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Original Research Article

PREVALENCE AND SPECTRUM OF ANTINUCLEAR ANTIBODIES USING INDIRECT IMMUNOFLUORESCENCE IN SUSPECTED CONNECTIVE TISSUE DISORDERS

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ABSTRACT

Background: Antinuclear antibodies (ANAs) are key serological markers in connective tissue disorders (CTDs). Indirect immunofluorescence assay (IIFA) on HEp-20-10 cells remains widely used because it provides both ANA positivity and clinically informative staining patterns. The objective is to determine the prevalence of ANA positivity by IIFA on HEp-20-10 cells in patients with clinically suspected CTDs at a tertiary care center in Western India and to describe the spectrum of ANA patterns and end-point titers.

Materials and Methods: This observational cross-sectional study was conducted over nine months at a tertiary care center in Pune, India. Consecutive patients with clinical suspicion of CTDs for whom ANA testing was ordered were included (N = 226). ANA testing was performed by IIFA on HEp-20-10 cells. Patterns were classified as per International Consensus on ANA Patterns (ICAP), and positive samples underwent serial dilutions to determine end-point titers. Descriptive statistics were reported with 95% confidence intervals (CI).

Results: Of 226 patients, 134 were ANA positive (59.29%; 95% CI, 52.78–65.49) and 92 were ANA negative (40.71%; 95% CI, 34.51–47.22). Fever (32.74%), generalized weakness (26.99%), breathlessness (19.47%), and joint swelling (15.04%) were the most frequent presenting symptoms. Among ANA-positive patients, nuclear-only staining occurred in 62.69%, cytoplasmic-only in 16.42%, and combined nuclear–cytoplasmic staining in 20.90%. The most common ICAP patterns were nuclear speckled coarse (AC-5; 42.54%) and nuclear speckled fine (AC-4; 31.34%); cytoplasmic reticular/AMA (AC-21) was observed in 26.12%. Most reactivities occurred at a titer of 1:100.

Conclusion: ANA positivity by IIFA was common among patients with suspected CTDs. Nuclear speckled patterns predominated, and cytoplasmic patterns were also frequent, supporting the utility of IIFA pattern interpretation and titer reporting in routine CTD evaluation.

Keywords: Antinuclear antibodies, Indirect immunofluorescence assay, HEp-20-10 cells, Connective tissue disorders, ICAP patterns.

INTRODUCTION

Connective tissue disorders (CTDs) are a heterogeneous group of autoimmune diseases characterized by multifactorial etiologies, overlapping clinical features, and multisystem involvement, with an estimated prevalence of 3–5% in the general population.[1] In India, CTDs are increasingly recognized, with reported estimates of 14–16 cases per 100,000 population, and systemic

lupus erythematosus (SLE) being the most frequently encountered entity.[2]

Antinuclear antibodies (ANAs) represent a key serological hallmark of CTDs and are routinely used for screening, diagnosis, and monitoring of these conditions. The ANA test supports the differential diagnosis of systemic autoimmune rheumatic diseases such as SLE, systemic sclerosis (SSc), rheumatoid arthritis (RA), and Sjögren's syndrome, and is generally requested when an autoimmune CTD is clinically suspected.[3] ANAs can be detected using several platforms, including indirect immunofluorescence assay (IIFA), enzyme-linked immunosorbent assay (ELISA), chemiluminescence, dot-blot immunoblot, and multiplex bead-based assays.[3] Despite the growing availability of antigen-specific and multiplex immunoassays, IIFA on HEp-2/HEp-20-10 substrates remains widely used as a screening method because it provides pattern-based information that may guide subsequent antigen-specific testing.[4]

The International Consensus on ANA Patterns (ICAP) has standardized nomenclature and classification of IIFA patterns on HEp-2/HEp-20-10 cells, thereby improving uniformity in reporting and facilitating clinical correlation.[5,6] In this context, understanding the distribution of ANA patterns and titers in local populations is essential for refining diagnostic algorithms and optimizing resource utilization.

The present study was undertaken to determine the prevalence of ANA positivity using IIFA on HEp-20-10 cells in patients with clinically suspected CTDs at a tertiary care center in Western India and to describe the spectrum of nuclear and cytoplasmic patterns and titers observed in this cohort.

MATERIALS AND METHODS

Study design and setting: This observational cross-sectional study was conducted over a period of nine months at a tertiary care center in Pune, Maharashtra, India. The study was approved by the Institutional Ethics Committee (IESC/PGS/2025/207), and all procedures conformed to the principles outlined in the Declaration of Helsinki.

