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Original Research Article

Immunofluorescence Patterns of Antinuclear Antibodies in the Indian Population

Poulami Biswas[1], Partha Guchhait[2], Bhaskar Narayan Chaudhuri[2], Sayahnika Dutta[1], Satadal Das[2*]

[1]Department of Biotechnology, Vellore Institute of Technology, Vellore, Tamil Nadu, India
[2]Department of Microbiology and Molecular Biology, Peerless Hospitex Hospital and Research Centre Limited, Kolkata, India

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Abstract: Autoimmune diseases are chronic hypersensitive diseases that occur due to an inability of the immune system to recognize self-antigens as part of its system. Antinuclear autoantibodies (ANA) are antibodies that target the proteins located in the nucleus, mitotic spindle, nuclear membrane, and cytoplasmic substructures. Detection of ANA is done using either the ANA-IIF or by ELISA method; the latter might be slightly more sensitive as compared to the ANA-IIF but the specificity is however low. ANA patterns such as nuclear, cytoplasmic, and mitotic can be important to clinical practice because they suggest the significance, as well as the nature of autoimmune Connective Tissue Disorder (CTDs). ANA is a predictor of autoimmune diseases, and the risk is greater in female individuals. The specific objective of this study was to assess the prevalence of ANA and the detection of demographic correlations with them; thereby it would help in the identification of the various autoantibody subclasses in the Indian people. The present cross-sectional descriptive study included 351 patient records from a tertiary care hospital in Kolkata, India expounds on the association of multiple ANA patterns with certain autoantibodies, and demography of the ANA-positive patients. Knowledge of such regional variations of autoimmune diseases would be useful to formulate a strategy for proper diagnosis of systemic autoimmune rheumatic diseases (SARDs) in India.
Keywords: Anti-Nuclear Antibody (ANA), Auto immunity, Connective Tissue Disorder (CTD), ANA-IIF.

INTRODUCTION

In autoimmune diseases (ADs), the immune system's inability to tolerate self-antigens can result in chronic inflammatory conditions that could lead to individual organ or generalized body damage [1]. In recent years, these diseases have substantially increased, reportedly affecting a total of 10% population in India [1] as compared to 4.5% of the global population [2]. These illnesses can thus be mediated by immune complexes including auto antibodies. One such group of auto antibodies are the antinuclear antibodies, also known as ANA, which have a potential to attack self-proteins situated in the nucleus of a cell including the mitotic spindle, nuclear envelope, cytoplasmic organelles, machinery cytosol and cell membranes [3].

Confirmation of ANA can be done by the following tests, namely, ANA-indirect immunofluorescence test or ANA-IIF and or the Enzyme-linked immunosorbent assay/ANA-ELISA. ANA-IIF is valuable as a primary method of screening for autoimmune connective tissue diseases, despite being non-specific. On the other hand, not only possible positive outcomes of AA diseases, but also of malignancy or other non-AA diseases and infections, Hashimoto thyroiditis or autoimmune hepatitis, are possible [4]. Since ANA is associated with malignancy, it has been found out that patient, who is positive to ANA, has better prognosis than Patients, negative to the test. This better outlook may be due to enhanced immune activity aimed at the neoplastic cells themselves [8]. It should also be mentioned that various influences can lead to false positive results including a particular range of medications, as well as first level relatives with no signs of autoimmune diseases [4].

ANA-IIF, which is supported by the American and European rheumatology associations (ACR and EULAR), lacks specificity and has a low positive predictive value at low titers. A titer of 1:40 could yield positive results in 25-30% of all healthy persons, with

***Corresponding Author:** Satadal Das
Department of Microbiology and Molecular Biology, Peerless Hospitex Hospital and Research Centre Limited, Kolkata, India

this proportion increasing as they grow older. It is advisable to employ a serum dilution yielding 95% specificity for this test among chronically normal population samples. The test's clinical importance increases with higher titers and the identification of certain autoantigens [4].

