCA pattern is in fact an artifact of ethanol fixation, which is a result of the migration of positively charged cytoplasmic proteins onto the surface of the negatively charged nucleus. Hence typical p-ANCA exhibits perinuclear pattern on ethanol fixation and diffused cytoplasmic pattern on formalin fixation where in the migration is restricted. On the other hand, the target antigens of atypical p-ANCA have not been yet identified. They may include lactoferrin, cathepsin G, elastase, lysozyme, bacterial permeability increasing protein, catalase, α-enolase, and lamin B1 [20]. Various studies have suggested these are not cytoplasmic antigens, like those for typical p-ANCA, but nuclear antigens, associated with the inner side of the neutrophils’ nuclear membrane. Few granular and non-histone chromosomal proteins; High Mobility Group Proteins (HMG1 and HMG2) and a myeloid cell-specific protein that is localized in the nuclear periphery are suspected to be the potential target antigens [21,22]. Hence atypical p-ANCA shows no change in the ANCA IIF pattern when fixed with different fixatives like ethanol and formalin. This is the differentiating criteria employed to distinguish atypical p-ANCA from typical p-ANCA.

The physical symptoms for Wegener’s granulomatosis include persistent runny nose, cough, ear infections, joint aches, nosebleeds, shortness of breath, sinusitis, skin sores, eye pain, burning sensation in the eyes, eye redness, vision problems, etc. The physical symptoms for Microscopic polyangiitis are rashes (usually over the legs), muscle aches, joint pain, cough and shortness of breath. On the other hand, the physical symptoms for UC are diarrhea-often with blood or pus, abdominal pain and cramping, rectal pain, rectal bleeding-passing small amount of blood with stool, urgency to defecate, inability to defecate despite urgency and weight loss. As can be noted, the physical symptoms of UC are categorically distinct as compared to ANCA related Vasculitits, namely, Wegener’s granulomatosis and Microscopic polyangiitis.

Although the target antigens for p-ANCA are known to be different from those of atypical p-ANCA, their fluorescence patterns on IIF staining are very similar to each other giving rise to significant discrepancies among laboratories with regards to interpretative criteria and reporting schemes. Hence considerable amount of technical expertise is required to differentiate between p-ANCA and atypical p-ANCA patterns. The ANCA IIF assay is not used till date as a diagnostic test for UC due to its low sensitivity and variability in interpretation of the ANCA patterns. A few commercial IIF assays are available in the market which detect different ANCA patterns like c-ANCA and typical p-ANCA. But none of these assays are used as stand-alone tools in clinics, and hence are only recommended as an adjunct to other clinical findings in diagnosis and prognosis of Wegener’s granulomatosis and Microscopic polyangiitis [23–25]. Moreover, these assays are mainly manufactured in the western countries and imported in India, thus leading to very high cost of the diagnostic tests. In a country like India where more than half of its population lives near the poverty line, it becomes very difficult for patients to afford the expensive diagnostic tests, thus leading to delayed and improper diagnosis and increase in the mortality rate.

In the present study, we aim to develop a novel UC-ANCA IIF assay using intact nuclei of human neutrophils and not the whole neutrophils for the detection of atypical p-ANCA. This assay will potentially eliminate the need to interpret the different ANCA patterns since only the atypical p-ANCA stains the nucleus and the cytoplasmic antigens MPO and PR3 do not come into play in UC. By virtue of substantially low cost, UC-ANCA assay may be used as a screening test or as a confirmatory test in conjunct with physical symptoms and other clinical findings to confirm the presence of UC in patients reporting to the clinic with symptoms similar to IBD. Thus the novel assay can aid in the diagnosis of UC in a resource-limited Indian scenario, which will dramatically reduce the cost per test in order to enable UC diagnostic tests reach the underprivileged masses.