Sample size and participants: The sample size was calculated assuming an ANA positivity prevalence of 36.8% based on a prior study.[4] With an absolute precision of 8% and a 95% confidence level, using WinPepi version 11.38, the minimum required sample size was estimated as 140. A total of 226 consecutive patients attending inpatient and outpatient services with clinical suspicion of CTDs, for whom ANA testing by IIFA on HEp-20-10 cells had been ordered, were included.

Eligibility criteria: Patients of any age and sex with clinical suspicion of autoimmune connective tissue disease (CTD) who had serum samples tested for antinuclear antibodies (ANA) by indirect immunofluorescence assay (IIFA) on HEp-20-10 cells during the study period were eligible for inclusion. Exclusion criteria comprised suspected or confirmed malignancy, active infectious diseases (e.g., tuberculosis), and serum samples exhibiting hemolysis, icterus, lipemia, or microbial contamination. Clinical symptoms, along with provisional or final diagnoses, were obtained from medical records.

ANA testing by IIFA: Screening for ANAs was performed using IIFA on mosaic HEp-20-10 cells with monkey liver substrate, processed on an automated immunofluorescence analyzer (FluoroMAT-50). Venous blood was collected in a plain vacutainer to obtain serum, which was transported to the central clinical laboratory.

For each patient, 11.1 µl of serum was diluted in 1.0 ml of sample buffer and mixed by vortexing. A volume of 30 µl of the diluted sample was applied to the designated field of the BIOCHIP tray and incubated for 30 minutes. Following incubation, BIOCHIP slides were rinsed with PBS-Tween buffer and then incubated for an additional 30 minutes with fluorescein-labeled anti-human globulin conjugate.

At the end of the assay, slides were examined under a fluorescence microscope by two experienced pathologists who independently assessed the presence, intensity, and pattern of fluorescence. In case of disagreement, a third senior pathologist adjudicated the findings to minimize observer bias. ANA positivity was defined as nuclear and/or cytoplasmic immunofluorescence at the screening dilution, and patterns were classified according to ICAP recommendations. Serial dilutions of positive samples were performed to establish end-point titers. Staining patterns were broadly categorized as nuclear, cytoplasmic, or mitotic, and fluorescence intensity was graded qualitatively from + to ++++ as a surrogate for relative antibody concentration and, indirectly, disease activity.

Data management and statistical analysis: Data were entered into Microsoft Excel and analyzed using SPSS version 20 and/or Epi Info, Primer, and WinPepi as required. Categorical variables were summarized as frequencies and percentages, and continuous variables as means and standard deviations (SD). No formal multivariable modeling was planned, as the primary objective was descriptive.

RESULTS

ANA positivity and participant characteristics: A total of 226 patients underwent ANA testing by IIFA. Overall, 134 patients were ANA positive (59.29%; 95% CI, 52.78–65.49) and 92 were ANA negative (40.71%; 95% CI, 34.51–47.22) [Table 1]. ANA positivity was higher than the reference prevalence of 36.8% used for sample size assumptions (one-sample proportion z test: $z = 7.01$; $p < 0.001$) [Table 1]. The mean age of the study population was 45.71 years; among ANA-positive patients, the mean age was 48.2

years, with the highest frequency observed in the fifth decade. The female-to-male ratio among ANA-positive patients was 1.6:1.

Clinical characteristics: Fever was the most common presenting symptom, reported in 74 of 226 patients (32.74%; 95% CI, 26.96–39.11), followed by generalized weakness in 61 (26.99%; 95% CI, 21.62–33.13), breathlessness in 44 (19.47%; 95% CI, 14.83–25.12), and joint swelling in 34 (15.04%; 95% CI, 10.97–20.29) [Table 2, Figure 4-6]. Evidence of systemic involvement was documented across multiple organ systems, including cardiovascular, renal, gastrointestinal, central nervous system, and pulmonary manifestations. A history of infertility was noted in two patients.

ANA staining categories, ICAP patterns, and end-point titers: Among the 134 ANA-positive patients, nuclear-only staining was observed in 84 (62.69%; 95% CI, 54.25–70.41), cytoplasmic-only staining in 22 (16.42%; 95% CI, 11.10–23.61), and combined nuclear plus cytoplasmic staining in 28 (20.90%; 95% CI, 14.87–28.54) [Table 3].

