Original Article

Antinuclear antibody (ANA) positivity pattern by line immunoassay in a hospital from eastern India: Update from a laboratory perspective

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ABSTRACT

Context: The existence of more than one antibody in systemic autoimmune rheumatic diseases (SARDs) or connective tissue disease (CTD) along with features of more than one autoimmune disease (AD) in an individual is suggestive of overlap syndrome (OS). Line immunoassay (LIA) can target many autoantibodies in a single approach, thus making the identification of OS feasible. **Aims and Objectives:** This study aimed to identify the pattern of distribution of antinuclear antibodies by LIA prevalent in a hospital population in eastern India and identify common forms of SARD in this belt based on laboratory findings. **Material and Methods:** A total of 1660 samples received for ANA profile testing by LIA were analysed. **Statistical Analysis:** Factor analysis was performed with factor loading scores used in the k-means algorithm to identify clustering of various autoantibodies. **Results:** U1-snRNP positivity was the highest at 16.69%, and the least frequent autoantibody noted was anti-Jo-1 at 0.71% positivity. Based on the outcome of factor analysis, three clusters were determined. Cluster 1 showed a predominance of anti-PM/Scl antibodies, cluster 2 showed a predominance of anti-dsDNA, anti-histone, anti-SmD1, anti-nucleosomes, anti-PCNA, anti-Po, anti-SSA/Ro52, anti-SSA-Ro60, anti-SSB/La, anti-Scl-70, anti-Mi-2, anti-Ku and anti-AMA-M2, and cluster 3 showed a predominance of anti-U1-snRNP. **Conclusions:** Mixed connective tissue disease (MCTD) and overlap syndrome (OS) are prevalent more than pure form of an AD in our study population. OS may be missed out by monospecific immunoassays and hence adds to diagnostic challenges. LIA may be more useful in identifying specific autoantibodies by a single approach rather than monospecific immunoassays in populations after a positive screen by indirect immunofluorescence (IIF).

Keywords: ANA profile, autoimmune diseases (ADs), line immunoassay (LIA), MCTD, overlap syndromes

Introduction

The incidence of systemic autoimmune diseases (ADs) seems to be on the rise in the past few years not only in Western countries but also in developing countries.[1] However, in Asian countries such as India, the picture is still not clear as, besides a few clinical studies on individual disease entities, no major work has been done to find out the extent and changing trends in the occurrence of connective tissue diseases (CTDs). A study in India shows that 11% of the causes for pyrexia of unknown origin (PUO) are CTDs, which mostly remain undiagnosed.[2] As the clinical presentation of CTDs is highly variable, the data collected from individual departments in hospitals may not represent the true prevalence of any particular CTD, in particular, if a hospital-based population is taken as the study group. Therein comes the role of laboratory biomarkers, which may play a significant role in supporting early and accurate diagnosis, monitoring disease activity and progression, selecting drugs and

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assessing treatment response. This paper aims to contribute to the study of CTDs through three specific objectives. First, we describe the pattern of antibody distribution in a hospital-based sample of the general population as reflected by the ANA profile based on laboratory findings in a state of eastern India. We also attempt to assess the demographic profile of patients with CTDs in our study population. We also try to evaluate the role of line immunoassay (LIA) for multiparametric detection of autoantibodies keeping in mind the increased frequency of occurrence of one or more CTDs together in a given individual contributing towards the entity of OS and/or undifferentiated connective tissue disease (UCTD).