Nuclear patterns like homogeneous, speckled (fine/coarse), nucleolar, centromere and others are part of the various ANA staining patterns. Besides, Cytoplasmic patterns may includespeckled, mitochondrial-like, ribosomal-like, lysosomal-like or Golgi apparatus and cytoskeletal filaments, which differ from Mitotic patterns which includes mitotic spindle fiber, mitotic chromosomal, centrioles and nuclear mitotic apparatus [10]. It is generally agreed upon internationally that all staining patterns observed on IIF should be recorded, even if the phrase anti-nuclear antibody would only refer to nuclear staining patterns. Members of the International Consensus on ANA Patterns (ICAP) have defined 29 different HEp-2 cell patterns and discussed their clinical significance [3].

These fluorescence intensity patterns are clinically important since they usually reflect antibody concentration and are predictive of the severity of Connective Tissue Disorders (CTDs) [9]. A high titer helps in diagnosing CTDs, yet a low titer can still be found in some healthy people. The fluorescence intensity is presented qualitatively using a scale that ranges from 1+ to 4+. It is crucial to accurately interpret the IIF-ANA results and to consistently compare them with the patient's symptoms and indications [3]. Elevated ANA titers have been linked to a higher risk of AIDS development, with ANA positivity often appearing long before the clinical onset of autoimmune diseases. However, ANA can also be detected in over 20% of healthy individuals due to associations with various non-autoimmune conditions, such as cancers, infections, certain medications, and environmental factors. ANA prevalence is higher in women as compared to men and the reasons for which are believed to be genetic, hormonal and reproductive conditions and the ways still unknown [7].

Although ANA-IIF has considerable high sensitivity, it requires technical proficiency and is time-consuming, operator-dependent and labor-intensive. Over the last two decades, the ELISA method has been brought in to save time and effort compared to ANA-IIF. But, the American College of Rheumatology (ACR) declared the HEp-2 IFA was the “gold standard” to screen for ANA, in the year 2010 [4].

The importance of screening and quantifying antinuclear antibodies (ANA) react with intra-cellular antigens has been established in screening of SARD included SLE, SjS, MCTD, SSc and IIM [5].

In SLE, most autoantibodies are formed against intracellular antigens of the cell nucleus including double stranded DNA (dsDNA), histones, and other extractable nuclear antigens (ENA) and cell cytoplasm (ribosomal P protein). It is an acute septic inflammatory disease with some chronic features characterized by autoimmune inflammation of various organs. The disease primarily affects women, with a female-to-male ratio of 6-12:1 and the highest prevalence among the population with the reproductive age [6].

The objective of this study is to highlight the ANA patterns, particular antibody profile positive prevalence, and correlation with certain demographic information of patients visiting a hospital in Eastern India.

MATERIALS AND METHODS

This is a retrospective cross-sectional data analysis study. 351 ANA test records were collected from Peerless Hospital, Kolkata, India from January to June 2024, irrespective of age or gender and then evaluated. The data was encoded into Microsoft Excel 2021. All the patients were kept anonymous without revealing their identities as per hospital ethical committee guidelines.

Serum samples were collected from suspected autoimmune disease patients and subjected to ANA assessment using the EUROIMMUN ANA IFA biochip slides. This test kit provides a qualitative or quantitative semi in-vitro procedure for the detection of human IgG anti-nuclear antibodies in patients’ samples to help in the diagnosis of an autoimmune disease, especially rheumatic lupus. The degree of fluorescence is measured employing a fluorescence microscope. The principle is incubating test fields with diluted patient samples. If positive, specific antibodies (IgA, IgG, and IgM) bind to the antigens. These bound antibodies are then detected with FITC-labeled anti-human antibodies and visualized using a fluorescence microscope.