The distribution of ICAP patterns among ANA-positive patients is shown in [Table 4]. The most frequent pattern was nuclear speckled coarse (AC-5), observed in 57 patients (42.54%; 95% CI, 34.49–51.00), followed by nuclear speckled fine (AC-4) in 42 (31.34%; 95% CI, 24.10–39.62). Cytoplasmic reticular/anti-mitochondrial antibody (AMA) pattern (AC-21) was identified in 35 patients (26.12%; 95% CI, 19.42–34.15), and cytoplasmic speckled (AC-20) in 13 (9.70%; 95% CI, 5.76–15.89). Less frequent patterns included nucleolar homogeneous (AC-8) in 10 (7.46%; 95% CI, 4.10–13.19), cytoplasmic rods and rings (AC-23) in 2 (1.49%; 95% CI, 0.41–5.28), and nuclear discrete multiple (AC-6) and nuclear homogeneous (AC-1) in 1 patient each (0.75%; 95% CI, 0.13–4.11) (Table 4). Representative nuclear and cytoplasmic staining patterns are illustrated in [Figure 2 and 3].

End-point titer distribution by staining compartment is summarized in [Table 5]. Among nuclear-only cases (n = 84), 73 were reactive at 1:100, 9 at 1:320, and 2 at 1:1000 [Table 5]. All cytoplasmic-only cases (n = 22) were reactive at 1:100 [Table 5]. Among patients demonstrating both nuclear and cytoplasmic staining (n = 28), nuclear reactivity was observed at titers of 1:100 (n = 22), 1:320 (n = 2), and 1:1000 (n = 4), while cytoplasmic reactivity was observed at 1:100 (n = 21) and 1:320 (n = 7), with no cytoplasmic reactivity at 1:1000 [Table 5]. Overall, most reactivities occurred at the 1:100 dilution, with fewer at 1:320 and 1:1000 [Table 5 Figure 4-6].

Table 1: Prevalence of ANA positivity by IIFA among clinically suspected CTD patients (N = 226)

Outcome	n/N	%	95% CI (proportion)	Statistical test*	Test statistic	p value
ANA positive	134/226	59.29	52.78–65.49	One-sample proportion z-test (vs 36.8%)	z = 7.01	<0.001
ANA negative	92/226	40.71	34.51–47.22			

**Reference prevalence (36.8%) used for comparison corresponds to the prior study used for sample size assumption.*

Table 2: Presenting clinical features in the study cohort (N = 226)

Clinical feature	n/N	%	95% CI (proportion)
Fever	74/226	32.74	26.96–39.11
Generalized weakness	61/226	26.99	21.62–33.13
Breathlessness	44/226	19.47	14.83–25.12
Joint swelling	34/226	15.04	10.97–20.29

Table 3: Distribution of ANA staining categories on IIFA among ANA-positive patients (n = 134)

ANA staining category	n/N	%	95% CI (proportion)
Nuclear only	84/134	62.69	54.25–70.41
Cytoplasmic only	22/134	16.42	11.10–23.61
Nuclear + cytoplasmic	28/134	20.90	14.87–28.54

Table 4: ICAP pattern distribution among ANA-positive patients (n = 134)†

ANA IIFA pattern (ICAP code)	n/N	%	95% CI (proportion)
Nuclear speckled coarse (AC-5)	57/134	42.54	34.49–51.00
Nuclear speckled fine (AC-4)	42/134	31.34	24.10–39.62
Nucleolar homogeneous (AC-8)	10/134	7.46	4.10–13.19
Nuclear discrete multiple (AC-6)	1/134	0.75	0.13–4.11
Nuclear homogeneous (AC-1)	1/134	0.75	0.13–4.11
Cytoplasmic reticular/AMA (AC-21)	35/134	26.12	19.42–34.15
Cytoplasmic speckled (AC-20)	13/134	9.70	5.76–15.89
Cytoplasmic rods and rings (AC-23)	2/134	1.49	0.41–5.28

†If more than one pattern could occur in the same patient, please confirm whether the counts above reflect primary pattern only or all observed patterns (non-mutually exclusive).