Material and Methods

This was a retrospective observational study conducted after obtaining ethical clearance from the Institute Ethical Committee. Samples included in the study were sent to the biochemistry wing of the central laboratory of a hospital in eastern India spanning the period from January 2019 to May 2020 for antibody profile testing against nuclear antigens by LIA for the evaluation of CTDs as a part of routine patient investigation. A total of 1660 venous blood samples (serum) received during this time frame were subjected to ANA profile testing against 17 nuclear antigens by LIA. LIA is a qualitative test that reveals antibody reactivity to antigens that are applied as distinct lines on a membrane. Specific nuclear antigens are applied to nitrocellulose strips at equal distances. The required number of strips is placed in the respective row of the incubation tray. To rehydrate and to block free binding sites against unspecific binding, the strips are incubated with buffer, containing blocking protein. After discarding the blocking buffer, the membrane strips are incubated with prediluted serum samples. According to their specificity, autoantibodies, if present in the patient's sample, bind to the antigens and are traced by alkaline phosphatase-conjugated anti-human-IgG antibodies that appear as blue-stained bands on the strips. Like dot blot, LIA is also easy to use and requires less processing time and is comparable to enzyme-linked immunoassay (ELISA) in sensitivity and specificity. Automated interpretation is also possible. It is possible to process any particular sample for autoantibodies to a large number of nuclear antigens (as high as 17 in our case) in a single step [Figure 1]. The nuclear and associated cytosolic antigens applied as lines on a nitrocellulose membrane in our study included dsDNA, nucleosome, histone, SmD1 (Smith), proliferating cell nuclear antigen (PCNA), P0 (ribosomal *P* protein (RPP)), SSA/Ro60, SSB/Ro52, SSB/La (Sjogren syndrome), centromere protein (CENP)-B, scleroderma (Scl)-70, U1-snRNP (small nuclear ribonucleoprotein), anti-mitochondrial antibody (AMA)-M2, Jo-1, polymyositis (PM)/Scl, Mi-2 and Ku. Qualitative measurement of IgG class of antibodies found in human serum against these antigens helps in diagnosis of a wide number of diseases that constitute systemic autoimmune rheumatic disease (SARD), which include systemic lupus erythematous (SLE), mixed connective tissue disease (MCTD), Sjogren's syndrome (SS), systemic sclerosis (SSc), primary biliary cirrhosis (PBC), PM and dermatomyositis (DM).

Patient's result data were categorized as age- and sex-wise. The positivity of different antinuclear antibodies was graded as negative (-), equivocal (0), (+), (++) and (+++) depending on the intensity of the band with reference to cut-off control. The interpretation of the test results takes place exclusively on the basis of the respective cut-off control regarded for each strip:

a) The test result is negative if no band is to be recognized or if the band exhibits a smaller intensity in comparison with the cut-off control.

b) The test is equivocal if the intensity of the band and the intensity of the cut-off control do not significantly differ.

c) The test result is positive if a band exhibits a stronger staining in comparison with the cut-off control.

The positivity of various antinuclear antibodies as mentioned above was recorded as single positivity or positivity along with other antibodies comprising the ANA profile to look for clustering and further associations. The kits used are provided by Human Diagnostics (IMTEC-ANA-LIA MAXX, Germany), and the instrument used is semiautomated analyser OZOBLOT 40M provided by Medsource Ozone Biomedicals.

Results

A total of 1660 samples received for ANA profile testing by LIA were analysed, of which 1109 were females and 557 were males. The total number of patients who tested either equivocal or positive was 962 (57.9%). A total of 650 females (39.2%) tested positive or equivocal for one or more antibodies and 312 males (18.8%) tested positive or equivocal for one antibody or more. The overall mean age of patients who tested equivocal or positive was 34.87 years. The mean age of males who tested positive or equivocal was 36.11 years, and for females, it was 32.37 years. The total number of patients who tested positive excluding equivocal results was 755 (45.5%). Around 12.4% of the patients tested equivocal. A total of 1414 antibodies were found to be positive during the duration of our study keeping in mind the fact that one person may test positive for single or many antibodies.

As far as individual antibodies were concerned, U1-snRNP positivity was found to be the highest at 16.69% with 236 individuals testing positive. This was followed by SSB/La at 11.10% with 157 individuals testing positive for it. Mi-2 with 9.26% positivity was the third most frequently seen antibody with 131 individuals testing positive for it. The least frequently encountered antibody was Jo-1 at 0.71% positivity with 10 patients testing positive for it. Table 1 shows the standard error of distribution with 95% CI for the 17 antibodies detected by LIA at our laboratory.

We attempted towards finding if any significant clustering occurs among various antibodies based on distribution pattern irrespective of any specific disease. All statistical analyses for this purpose were conducted using the Statistical Package for the Social Sciences (SPSS), version 22.

As the presence of autoantibodies was categorical variables, hence, factor analysis was performed with factor loading scores used in the k-means algorithm. Based on the outcome of factor analysis, three clusters were determined. The frequencies of the presence of 17 autoantibodies and demographic characteristics among clusters were compared using the Chi-square tests with Yates' corrections for overall *P* values and Fisher's exact test to compare between individual clusters. Bonferroni corrections were used, and only *P* values less than <0.05 were considered significant. The number

of clusters was determined using score plots and factor loading plots as depicted in Figures 2 and 3, respectively. Both graphs indicate that total antibodies can be grouped into three clusters. The frequencies of the presence of individual autoantibodies and demographic characteristics in each cluster are presented in Table 2.