2. Materials and methods

2.1. Reagents

The materials used for the Assay preparation comprised of reagents required for pre-processing of the ANCA IIF slides and reagents required for the Assay. IIF slides were prepared using an in-house developed protocol. Blood was collected from healthy volunteers as source of neutrophils. Polymorph prep was purchased from Fresenius Kabi, Norway. Sodium chloride and Magnesium chloride were purchased from Rankem, India. Sucrose, Sodium phosphate dibasic, Potassium phosphate monobasic, n-propyl gallate were purchased from SRL, India. HEPES (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid), Glycerol, Tris and Evan’s Blue Dye were sourced from Sigma Aldrich, USA and Human Serum Albumin from Reliance, India. Molecular Grade Ethanol was purchased from Merck, USA. Goat Anti-human FITC (Fluorescein isothiocyanate) Conjugated IgG was procured from GeNei, India. Commercially available ANCA IIF and ANA Hep-2 Assay was purchased from BioRad, USA.

2.2. Specimen collection and storage

45 UC patients from MGM Hospital, Navi Mumbai were selected for the present experiment. The experimental protocol of this study was approved by the Institutional Research Review Committee and Ethical Review Committee of MGM Institute of Health Sciences, Navi-Mumbai. All the study subjects were enrolled after obtaining their informed consent. The UC diagnosis was confirmed by physical symptoms, endoscopy results and supplementary clinical findings. 45 healthy individuals were included in the study as negative controls. One Autoimmune Vasculitis specimen was included as a related autoimmune disease control. The serum specimens of the subjects were collected and preserved at –20°C till further experimentation. All the samples had been tested negative for antinuclear antibodies which are known to interfere in ANCA detection.

ANCA detection was performed using two IIF Assays. One Assay, viz. commercially recommended ANCA IIF Assay from BioRad, USA, was used as the IIF reference standard for ANCA detection and the in-house developed assay UC-ANCA was used to test its efficacy in comparison with the former. The test samples were diluted 1:20 with sample diluents and visualized using UV light (490 nm wavelength) by conjugating them with FITC labeled IgG secondary antibodies.

2.3. UC-ANCA assay development

The UC-ANCA assay made use of intact nuclei from human neutrophils coated onto IIF slides. Due to the absence of whole neutrophils, the result interpretation was simplified and could be stated as positive for fluorescence observed in the nuclei and negative for absence of fluorescence. The methodology for assay development is as follows:

2.3.1. IIF slide preparation

5 ml blood sample was collected and treated with 0.5 M EDTA (50 µl) and layered on 5 ml polymorph prep. The tubes were centrifuged at 1500 rpm for 30 min at 15°C. The neutrophil layer (buffy coat) was separated and washed with 5 ml Hank’s basal salt solution
Table 1

<table>
<thead>
<tr>
<th>Ulcerative Colitis (n = 45)</th>
<th>Healthy Controls (n = 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BioRad ANCA</td>
<td>UC-ANCA</td>
</tr>
<tr>
<td>32 (71.1%)</td>
<td>39 (86.6%)</td>
</tr>
</tbody>
</table>

(HBSS). It was re-centrifuged at 1500 rpm for 15 min at 15°C. The supernatant was decanted and 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 min. Then it was centrifuged at 1500 rpm for 15 min at 15°C. The above process was repeated 3–4 times till the pellet turned white. This pellet was washed with 5 ml HBSS and centrifuged at 1500 rpm for 15 min at 15°C. Supernatant was decanted and the pellet was resuspended in 1 ml of ice cold Buffer A (250 mM Sucrose, 5 mM MgCl₂, 10 mM Tris–HCl at pH 7.4) and centrifuged at 600 g for 10 min. The supernatant was discarded and previous step was repeated for the pellet. After this step the pellet was resuspended in 1 ml of ice cold Buffer B (1.8 M Sucrose, 1 mM MgCl₂, 10 mM Tris–HCl at pH 7.4) and centrifuged at 16,000 g for 30 min. The supernatant was discarded and pellet containing the intact nuclei was resuspended in 1 ml Buffer A. This nuclei suspension was coated on IIF slide (30 μl per well) and the slide was incubated at room temperature in a humid chamber for 30 min. The slide was blotted on tissue paper to remove excess liquid and then immersed in molecular grade ethanol (100%) for 2 h. The slide was dried with a dryer and used for UC-ANCA staining. The slides were stored at 4°C in dry condition for future experimentation.