Table 5: ANA end-point titers by staining compartment among ANA-positive patients (n = 134)

ANA staining compartment	Total, n (%)	1:100, n (%)	1:320, n (%)	1:1000, n (%)
Nuclear only	84 (62.69)	73 (54.48)	9 (6.72)	2 (1.49)
Cytoplasmic only	22 (16.42)	22 (16.42)	0 (0.00)	0 (0.00)

Both nuclear + cytoplasmic	28 (20.90)	Nuclear: 22 (16.42) Cytoplasmic: 21 (15.67)	Nuclear: 2 (1.49) Cytoplasmic: 7 (5.22)	Nuclear: 4 (2.99) Cytoplasmic: 0 (0.00)
Total reactivities*	—	138	18	6

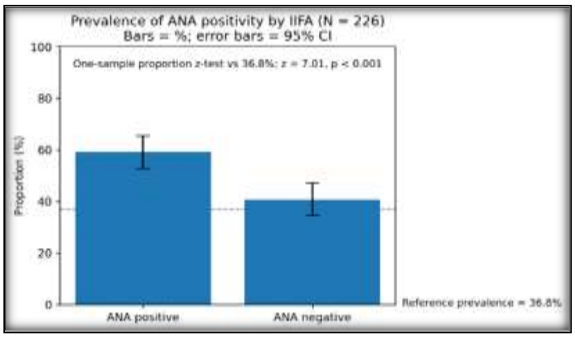

Figure 1: Prevalence of ANA positivity by IIFA (N = 226). ***Bar plot showing the proportion of participants classified as ANA-positive and ANA-negative by indirect immunofluorescence assay (IIFA). Bars represent percentages and error bars indicate 95% confidence intervals (CI). The dashed horizontal line denotes the reference prevalence (36.8%). A one-sample proportion z-test compares the observed ANA-positivity rate with the reference prevalence (z = 7.01, p < 0.001).***

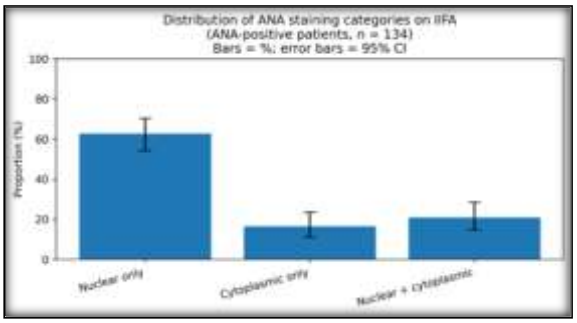

Figure 2: Distribution of ANA staining categories on IIFA among ANA-positive patients (n = 134). ***Proportions of ANA-positive samples exhibiting nuclear-only, cytoplasmic-only, or combined nuclear + cytoplasmic staining patterns on IIFA. Bars represent percentages and error bars indicate 95% CIs.***

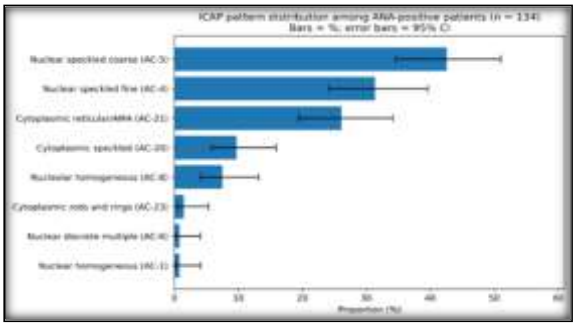

Figure 3: ICAP pattern distribution among ANA-positive patients (n = 134). ***Horizontal bar chart showing the frequency of International Consensus on ANA Patterns (ICAP) categories identified in ANA-positive samples, including nuclear speckled coarse (AC-5), nuclear speckled fine (AC-4), cytoplasmic reticular/AMA (AC-21), cytoplasmic speckled (AC-20), nucleolar homogeneous (AC-8), cytoplasmic rods and rings (AC-23), nuclear discrete multiple (AC-6), and nuclear homogeneous (AC-1). Bars represent percentages and error bars indicate 95% CIs.***

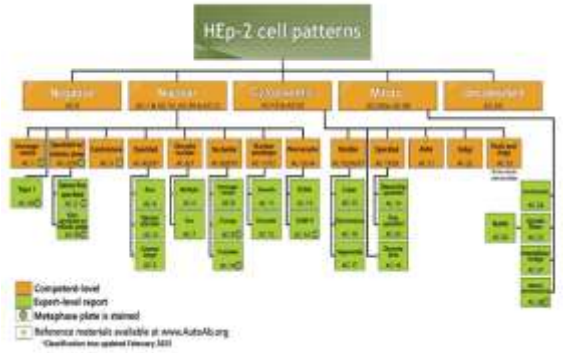

Figure 4: Indirect immunofluorescence assay (IIFA) antinuclear antibody (ANA) nomenclature based on the International Consensus on ANA Patterns (ICAP)