Cluster 1 consisted of 643 subjects represented by 12.9% prevalence of PM/Scl antibodies, which was significantly different overall (*P* value = 0.0117) among three clusters;

Table 1: Depicting 95% CI for various autoantibodies											
Name of antibody	Identity	0 (equivocal)	1+	2+	3+	Total positives	*P*	Q	PQ/*n*	SE (P^)	Lower limit	Upper limit
dsDNA	A1	8	20	4	16	40	2.83	97.17	0.436911	0.856345	1.97	3.69
Nucleosome	A2	15	24	8	11	43	3.04	96.96	0.452504	0.886908	2.15	3.93
Histone	A3	10	19	6	12	37	2.62	97.38	0.420666	0.824505	1.79	3.44
SmD1	A4	19	25	12	25	62	4.38	95.62	0.539577	1.057571	3.33	5.44
PCNA	A5	59	95	17	6	118	8.35	91.65	0.728808	1.428463	6.92	9.77
PO (RPP)	A6	6	12	7	19	38	2.69	97.31	0.426158	0.835269	1.85	3.52
SSA/Ro60	A7	16	48	16	66	130	9.19	90.81	0.761419	1.492381	7.70	10.69
SSA-Ro52	A8	6	17	14	43	74	5.23	94.77	0.586864	1.150254	4.08	6.38
SSB-La	A9	27	82	27	48	157	11.10	88.90	0.827917	1.622717	9.48	12.73
CENP-B	A10	8	12	6	5	23	1.63	98.37	0.333347	0.653361	0.97	2.28
Scl-70	A11	5	23	5	12	40	2.83	97.17	0.436911	0.856345	1.97	3.69
U1-snRNP	A12	54	146	38	52	236	16.69	83.31	0.982648	1.92599	14.76	18.62
AMA-M2	A13	13	37	10	10	57	4.03	95.97	0.518318	1.015904	3.02	5.05
Jo-1	A14	3	7	1	2	10	0.71	99.29	0.220827	0.432822	0.27	1.14
PM/Scl	A15	32	59	25	20	104	7.36	92.64	0.687894	1.348273	6.01	8.70
Mi-2	A16	32	92	30	9	131	9.26	90.74	0.764044	1.497526	7.77	10.76
Ku	A17	47	80	19	15	114	8.06	91.94	0.717453	1.406208	6.66	9.47