2.3.2. UC-ANCA protocol

UC specimens were diluted 1:20 using phosphate buffered saline. The UC-ANCA slides were coated with diluted specimen solution (30 μl/well) and incubated at room temperature for 20 min in a humid chamber. The slides were blotted at the edges to remove excess liquid. 30 μl HTIC conjugate per well was added and incubated at room temperature for 20 min in a humid chamber. Excess liquid was discarded and the slides were washed gently with PBS. The slides were further immersed in phosphate buffered saline (PBS) with 2–3 drops of Evan’s Blue counter stain for 5 min. The slides were blotted on tissue paper to remove excess liquid. 1 drop of mounting medium (Glycerol) was placed on each well and observed under UV fluorescent microscope at 40× magnification of the objective.

3. Results

As per the BioRad ANCA Test, the specimen results were interpreted based upon the pattern showed by them on the ethanol fixed BioRad ANCA slides. The ANCA patterns seen were: c-ANCA pattern was presented as coarse speckled granular cytoplasmic staining; p-ANCA pattern was seen as fine rimmed perinuclear staining and atypical p-ANCA pattern was observed as thick peri-nuclear staining along with infrequent nuclear extensions (Fig. 1). The in-house developed UC-ANCA has a rather simplified interpretation of the results with positive result being fluorescence observed at the nuclear periphery and negative result being absence of fluorescence on the UC-ANCA slide (Fig. 2).

As per the results shown in Table 1, the BioRad ANCA IIF Assay, had a sensitivity of 71.1% and a specificity of 100%. On the other hand, In-house developed UC-ANCA Assay had a sensitivity of 86.6% and a specificity of 100%. None of the healthy controls showed positivity for ANCA pattern. The Autoimmune vasculitis disease control was observed to be negative on both the assays. The occurrence of atypical p-ANCA amongst UC patients in Indian population using BioRad ANCA assay was found to be 71.1% and using UC-ANCA assay was found to be 86.6%.

The test outcome results of ANCA IIF Assay from BioRad, USA were compared to in-house developed ANCA IIF Assay as shown in Table 2. The comparative analysis of Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of UC-ANCA assay and BioRad ANCA assay is illustrated in Fig. 3. The in-house ANCA IIF Assay had better sensitivity and negative predictive value as compared to ANCA IIF Assay from BioRad. To test the intra and inter assay variability of the In-house UC-ANCA Assay, three UC positive and three healthy control specimens were tested at variable dilutions (1:20, 1:40 and 1:60) over a period of one month using different batches of UC-ANCA slides. The positives were observed as positives and negatives were observed as negatives.

4. Discussion

The role of ANCA in UC has been suggested in many studies [26–29]. In serological testing, IIF remains one of the most reliable techniques to detect ANCA in UC [3,17,30]. However, considerable amount of expertise is required to correctly distinguish between the various patterns of ANCA. Differentiating the UC related atypical p-ANCA pattern from the non-UC autoimmune typical p-ANCA pattern on ethanol fixed neutrophils by IIF presents various challenges [3,17]. Nevertheless, given the major differences in diagnostic implications, this distinction is vital. The microscopic criteria suggested by Terjung et al. [31] has not been extensively adopted in IBD detection [32,33]. Serological specimens depicting the p-ANCA pattern on ethanol fixation and a faint or negative pattern using formalin fixation, determined the atypical p-ANCA pattern associated with IBD [3,17]. Previous study has reported that atypical IIF-ANCA samples, defined as being positive on ethanol- but negative on formalin-fixed neutrophils, had a very low ( < 1%) positivity rate for anti-PR3 or anti-MPO antibodies [34].

