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PREVALENCE OF ANTI-NUCLEAR ANTIBODY IN THE INDIAN POPULATION: A TERTIARY REFERENCE LABORATORY STUDY

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ABSTRACT **Background:** Autoimmune diseases are known to be the third leading cause of fatality and morbidity amongst the population of industrialized world. They account for 3-9% of health burden in general people, but information regarding prevalence of autoantibodies and autoimmune diseases in developing nations is scarce. **Aim:** To study the prevalence of Anti-nuclear antibodies, the total number and distribution of different tests used in the diagnosis of anti-nuclear antibody amongst Indian population, and correlate the findings from these tests with the clinical characteristics of the patients. **Method:** Retrospective data was evaluated from a Global Reference Diagnostic Laboratory in Mumbai, for a period of 6 years. This included a total of 285095 cases tested for ANA. ANA-IFA and ANA-ELISA were the screening tests used while ANA-ELISA Profile and ANA Blot were the confirmatory tests. **Results:** ANA by IFA was the most preferred screening test (88.73%) and ANA by Blot was the most preferred Confirmatory test (67.13%) based on their sensitivity and positive predictive value respectively. ANA-IFA showed positivity of 36.48% and ANA by ELISA test had positivity of 11.46%. In confirmatory testing, ANA Blot showed a positivity of 31.90% and ELISA Profile had 23.36% positivity. Females showed significantly higher positivity for both the screening test and Confirmatory tests than males ($p<0.001$). **Conclusion:** Screening by ANA IFA and Confirmatory by ANA Blot was the most preferred tests in our study population. These tests were found to be better for diagnosis, sub-syndrome categorization, prognosis, clinical follow-up and therapeutic strategies in various autoimmune disorders.

KEYWORDS : Antinuclear antibody, ANA IFA, ELISA, ANA Blot

INTRODUCTION

Autoimmune diseases are known to be the third leading cause of fatality and morbidity amongst the population of industrialized world [30]. A healthy immune system is tolerant and is bound to successfully distinguish between self and non-self-antigens [30]. The downfall of self-tolerance and immune regulatory circuit, gives rise to a deleterious effect known as 'Autoimmunity' [12] [30]. Generally, the function of antibodies in humans is to fight off any invading infection but in case of Autoimmunity, the antibodies end up identifying "naturally-occurring" proteins as foreign molecules. These antibodies are termed as 'Autoantibodies' and their presence in large number leads to abnormal 'anti-self-response' called as 'autoimmune diseases'. Unlike its name Antinuclear antibodies (ANA) it does not only attack proteins within the nucleus but also target cell membrane, cytoplasm and nucleoli. Though Indirect Immunofluorescence Assay is considered as "gold standard" test for detection of Antinuclear Antibodies, development of various other diagnostic tests like ELISA and Immunoblot assay have been witnessed in recent years. In the western countries, autoimmune diseases are responsible for almost 5% of morbidity and mortality on a yearly basis. But the actual burden of various autoimmune diseases in different populations specially amongst the people of developing countries is still unspecified. Poverty, limited awareness about autoimmunity in general public, lack of proper infrastructure and skilled staff involved in diagnosis and treatment of these diseases are possible reasons for this [9] [21]. In this study, we aim to evaluate the prevalence of anti-nuclear antibodies testing in the Indian population by conducting a comparative study between the two ANA screening tests – ANA IFA and ANA-ELISA; a comparative study between the two confirmatory tests – ELISA Profile and ANA-Blot; and to correlate the findings of these tests with clinical characteristics of patients.

MATERIALS AND METHODS

Study design:

A retrospective study was conducted in a Global Reference Diagnostic Laboratory in Mumbai, over a period of six years, from January 2015 to February 2021. In total, 285095 cases were included in our study during the study period. The tests used for ANA detection in our study were classified into two groups – Screening tests (ANA-IFA and ANA-ELISA) and Confirmatory tests (ELISA Profile and ANA Blot).