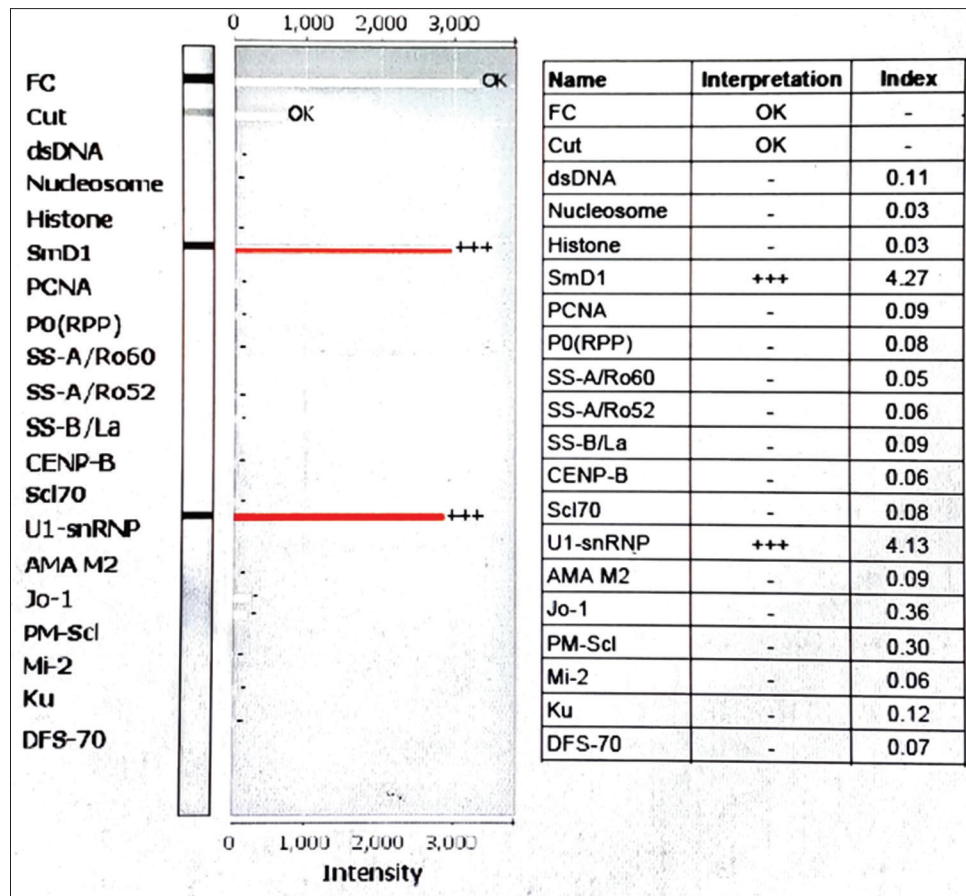| Table 2: Clustering of antibodies among a sample of 962 subjects into three clusters						
Characteristics/autoantibody	Cluster 1 (*n*=643)	Cluster 2 (*n*=55)	Cluster 3 (*n*=264)	Overall *P*	*P* between individual clusters		
					1 vs 2	1 vs 3	2 vs 3
Females, *n* (%)	429 (66.7)	45 (81.8)	176 (66.7)	0.067	-	-	-
Age in years Mean (SD)	35.58 (17.31)	31.63 (17.47)	35.92 (16.96)	0.231	0.310	1.00	0.282
Comparison of frequencies of different antibodies among cluster							
dsDNA	10 (1.56)	25 (45.45)	5 (1.89)	0.0001	0.001	0.7163	0.001
Nucleosomes	7 (1.09)	31 (56.4)	5 (1.89)	0.0001	0.001	0.3349	0.001
Histones	5 (0.78)	30 (54.55)	2 (0.76)	0.0001	0.001	0.975	0.0011
SmD1	23 (3.58)	33 (60.0)	6 (2.27)	0.0001	0.001	0.310	0.001
PCNA	70 (10.9)	14 (25.45)	34 (12.88)	0.0063	0.0014	0.392	0.0176
PO (RPP)	19 (2.95)	13 (23.64)	6 (2.27)	0.0001	0.001	0.5686	0.008
SSA-Ro60	84 (13.06)	22 (40.0)	24 (9.09)	0.0001	0.002	0.093	0.001
SSA-Ro52	51 (7.93)	13 (23.64)	10 (3.79)	0.0001	0.0001	0.0236	0.0011
SSB-LA	113 (17.57)	18 (32.7)	26 (9.85)	0.001	0.0057	0.0033	0.0001
CENP-B	15 (2.33)	4 (7.27)	4 (1.52)	0.0388	0.030	0.4347	0.0129
Scl-70	28 (4.35)	6 (10.91)	6 (2.27)	0.0128	0.0302	0.133	0.0022
U1-snRNP	40 (6.2)	32 (58.2)	164 (62.1)	0.0001	0.001	0.001	0.5854
AMA-A2	30 (4.67)	9 (16.36)	18 (6.82)	0.0015	0.0002	0.188	0.0206
Jo-1	5 (0.78)	2 (3.64)	1 (0.38)	0.0516	0.041	0.5093	0.0227
PM/Scl	83 (12.91)	4 (7.27)	17 (6.44)	0.0117	0.224	0.0047	0.8206
Mi-2	83 (12.91)	16 (29.09)	32 (12.12)	0.0025	0.0001	0.746	0.0014
Ku	68 (10.58)	19 (34.55)	27 (10.23)	0.0001	0.001	0.8763	0.001

Name	Interpretation	Index
FC	OK	-
Cut	OK	-
dsDNA	-	0.11
Nucleosome	-	0.03
Histone	-	0.03
SmD1	+++	4.27
PCNA	-	0.09
P0(RPP)	-	0.08
SS-A/Ro60	-	0.05
SS-A/Ro52	-	0.06
SS-B/La	-	0.09
CENP-B	-	0.06
Scl70	-	0.08
U1-snRNP	+++	4.13
AMA M2	-	0.09
Jo-1	-	0.36
PM-Scl	-	0.30
Mi-2	-	0.06
Ku	-	0.12
DFS-70	-	0.07

Figure 1: Depicting LIA findings with antigens on the y-axis and intensity scale on the x-axis
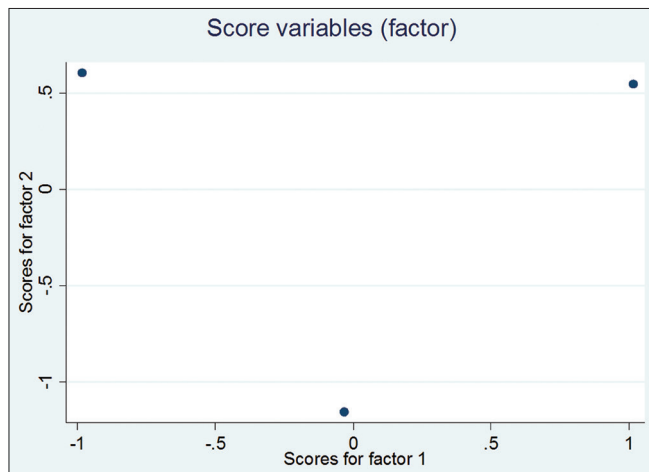

Figure 2: Score variables (factor) of three clusters

however, it was more prevalent in cluster 1 as compared to cluster 3 ($P = 0.0047$) and but not with cluster 2 (P value $= 0.224$) after Bonferroni correction.