The TITERPLANE Technique, developed by EUROIMMUN, was used to standardize immunological analyses. 30 µl of diluted samples were applied to reaction fields on reagent trays, avoiding air bubbles. BIOCHIP slides were placed into tray recesses to initiate reactions and incubated for 30 minutes at room temperature (18°C to 25°C). BIOCHIP slides were rinsed with PBS-Tween and immersed in PBS-Tween for at least 5 minutes, using a rotary shaker. 25 µl of conjugate was applied to clean reagent trays using a stepper pipette. After blotting, BIOCHIP slides were placed into tray recesses and incubated for another 30 minutes at room temperature. Slides were rinsed again with PBS-Tween and immersed in fresh PBS-Tween for at least 5 minutes. The cover glasses were coated with a mounting medium, and then the BIOCHIP slides were oriented face down on the medium. The obtained was then slides analyzed under the fluorescence microscope

at 10X and 40X magnification. Each sample's fluorescent intensity was compared to the negative and positive controls in the kit. As per the Group 2 dilution scheme, samples were diluted from 1:100 to 1:10000 and the fluorescence intensities were graded from + to ++++. A titer of ≥1:80 were also used as the reference for defining ANA positivity as recommended by the manufacturer. ANA was categorized into subgroups based on titer levels: Negative, week positive, 1:40 – 1:80 positive, moderately positive 1:160 to 1:320 positive and strongly positive at a dilution of 1:640 or more. Finally, the relations between the frequency and pattern of ANA and gender and age of participants were explored; more precisely, the ANA occurrence was compared among participants in the age groups of 0 to 20; 21 to 40; 41 to 60; and over 60 years. The ANA patterns were divided into groups and sorted based on the classification system that was highlighted in the International Congress on Antinuclear Antibody Patterns (ICAP).

RESULT

A total of 351 patients were analyzed, with 18.23% (64 patients) tested positive for ANA. Among 64 positive cases, 49 cases were found to be female, and 15 cases were male. ANA positivity was highest in the 41 to 60 age group, with a mean age of 50 years. Women made up the majority of ANA-positive cases, accounting for 76.56%. In all age groups, females (76.56%) had a higher positivity rate than males (23.44%).

In the 0-20 age group, all affected individuals were female, accounting for 100% of the cases. In the 21-40 age group, 77.78% were female and 22.22% were male. Among those aged 41-60, 86.36% were female and 13.64% were male. Among those over 60, 63.64% were female, while 36.36% were male (Table 1).

Table 1: Distribution of ANA Positivity by Age and Gender, gender based distribution of different age groups are not statistically significant

Age group	Total affected	Total %	Male	Female
0-20	2	3%	0%	100%
21-40	18	28%	22.22%	77.78%
41-60	22	34%	13.64%	86.36%
>60	22	34%	36.36%	63.64%

Six different patterns of nuclear fluorescence were observed. Among the fluorescence-positive samples, the Nucleoplasm granular pattern was the most common, found in 37.5% of the cases. This was followed by the mixed pattern (20%), nuclear homogeneous & nuclear dotted pattern (17.18%), Cytoplasmic pattern (9.37%), other patterns (9.375%), and Nucleolar pattern (6.25%) (Fig 1, 3-5). In the Cytoplasmic pattern, which includes both granular and filamentous types, 66.67% of the affected individuals were female. In Nucleoplasm pattern (including both granular and speckled types), 75% of its affected patients were female. The Nuclear pattern, which comprised of homogeneous, dots, and speckled types, had a 90% female preponderance. In the Mixed pattern, 84% of those affected were female. The Other patterns, which include autoantibodies against spindle fibers, centromere, mitosin, and lysosome, had an equal gender distribution, with 50% being female (Table 2).