Sample flow:

Barcoded patient samples from all over India were received in the accession department of the laboratory. The samples were centrifuged and labelled (3000rpm x 10 min). The samples were then loaded onto the pre-analytical system "Automate 2550" which is connected to Laboratory Information System (LIS).Tubes further received, scanned and sorted according to the tests department. The respective Scientific Officer collected and arranged the samples according to the worksheet generated by LIS. The samples were stored at 2-8°C and were processed as per the batch schedule.

Procedure:

The tests used for ANA detection in our study is broadly classified into two groups – Screening tests (ANA-IFA and ANA-ELISA) and Confirmatory tests (ANA-ELISA Profile and ANA Blot). Information on the patients' age, gender, location and clinical characteristics were available and was collected from their Test Requisition Form (TRF).

ANA-IFA was performed using Automated Immunofluorescence Assay Processor Euroimmun IF- SPRINTER XL and Euroimmun Mosaic HEp20-10/liver (monkey)® kit. The fluorescent pattern was then viewed at 40X magnification under EUROstar III PLUS fluorescent microscope.

ANA by ELISA was performed using barcoded Alegria® test strip along with Alegria® Analyzer, and the results were displayed as "Index Value". ELISA Profile test was performed using Euroimmun Analyzer I system which comprises of Euroimmun Analyzer I instrument and software of the same name. As per the protocol of the reference laboratory, ANA-ELISA Profile panel consists of dsDNA, Sm, SSA, SSB, RNP-Sm, U1RNP, Centromere, Scl-70, Jo-1 antigen coated wells.

ANA Blot was performed using EuroBlotOne Processor and Euroline ANA Profile® kit. The blot strips are costed with bands of dsDNA, SSA, SSB, Sm, nRNPsm, Ro-52, Scl-70, Jo-1, CENP-A/B, PCNA, rib.P-protein, PM-Scl100, Nucleosomes, Histones, Mi-2, Ku, AMA-M2 and DFS70 autoantigens. The dried test strips were then photographed automatically and scanned by EUROline Scan program to detect the presence of blue coloured autoantibody bands against the 18 coated autoantigens.

Statistical Analysis:

Data recording was done in MS Excel. Categorical variables are reported as frequency and percentage. For comparison of categorised

variables Chi-square test or Fisher's-exact test has been used. All statistical analysis was performed using "R Studio version 1.4.1103". A two-tailed p value of <0.05 was considered to be statistically significant.

RESULT:

Total of 285095 patients were screened during the period of 6 years. Maximum cases belonged to the age group 31-40 years (22.7%). The female percentage was higher than that of males (69.16% vs 30.84%) (Table 1)

Table 1: Demographic details

	Frequency	Percentage
Age group		
<=10	6644	2.40%
11-20	18987	7.00%
21-30	56349	20.70%
31-40	61846	22.70%
41-50	51190	18.80%
51-60	40844	15.00%
>60	36528	13.40%
Not Given	12406	-
Gender		
Female	196968	69.16%
Male	87825	30.84%

Amongst the screening tests, ANA IFA was the preferred test ordered (88.73%) over ANA by ELISA method (11.27 %.). ANA blot was found to be the preferred confirmatory test over ANA profile by ELISA method (67.13% and 32.87%, respectively). (Table 2)

Table 2: Frequency of test done in each of the Screening and confirmatory tests

Test		Frequency	Percenta ge
Screenin g tests	ANA by IFA	245413	88.73%
	ANA by ELISA	31178	11.27%
Confirm tests atory	ANA Blot	24173	67.13%
	Elisa Profile	11838	32.87%

It was observed that, the patients tested with ANA by IFA showed more positivity (36.48%) than those who tested using ANA by ELISA test (11.46%). However, confirmatory testing ANA Blot showed a positivity of 31.90% and ELISA Profile 23.36%. About one in every three patients requested for ANA screening test based on clinical suspicion turned out to be positive. (Table 3.1 and 3.2)

Table 3.1: Positivity for each of the screening tests

Screening test	Frequency	Percentage
ANA by IFA		
Negative	155882	63.52%
Positive	89531	36.48%
ANA by ELISA		
Negative	26644	85.46%
Borderline	961	3.08%
Positive	3573	11.46%