Cluster 2 consisted of 55 subjects with high prevalence of almost all autoantibodies. The prevalence of antibody to dsDNA was 45.5% in cluster 2 and was significantly different overall ($P < 0.0001$) with respect to the prevalence of the same in clusters 1 and 3. The prevalence of antibody to nucleosomes was 56.4% in cluster

2 and was significantly different overall ($P < 0.0001$) with respect to the prevalence of the same in clusters 1 and 3.

The prevalence of antibody to histone was 54.5% in cluster 2 and was significantly different overall ($P < 0.0001$) with respect to the prevalence of the same in clusters 1 and 3.

The prevalence of antibody to SmD1 was 60.0% in cluster 2 and was significantly different overall ($P < 0.0001$) with respect to the prevalence of the same in clusters 1 and 3.

The prevalence of antibody to PCNA was 25.45% in cluster 2 and was significantly different overall ($P = 0.0063$) with respect to the prevalence of the same in clusters 1 and 3.

The prevalence of antibody to Po-RPP was 23.6% in cluster 2 and was significantly different overall ($P = 0.0001$) with respect to the prevalence of the same in clusters 1 and 3.

The prevalence of antibody to SSA-A Ro60 was 40.0% in cluster 2 and was significantly different overall ($P = 0.0001$) with respect to the prevalence of the same in clusters 1 and 3.

The prevalence of antibody to SSA-Ro52 was 23.6% in cluster 2 and was significantly different overall ($P = 0.0001$) with respect to the prevalence of the same in clusters 1 and 3.

Figure 3: Factor loadings of three clusters

The prevalence of antibody to SSB-La was 32.7% in cluster 2 and was significantly different overall ($P = 0.001$) with respect to the prevalence of the same in clusters 1 and 3.

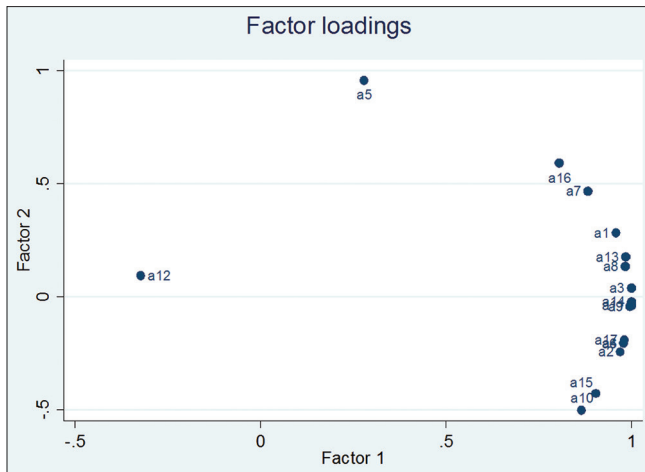The prevalence of antibody to AMA-M2 (16.36%) and Mi-2 (29.09%) was high in cluster 2 and significantly higher as compared to cluster 1 but not with cluster 3.

The prevalence of antibody to Ku (34.55%) was high in cluster 2 compared to clusters 1 and 3 with overall significant difference ($P = 0.0001$).

Cluster 3 consisted of 243 subjects represented by the highest prevalence of only one antibody U1-snRNP, which was significantly higher as compared to cluster 1.

No significant difference in the distribution of antibodies CENP-B and Jo-1 was observed among three clusters.