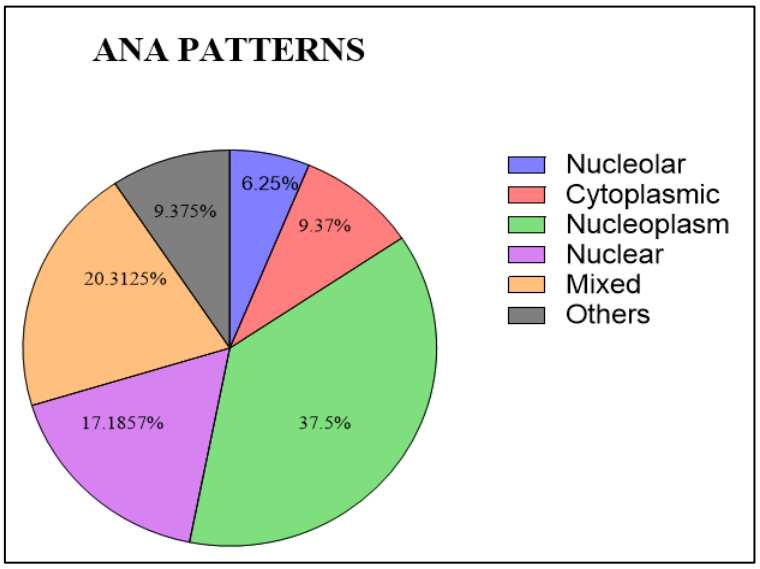

Fig 1: Distribution of different ANA Patterns among ANA positive individuals

Table 2: Distribution of ANA Patterns by Gender

Pattern	Female	Male	Total
Nucleolar	3	1	4
Cytoplasmic	4	2	6
Nucleoplasm	18	6	24
Nuclear	10	1	11
Mixed	10	1	11
Others	3	3	6

Additionally, we examined 51 patients for dsDNA by IFA, and 5 of them were tested positive for anti dsDNA antibody. All dsDNA Positive patients were female. High positivity seen in 21-40 aged individuals. Three (60%) out of the five positive dsDNA cases showed nuclear homogeneous pattern by the ANA-IFA test (Fig 2). DsDNA titer has a high positive predictive value in estimating disease severity and in treatment follow up.

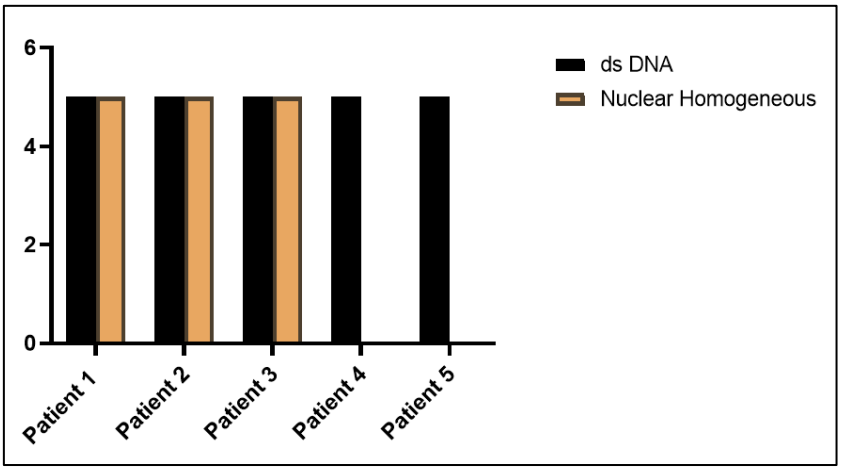

Fig 2: Concurrence of ds-DNA with Nuclear Homogeneous Pattern

DISCUSSION

The use of human cancer cell lines, such as Hep-2, in ANA testing has significantly increased sensitivity but also led to high false-positive rates. To address this, a biochip combining Hep-2 cells with primate liver was developed. This approach required the high sensitivity of Hep-2 cells to be confirmed by the primate liver response, thereby significantly reduce false positives. In India, where reliable and cost-effective screening tests are essential, ANA-IIF stands out for its ease of performance and affordability. Despite some subjectivity in interpretation, specific ANA patterns provide valuable clinical insights. With the rising prevalence of autoimmune disorders in India and limited prevalence data, documenting their occurrence and clinical significance is crucial.