Table 3.2: Positivity for each of the confirmatory tests

Confirmatory tests	Frequency	Percentage
ANA Blot		
Negative	16463	68.10%
Positive	7710	31.90%
Elisa Profile		
Negative	9073	76.64%
Positive	2765	23.36%

The females had shown significantly higher positivity for ANA screening test and confirmatory tests than males (p<0.001). (Table 4)

Table 4: Gender versus ANA screening and confirmatory tests

Gender		Screening tests				Confirmatory tests			
		ANA-IFA		ANA-ELISA		ELISA Profile		ANA Blot	
		Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Female	N	68734	100984	3798	18201	1964	3934	6647	11209
	%	40.50%	59.50%	17.26%	82.74%	33.30%	66.70%	37.23%	62.77%
Male	N	20631	54780	716	8397	171	990	1059	5249
	%	27.36%	72.64%	7.86%	92.14%	14.73%	85.27%	16.79%	83.21%
p value		0.0001		0.0001		0.0001		0.0001	

Statistical significant association was seen between age group and both the screening tests and both the confirmatory tests. Age group of >60 years had the maximum positivity in ANA by IFA whereas age group of 41 – 50 years had the maximum

positivity in ANA by ELISA. In both confirmatory testing age group of 21 – 30 years was observed to have highest positivity. (Table 5)

Table 5: Age versus ANA screening and confirmatory tests

Age group		Screening tests				Confirmatory tests			
		ANA by IFA		ANA by ELISA		Elisa Profile		ANA Blot	
		POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	Positive	Negative	Positive	Negative
<=10	N	1481	4468	67	530	35	160	52	194
	%	24.89%	75.11%	11.22%	88.78%	17.95%	82.05%	21.14%	78.86%
11-20	N	5387	11057	278	1614	223	672	498	1017
	%	32.76%	67.24%	14.69%	85.31%	24.92%	75.08%	32.87%	67.13%
21-30	N	16258	32159	952	5749	669	1555	1597	2983
	%	33.58%	66.42%	14.21%	85.79%	30.08%	69.92%	34.87%	65.13%
31-40	N	18663	34156	1048	6105	656	2022	1822	3794
	%	35.33%	64.67%	14.65%	85.35%	24.50%	75.50%	32.44%	67.56%
41-50	N	16647	27366	840	4717	534	1747	1489	3123
	%	37.82%	62.18%	15.12%	84.88%	23.41%	76.59%	32.29%	67.71%
51-60	N	14036	21386	577	3527	379	1404	1098	2374
	%	39.63%	60.37%	14.06%	85.94%	21.26%	78.74%	31.62%	68.38%
>60	N	13250	18420	474	2848	223	1404	877	2314
	%	41.84%	58.16%	14.27%	85.73%	13.71%	86.29%	27.48%	72.52%
p value		<0.00 01		0.2192		<0.0001		<0.0001	

ANA IFA test had sensitivity 54.254% (95% CI: 52.04% - 56.456%), Specificity 57.224% (95% CI: 55.778% - 58.660%) when compared to ELISA profile whereas ANA by ELISA had sensitivity 32.373% (95% CI: 28.071% - 36.907%), Specificity 83.625% (95% CI: 81.145% - 85.899%) when compared to ELISA profile. (Table 6)

Table 6: Comparison Both Screening test vs. ELISA Profile (Confirmatory test):

	ELISA Profile				
	Positive		Negative		p value
	N	%	N	%	
ANA by IFA					
Positive	1084	16.44%	1966	29.81%	<0.0001
Negative	914	13.86%	2630	39.88%	
ANA by ELISA					
Positive	146	10.27%	159	11.18%	<0.0001
Negative	305	21.45%	812	57.10%	

Frequency of IFA patterns with Mono-specific Antigens using ELISA Profile:

Nuclear pattern was observed in majority of the cases. Cytoplasmic pattern and mixed patterns were observed subsequently. Mitotic pattern was the least prevalent one.

Nuclear patterns:

The prevalence of subtypes of nuclear patterns corresponding to auto-antigens were Speckled, Homogenous, Homogenous Nucleolar, Centromere, Nucleolar & speckled patterns, and Nucleolar. Speckled pattern was majorly observed in RNP/Sm (IgG) positive (23.73%)

patients, followed by patients positive for SSA (21.19%) and UIRNP (19.49%).