Discussion

CTDs are ADs characterized by the involvement of several organs and the presence of various autoantibodies. Autoimmune rheumatic diseases are classified using internationally accepted criteria, which frequently incorporate the detection of specific autoantibodies as unique diagnostic markers. Anti-double-stranded DNA (anti-dsDNA) and anti-Smith antigen (anti-Sm) are used as a part of the American College of Rheumatology/European League against Rheumatism (ACR/EULAR) and Systemic Lupus Erythematosus International Collaborating Clinics (SLICC) criteria for classifying SLE. ANA screen assay by indirect immunofluorescence (IIF) shows high diagnostic sensitivity for certain CTDs, for example, SLE (90-95%), primary SS (75%), Scl (85-90%) and MCTD (100%), but it has relatively low specificity.[3]

However, as per current observations, many patients cannot be assigned to a single disease category. This observation has led to the concept of OS, which implies the occurrence of two or more well-defined CTD in the same patient. These conditions share common immunopathogenic mechanisms and risk factors known as the autoimmune tautology, which explain the fact that one AD may coexist with others (i.e., polyautoimmunity (Poly A)). Previous research has shown the existence of the phenomena of overt Poly A, which correspond to the presence of more than one well-defined AD in a single patient, and latent Poly A, which correspond to the presence of several autoantibodies not directly related to the underlying AD but with predictive value for an additional AD.[4]

Our study aimed to see the grouping of various autoantibodies detected by LIA as part of ANA profile to identify the common patterns of distribution. This would broadly give a clearer picture about the type of ADs prevalent in this belt assuming the hospital population to be reflective of the state. Further validations of the study on different population subsets across different geographical locations in the state would identify the prevalence of the common ADs. It may also serve to help in the identification of new biomarkers common to a particular cluster, which may go a long way in improvement of our understanding of etiopathogenesis and treatment of AD. We also wish to propose a reflexive testing strategy with the incorporation of LIA along with existing techniques such as IIF and ELISA-based ANA detection to improve diagnostic reporting. The importance of LIA as a multianalyte solid phase assay cannot be underestimated in the detection of many autoantibodies simultaneously using a single approach. For example, SLE patients commonly have more than one autoantibody, typically reflecting what is known as 'linked sets' or B-cell responses to macromolecular complexes such as spliceosomes, nucleosomes and cytoplasmic ribonucleoprotein complexes.[5]

In our study population, cluster 2 had the highest prevalence of almost all antibodies. These antibodies were directed against dsDNA, nucleosomes, histones, SmD1, PCNA, Po-PRPP, SSA-Ro52, SSA-Ro 60, SSB/La, CENP-B, AMA-M2, Scl-70, Mi-2, Jo-1 and Ku. The first six antibodies are mainly associated with SLE. SSA/Ro52, SSA/Ro 60 and SSB/La are associated with SS. AMA-M2 is specific for PBC. Mi-2 and Ku are specific for myositis. The clustering of all these antibodies together signifies the simultaneous positivity for some or all of these antibodies in 55 patients comprising cluster 2. This indicates the existence of the 'OS' in our study population with serological evidence in the form of antibodies linked to SLE, SS, PBC and myositis.

Literature in the past has observed many combinations and permutations of autoantibodies in SLE, which has been found to be associated with unique phenotypes. For example, a combination of anti-dsDNA, anti-histone and anti-nucleosome antibodies is associated with a higher risk of severe lupus nephritis (LN).[6] In a study from France, to determine the diagnostic utility of anti-SSA/Ro52 and anti-SSA/Ro60 antibodies in AD detection, it was found that clinical and immunological associations differ depending

on either antibody' presence. In the Ro52-Ro60+ group, SLE was the most frequent diagnosis, with a possible association with antiphospholipid antibodies (APLA) and lupus anticoagulant. In the Ro52+Ro60+ group, primary SS (pSS) was the most probable diagnosis especially in patients with Ro52+Ro60+La+.[7]

In a study from Spain, a total of 322 patients presented more than one positivity for these antibodies, and the most prevalent disease was SLE and pSS, mainly associated with immunologic profile anti-Ro52+/anti-Ro60+/anti-La− and anti-Ro52+/anti-Ro60+/anti-La+, respectively. The presence of circulating antibodies anti-Ro52, anti-Ro60 and anti-La predisposed to xerostomia and xerophthalmia, and this finding was supported by numerous previous studies on pSS.[8-10] A combination of anti-Ro52+/anti-Ro60−/anti-La− exhibited negative association with photosensitivity, xerostomia and xerophthalmia; however, in previous studies, patients with anti-Ro52+ had higher frequency of cutaneous involvement.[11,12] Similarly, isolated positive anti-Ro52 was closely related to the main clinical, histopathological and immunological features of pSS.[13] Isolated anti-Ro60+ or anti-Ro60+/anti-Ro52+ increased the probability for SLE or overlap SLE/SS in many previous studies and was strongly associated with oral ulcers and arthritis in this study from Spain.[14] Anti-La reactivity was strongly associated with pSS and its main clinical manifestations (xerostomia and xerophthalmia) in this and many other studies in different populations.[14] These observations from many such studies in the past show that a single autoantibody or a combination of two or three cannot be used as a diagnostic criteria for any specific AD despite the existence of such defined criteria.