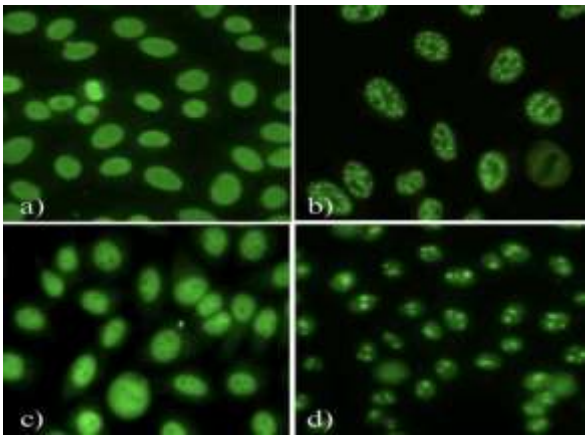

Figure 5: Various nuclear patterns of ANA positivity on IIFA: (a) Nuclear homogeneous, (b) Nuclear speckled coarse, (c) Nuclear speckled fine, (d) Nucleolar homogeneous.

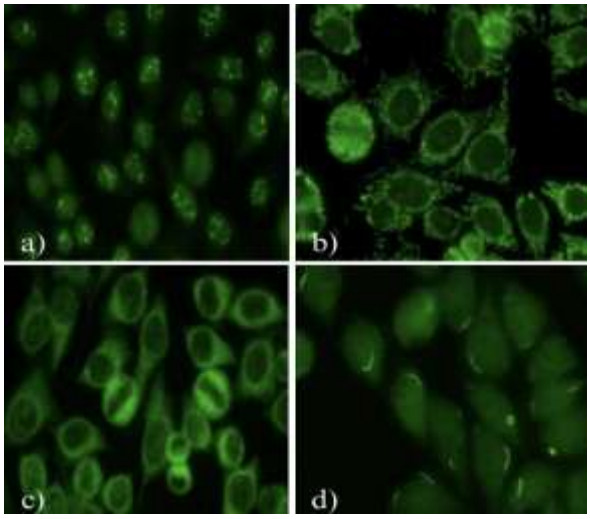

Figure 6: Various additional patterns of ANA positivity on IIFA: (a) Nuclear discrete multiple, (b) Cytoplasmic anti-mitochondrial antibody (AMA), (c) Cytoplasmic speckled, (d) Cytoplasmic filamentous (rods and rings).

DISCUSSION

This cross-sectional study from a tertiary care center in Western Maharashtra demonstrates that ANA positivity by IIFA on HEp-20-10 cells was present in 59.29% of patients with clinically suspected CTDs, supporting IIFA as a sensitive screening tool in routine practice.[4] Although many laboratories now employ antigen-specific immunoassays and multiplex methods, ANA testing by IIFA remains clinically valuable because it offers both screening sensitivity and pattern recognition that can support downstream diagnostic decision-making.[4,16]

A positive ANA result should be interpreted as an entry point into diagnostic evaluation rather than a stand-alone diagnosis; its clinical significance depends on correlation with patient phenotype and complementary investigations.[6] The mean age of ANA-positive patients in this cohort (48.2 years), with a peak in the fifth decade, is consistent with observations that CTDs commonly manifest in middle age.[7] The female-to-male ratio (1.6:1) in the present series is lower than ratios reported in Western cohorts (commonly 4:1 to 11:1), which may reflect referral patterns, selection bias, or case-mix differences.[8] The clustering of cases in the fifth decade aligns with the perimenopausal transition, during which endocrine changes have been linked to altered immune balance and cytokine profiles that may influence autoimmune activity.[9]

Clinically, fever, generalized weakness, breathlessness, and joint swelling were the predominant presenting features, underscoring that early CTD presentations can be non-specific and may mimic infectious or metabolic conditions. Similar reports have highlighted musculoskeletal symptoms and systemic complaints as common clinical cues prompting ANA testing in suspected CTDs.[2,7,10] The multisystem involvement documented in renal, pulmonary, cardiovascular, gastrointestinal, and central nervous system domains further reinforces the systemic nature of these disorders. The observation of infertility in a small subset is in keeping with literature describing reproductive implications of CTDs and their treatments, although this requires targeted evaluation in future studies.[7]