The ANA-IIF test is a low-cost, efficient screening tool for determining the clinical diagnosis of autoimmune illnesses. The present study examined 351 patients' ANA findings and found 18.233% positivity. A tertiary care hospital &research center conducted a similar study in Central India had a higher ANA positive percentage of 30.8% [11], while investigations conducted in Bangalore by Sebastian *et al.*, found a positivity rate of 38% [12]. Research from Delhi [13] and Chandigarh [14] found lower positive rates of 11.1% and 18.9%, respectively.

In a study conducted in Jaipur in 2021, Choudhary et al. looked at the frequency and patterns of Anti nuclear antibody (ANA) in autoimmune illnesses. They found similar things about how ANA positivity is commonly in women and how certain ANA patterns are linked to various autoimmune diseases [5]. Ramachandran *et al.*,'s (2023) retrospective analysis of ANA patterns in SLE patients revealed that the speckled pattern was more prevalent, similar to our finding of Nucleoplasm granular /Speckled variety [6]. The major variation in positive rates throughout the nation's regions might be attributed to climatic, genetic, and inter-individual differences in regulating the various molecular causes of immunity. This shows the need for more research on autoimmunity in various cultures and sectors within our nation.

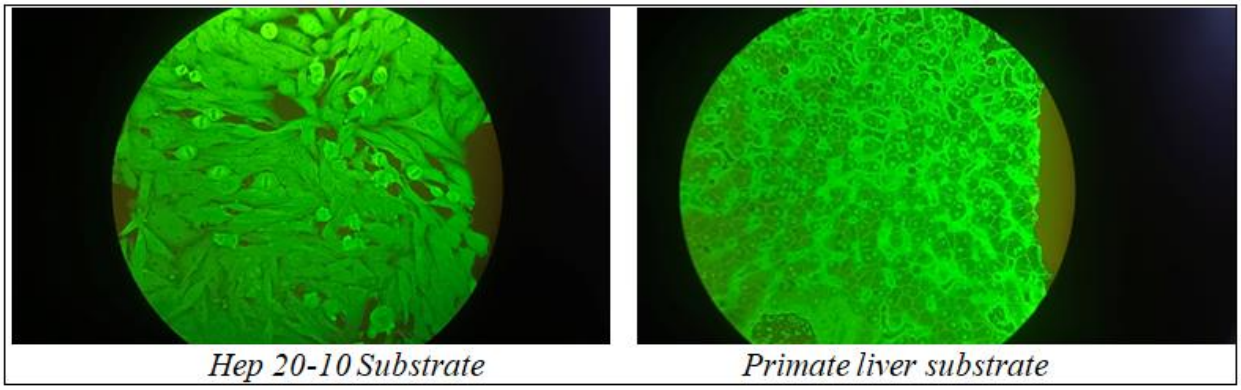

Fig 3: Nucleoplasm Granular Pattern

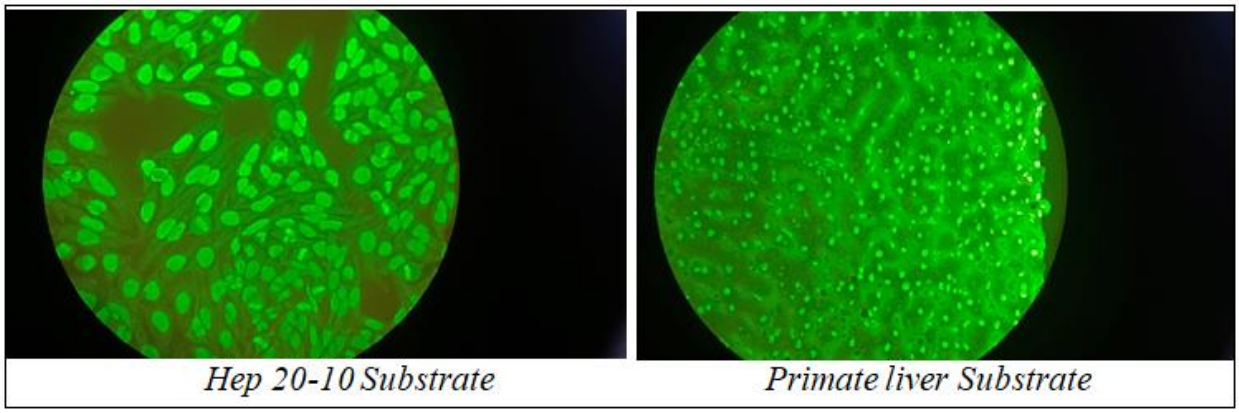

Fig 4: Nuclear Homogeneous pattern

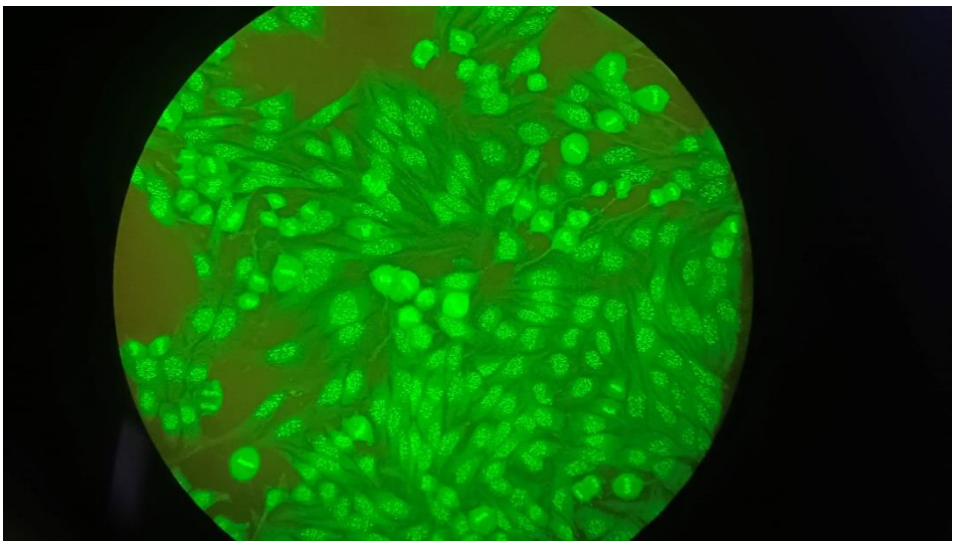

Fig 5: Nucleoplasm dotted pattern (46-92 dots; Antibody against Centromere)

CONCLUSION