In the present study, we aimed to simplify the detection of atypical p-ANCA in UC patients by using a novel IIF based assay. Atypical p-ANCA was the predominant pattern observed amongst UC patients using the UC-ANCA immunofluorescence assay. Similar results were found by other authors earlier [35,36]. The in-house developed UC-ANCA assay simplified the process of interpretation of test results by eliminating the possibility of a c-ANCA and p-ANCA type fluorescence pattern. The results were interpreted as positive for fluorescence observed in the nuclei and negative for absence of fluorescence. This test produced reasonably higher sensitivity (86.6%) as compared to that of BioRad ANCA IIF assay (71.1%). This result can be attributed to the fact that the atypical p-ANCA specific antigens, being localized on the inner periphery of the nuclei, may have been better exposed to the ANCA antibodies in the patient sera in the UC-ANCA slides containing intact nuclei rather than the BioRad IIF slides coated with whole neutrophils. The NPV of UC-ANCA assay was also seen to be significantly higher than that of BioRad ANCA assay. Also, none of the healthy controls or the related disease Autoimmune Vasculitis control showed positivity on the UC-ANCA assay, justifying its high specificity. Thus with a high specificity and an enhanced sensitivity the UC-ANCA assay will prove to be a better diagnostic test for UC as compared to other IIF assays available in the market. The use of this IIF ANCA test as a standalone may be insufficient for diagnosis of patients suspected to have UC [37]. However, the high sensitivity of this assay supports its use as a first line screening test for UC [38,39], which is generally not employed by gastroenterologists due to high costs involved. Also, the high specificity may also enable UC-ANCA assay to be used as a confirmatory test for UC after corroborating the test results with physical symptoms and other clinical findings.
Fig. 1. c-ANCA, typical p-ANCA and atypical p-ANCA staining patterns as observed on BioRad ANCA slide. Magnification of objective 40×.

Fig. 2. Atypical p-ANCA staining on UC-ANCA slides showing nuclear fluorescence and absence of fluorescence. Magnification of objective 40×.

Table 2
Comparison of test outcome of In-house developed UC-ANCA Assay with BioRad ANCA IIF Assay with respect to UC diagnosis.

<table>
<thead>
<tr>
<th>Ulcerative colitis⁵</th>
<th>Healthy controls⁶</th>
<th>UC Status of Subjects</th>
<th>UC-ANCA Assay</th>
<th>BioRad ANCA Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
<td>Positive</td>
</tr>
<tr>
<td>UC Status of Subjects</td>
<td>Positive</td>
<td>39</td>
<td>6</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>0</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>51</td>
<td>90</td>
<td>32</td>
</tr>
</tbody>
</table>

⁵ Total number of UC patients: 45.
⁶ Total number of control subjects: 45.

Application of UC-ANCA assay would not only improve diagnostic accuracy, but also minimize intra- and inter-laboratory variation, given its relatively straightforward and unambiguous interpretation as compared to the often subtly different staining patterns on an ethanol-fixed neutrophil slide.

The specific antigens have not been yet elucidated for atypical p-ANCA. Radice et al. [40] have stated that the specificity of atypical p-ANCA was not solely directed against either one of the following known antigens; Proteinase 3 (PR3) or Myeloperoxidase (MPO). Radice et al. [40] also studied serum samples selected on the basis of disease and antigenic specificity, and found that samples from patients with Vasculitis were positive for anti-PR3 or anti-MPO antibodies whereas serum samples from patients with UC were not. Our results suggest that performing the ANCA detection using
the in-house developed UC-ANCA assay, with the use of nuclei of neutrophils will prove to be a first line diagnostic test for patients suspected of UC. Also the determination of atypical p-ANCA may be useful to differentiate UC from non UC autoimmune gastrointestinal diseases. This will lead to early diagnosis and initiation of treatment for UC.

Our study has significant implications for the optimal cost-effective approach for detecting ANCA antibodies in human patients. India being a developing country, with almost half of its population living near the poverty line, majority of the population can rarely afford expensive tests for diagnosis of diseases followed by even more expensive treatment. It is essential to note that the projected cost of the in-house developed UC-ANCA Assay is one-tenth of the average cost of an ANCA-II Assay imported from western countries. Our study therefore will have significant cost implications for diagnostic laboratories, particularly due to the increasing number of patients now being screened for ANCA in India. The cost efficiency of UC-ANCA assay will prove to be a decisive factor in including this test in the first line of tests for the early diagnosis of UC.

5. Conclusion

A novel indirect immunofluorescence based UC-ANCA assay was developed and validated for the diagnosis of UC. The assay showed enhanced sensitivity and specificity at fraction of the cost of imported ANCA diagnostic assays. In a country of more than 1.2 billion population, this test will open up new avenues in autoimmune UC diagnostics to understand its pathophysiology and etiology. Further research in this topic may be carried out in elucidating the specific antigen or a panel of antigens corresponding to atypical p-ANCA which will dramatically change the way we look at UC and usher in a new era in its diagnosis and treatment.

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Conflict of interests

The authors declare no potential conflict of interests.

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