The disease is defined by a combination of clinical and immunological criteria, and many combinations of antibodies may exist in a defined AD, which also decide the varying clinical manifestations leading to subsets within an existing AD. As per a study conducted by the Barbara Volcker Center for Women and Rheumatic Disease (BVC), New York, it was reported that a second AD, rheumatic and non-rheumatic, occurs in 30%–52% of patients who have a diagnosis of SLE, rheumatoid arthritis (RA), SS or antiphospholipid syndrome (APS). Patients with overlapping disease differed from pure form of disease with respect to many variables such as age, sex, race and treatment. Three clinical patterns of overlap occurred with the most common pattern being the presence of two or more well-defined autoimmune rheumatic diagnoses simultaneously, such as 'rhupus' (RA+SLE). In the second pattern, a rheumatic AD coexisted with a non-rheumatic AD, such as Hashimoto's thyroiditis, multiple sclerosis (MS) or Crohn's disease. In the third pattern, onsets of the second AD were asynchronous, but in no fixed sequence: rheumatic and non-rheumatic with great variability in intervals between occurrences of two or more of the diagnoses. A few patients evolved from one rheumatic diagnosis (e.g., SLE) to another (e.g., RA) over many years with conversion being both clinical and serological.[15] SLE is most often associated with SS, as reported in 9%–33% of SLE

patients.[16] Systematic reviews and meta-analyses published more recently show a prevalence of SS in SLE patients of about 14%–17.8%.[17,18] SS constitutes a major cause of ocular and oral involvement in SLE.[19,20]

Patients of SLE with SS (sSS) and without SS differed with respect to clinical and serological profiles.[21] Careful analyses of the clinical features and autoantibody profile are important for the differential diagnosis between pSS and sSS. The possible development of sSS in SLE patients should be considered, especially in those aged 25 years at the onset of disease and with positive anti-Ro (SSA) antibody.[21]

The prevalence of SLE-SSc overlap in SSc cohort was reported to be 6.8% and was reported more common in the East Asian and South Asian populations compared to the Western population. Similarly, the development of inflammatory myositis with SLE is reported in 4%–16% of cases either simultaneously or following a gap of few months.[22] In a study by Rachna Aggarwal *et al.*, conducted on 2694 SLE subjects, 548 had sSS, while 71 of their 7390 SLE-unaffected relatives had pSS. None of the 1470 controls had SS (compared to SLE-unaffected relatives). Of the 71 SLE-unaffected relatives with pSS, 18 (25.3%) had an SLE-affected family member with sSS, while only 530 of the 7319 (7.2%) SLE-unaffected relatives without SS did so demonstrating that SLE and SS tend to occur in the same families.[23]

Similarly, idiopathic PM or DM is reported to complicate 4–16% of SLE patients. The clinical incidence of skeletal muscle involvement in SLE has been reported to be from 5% to 50%.[24] Thirty per cent of these patients had anti-Jo-1 antibodies. However, in our study population, the prevalence of anti-Jo-1 has been the least. Anti-Mi-2 and anti-Ku antibodies, which are also markers of myositis, have been noted more frequently.

In our study population, cluster 2 represented the existence of the phenomena of overlap syndrome (OS). LIA is a very important tool to detect many autoantibodies in a single approach. Though qualitative and not a very sensitive method, it provides a clue to the probable simultaneous existence of many systemic autoantibodies and thus directs the individual for further quantitative testing if required by other methods. Individual antibody estimation by ELISA based on a positive autoantibody screening test by IIF may be cumbersome and time-taking. Moreover, some antibodies may not be ordered for testing for lack of clinical suspicion and time involved on running individual samples for different antibodies.

Cluster 1 in our study showed a higher prevalence of anti-PM/Scl antibodies. In a study by Pakozdi *et al.* involving SSc and overlap patients of SSc, the most frequently appearing autoantibody in SSc/myositis was the polymyositis–scleroderma (PM/Scl) antibody, statistically more prevalent compared to SSc/RA or SSc/SS, whereas it was virtually absent in SSc/SLE. No other coexisting autoantibody than PM/Scl was detected in 94% of the cases.[25] In another study by Moinzadeh P *et al.* comprising 3240 patients, registered in the database of the German Network for Systemic

