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

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ABSTRACT

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 human neutrophils 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.

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-

Abbreviations: IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; ANCA, anti-neutrophil cytoplasmic antibodies; IIF, indirect immunofluorescence.

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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 Vasculitis, 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-piperazineethanesulfonic 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 investigation. 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 it 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