This study exemplify the need of examination of ANA patterns and how certain autoantibodies in the Indian population are related to them. The results show that ANA positivity is more among females, especially those between the ages of 41 to 60. The most common fluorescence pattern was found to be the nucleoplasm granular pattern, which was followed by the mixed and nuclear homogeneous patterns. These patterns are clinically significant as they reflect the concentration of antibodies and predict the severity of connective tissue disorders. The study also validates the ANA-IIF test as a sensitive and cost-effective screening tool for autoimmune disorders. Given the increased frequency of autoimmune illnesses in India, this study emphasizes the need for more research into the clinical significance of ANA patterns across diverse ethnic and demographic groups in the nation. This might help to develop more

precise diagnostic and therapy options for autoimmune diseases.

Limitation of the study

Although ANA by IFA may be considered as a gold standard screening tool for AIDs, any particular /doubtful ANA pattern needs to be confirmed either by mono-specific ELISA or immunoblotting, which has not been done due to costly reagents and delayed turn-around time of the test.

Conflict of Interest: The authors declare no conflict of interest.

Author's Contribution

Dr. Satadal Das designed the study procedure, analyzed the data and corrected the manuscript. Ms Poulami Biswas and, Ms Sayahnika Dutta performed the experiment and evaluated the data under guidance of Dr. Partha Guchhait, Dr. Bhaskar Narayan Chaudhuri and Mr. Arup Kumar Dawn who also analyzed the data and corrected the manuscript.

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