With respect to IIFA patterns, nuclear speckled patterns (coarse AC-5 and fine AC-4) predominated, aligning with prior Indian and international observations that speckled patterns are among the most frequent in CTD evaluations.[13–15] In our cohort, SLE was the most common suspected autoimmune disorder, consistent with reports from Malaviya et al. and Uramoto et al. describing SLE epidemiology and trends.[11,12] The clinical relevance of AC-code interpretation is supported by the ICAP perspective, where specific nuclear patterns contribute to recognizing systemic autoimmune rheumatic diseases and narrowing differentials (e.g., SLE, SSc, overlap syndromes).[16]

Cytoplasmic patterns were identified in more than one-third of ANA-positive patients, with cytoplasmic reticular/anti-mitochondrial antibody (AMA, AC-21) predominating, which is strongly suggestive of autoimmune liver disease (AILD), particularly primary biliary cholangitis and overlap syndromes. This reinforces the importance of systematically assessing cytoplasmic fluorescence—even when nuclear staining is absent or minimal—during IIFA interpretation.[17] The detection of rods and rings (AC-23) in a small proportion is notable given its reported association with hepatitis C virus infection and its treatment, and the described target antigens (IMPDH2 and CTPS1) in this phenomenon.[18,19] However, rods and rings patterns have also been reported outside HCV contexts, including in other autoimmune conditions and even in healthy individuals, emphasizing that this pattern must also be clinically contextualized.[19,20]

Most ANA-positive samples in this cohort had low titers (1:100), with fewer patients showing higher titers (1:320 and 1:1000). Low ANA titers may occur in a proportion of apparently healthy individuals and therefore do not invariably indicate clinically significant autoimmune disease.[21] Consequently, ANA results should be interpreted alongside clinical findings, and where indicated, supplemented by confirmatory serology (e.g., line immunoassay/immunoblot or antigen-specific assays) to improve diagnostic specificity.[22,23] In the present cohort, nuclear patterns occurred across titer categories, while cytoplasmic-only patterns were largely confined to 1:100, a distribution that may be compatible with early or less aggressive disease activity in some patients, though this requires longitudinal validation.[21] The co-occurrence of nuclear and cytoplasmic patterns in a subset may raise consideration of overlap syndromes, warranting careful clinical correlation and extended autoantibody profiling.[22]

Our findings are concordant with prior Indian studies emphasizing the importance of ANA testing in early CTD recognition and highlighting the diagnostic weight of speckled patterns.[22,23] The relatively higher cytoplasmic pattern recognition in recent studies has also been noted elsewhere and may reflect ethnic differences, patient selection, or improved reporting of cytoplasmic fluorescence in contemporary practice.[24]

Strengths of this study include its real-world tertiary-care cohort, broad clinical spectrum, and the use of an automated IIFA platform with expert microscopic review, which may reduce interobserver variability. Limitations include the lack of systematic correlation with antigen-specific assays or line immunoassay/immunoblot confirmation, which would strengthen diagnostic attribution to specific CTDs, and the absence of longitudinal follow-up to assess prognostic implications of particular patterns and titers.[22]

In summary, this study supports continued use of IIFA on HEp-20-10 cells as a sensitive screening tool

for CTDs in the Indian setting and emphasizes that careful interpretation of ANA patterns and titers, integrated with clinical context and supported by confirmatory assays when needed, is essential for accurate diagnosis and optimal patient management.[6,16,22] Larger multicentric studies incorporating comprehensive autoantibody profiling and follow-up are warranted to further clarify the clinical utility of specific ANA patterns in Indian patients with suspected CTDs.[22]

CONCLUSION

This cross-sectional study from a tertiary center in Western India demonstrates a high prevalence of ANA positivity among patients with clinically suspected connective tissue disorders, reinforcing indirect immunofluorescence on HEp 20 10 cells as a sensitive screening tool in routine practice. Nuclear speckled patterns (AC 4, AC 5) predominated, while cytoplasmic patterns, especially reticular/AMA (AC 21), were also frequent, underscoring the diagnostic value of systematic pattern assessment and titer reporting. Most reactivities occurred at low titers, highlighting the need for careful clinicopathological correlation and confirmatory antigen specific assays before assigning definitive diagnoses. Despite limitations, including lack of longitudinal follow up and extended autoantibody profiling, these findings support continued use of IIFA with structured ICAP based reporting to optimize early CTD recognition and guide targeted testing in Indian patients.

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