Scleroderma and followed between 2003 and 2013, a subgroup of patients with SSc/myositis overlap were observed to have creatine phosphokinase (CPK) elevation and PM/Scl antibodies (22.7%).[26] Scleroderma is a CTD characterized by tissue fibrosis, vasculopathy and immune dysregulation. The frequency of occurrence of scleroderma OS ranges from 10% to 38% in various studies.[27]

Recently, data from various scleroderma registries have shown that a significant proportion of scleroderma patients have predominant manifestations of other CTDs described as scleroderma OSs. In a Brazilian study conducted on 60 consecutive patients with scleroderma, polyautoimmunity was found in 43.3% of patients and the most frequently observed AD was Hashimoto's thyroiditis (53.8%) followed by SS (38.5%), inflammatory muscle disease (11.5%), APS (7.7%) and RA (3.8%). The majority of patients had only one concurrent AD (73.08%).[28] In a study involving interstitial lung disease (ILD) patients by Lega *et al.*, all anti-PM/Scl patients had symptomatic pulmonary involvement with a globally favourable prognosis comparable to that of patients with anti-tRNA synthetase antibodies. The extrapulmonary manifestations of anti-PM/Scl-related ILD were characterized by a similar prevalence of Raynaud's phenomenon, arthralgia and mechanic's hands and differed only by a significantly lower prevalence of clinical myositis in comparison with anti-tRNA synthetase antibody-related ILD.[29]

Anti-U1-snRNP antibodies have been seen predominantly in cluster 3 in our study group. Moreover, it was noted to be the most frequently occurring antibody at around 16.69%. Anti-RNP antibodies react against associated proteins such as U1RNA and the U1-snRNP form associated with spliceosome.[30] These antibodies are found in 25–50% of the patients with SLE, but they can also be found in different ADs. However, it is considered that high titres of this autoantibody are associated with MCTD especially when the presence of some other autoantibody has been ruled out, being part of the classification criteria for this entity. However, an association of this antibody with Raynaud's syndrome, oedema in the fingers of the hands and leukopenia has been found.[31] In one study, these antibodies were positive in 20–40% of patients with SLE, 2–14% of patients with SSc and 6–9% of patients with myositis.[32] In a prospective study on patients with anti-U1RNP antibodies, 60% of patients presented with symptoms compatible with a specific CTD other than MCTD. After a mean follow-up of 6 years, 90% fulfilled the criteria for MCTD.[33] A high titre of anti-RNP antibodies in any patient with features suggestive of UCTD is highly predictive of evolution into MCTD. MCTD evolves over time, and patients typically develop new clinical and laboratory features in the course of the disease. Thus, patients might display a few features of the disease and may not fulfil the classification criteria for MCTD at their initial presentation, as noted in a study by Rahmouni *et al.*[34] There is no single widely accepted set of classification criteria; several criterion sets have been tested successfully, including Sharp's criteria, the Kasukawa diagnostic criteria and the Alarcón–Segovia criteria. Diagnostic criteria may help define patients with MCTD, but in several cases, patients may not fulfil diagnostic criteria at their initial presentation.[35-37] The most common presenting features

of MCTD in a study by John *et al.* in the Indian population were arthritis, Raynaud's phenomenon,[38] ILD and sclerodactyly with anti-U1RNP antibody positivity in all patients. The expression of MCTD in the Indian population was different from that in the patients described in other studies with a more marked female preponderance, less deforming arthritis, with lesser prevalence of pulmonary Arterial hypertension (PAH) and renal involvement.

Limitations

As it was a retrospective observational study, clinical presentation, disease identification and correlation were not possible. Hence, the study of specific patterns obtained on ANA profile via LIA and their disease association could not be studied. The pattern of CTD distribution in this area of eastern India in a hospital-based population was not feasible in our study design.

Conclusions

(1) CTDs may not be classified as a definitive CTD (DCTD) in many cases despite the existence of defined diagnostic criteria for CTDs based on clinical and immunological findings due to the presentation with overlapping features of two or more diseases.

(2) Many subtypes exist within a DCTD due to difference in combinations and permutations of antibodies present, and a subclassification of a DCTD based on immunological criteria may be helpful in deciding future therapeutics and prognosis.

(3) MCTD may often pose a diagnostic dilemma due to a mixed range of symptoms. A high titre of anti-RNP antibodies in any patient with features of UCTD is highly predictive of evolution into MCTD. Patients develop new clinical features and laboratory markers as the disease evolves, thus emphasizing the need for regular follow-ups.

(5) LIA may prove to be important tool to identify specific antibodies in UCTD, DCTD, MCTD or OS after a screen positive by IIFT for ANA.

Conflicts of interest

There are no conflicts of interest.

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