Homogenous pattern was frequently observed in patients positive for dsDNA (28.26%), SSA (21.74%), RNP/Sm (IgG) (17.39%) and UIRNP (15.22%).

The auto-antigen corresponding to Centromere pattern are Centromere (80%) and SSA (20%). Mitotic patterns were the least prevalent pattern and showed positivity majorly for SSA antigen. Mixed patterns observed were Speckled & Cytoplasmic Speckled, Nucleolar & Cytoplasmic Speckled, Centromere & Cytoplasmic Speckled, and Nucleolar & Speckled pattern. SSA (50%) was the major positive antigen found amongst the patient who showed mixed positivity for Speckled & Cytoplasmic Speckled pattern; while dsDNA was major antigen found for Nucleolar & Cytoplasmic Speckled pattern and U1RNP (1 case) was the corresponding major antigen for Nucleolar & Speckled pattern.

Comparison of Both screening test with ANA Blot (Confirmatory test):

ANA IFA test had sensitivity 75.075% (95% CI: 73.637% - 76.473%), Specificity 49.879% (95% CI: 48.667% - 51.091%) when compared to ANA by Blot whereas ANA by ELISA had sensitivity 33.413% (95% CI: 28.908% - 38.154%), Specificity 80.978% (95% CI: 77.451% - 84.170%) when compared to ANA by Blot. (Table 7)

Table 7: Comparison of Both screening test vs. ANA Blot (Confirmatory test):

	ANA by Blot				
	Positive		Negative		p value
	N	%	N	%	
ANA by IFA					
Positive	2735	26.65%	3318	32.33%	<0.0001
Negative	908	8.85%	3302	32.17%	
ANA by ELISA					
Positive	140	14.42	105	10.81	<0.0001
Negative	279	28.73	447	46.04	

Frequency of IFA patterns with Mono-specific Antigens using ANA Blot:

Nuclear patterns:

Speckled pattern was majorly observed in Ro-52 (18.89%) positive patients followed by patients positive for RNP/Sm (IgG) positive (16.99%) and SSA (15.09%).

The most prevalent clinical symptom was Pain with 27% patients suffering from it. Pain is inclusive of joint pain, muscle pain, neuropathic pain and body ache. Some other clinical characteristics seen were nodular lesions, thrombocytopenia and imbalance while walking. dry cough/cough, swelling, dry/ red eyes, itching/skin infection, loss of sensation, breathlessness, numbness in hands/legs and burning sensation in limbs were also observed in Other less common complains included dry mouth, rash around mouth and skin, tingling in hands/legs and weakness.

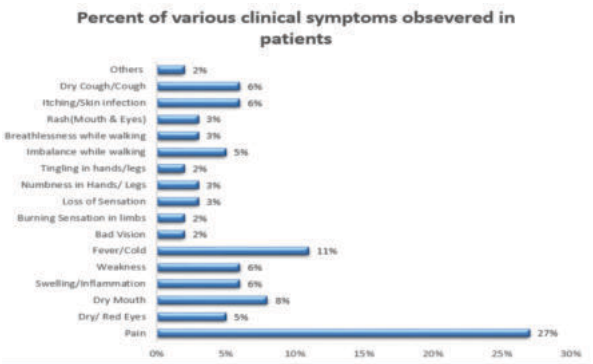

Figure 1: Clinical presentation of patients

DISCUSSION:

I) Age and Gender-wise prevalence of ANA positive and negative cases

As the age increases, the proportion of T cells decreases, immune cells tend to undergo senescence and self-tolerance suffers a downfall, causing elder population to have high levels of autoantibodies more frequently than children and younger population [36]. Age related rise in ANA levels can also be attributed to increased cellular damage and inflammatory reactions in response to other diseases [10]. In our study, higher percentage of positive cases during IFA screening was observed in patients with age group > 50 years. Our findings were similar to findings of Meier et al [10] and Patin Y.R et al [35] as in their study population, most of the positive patients are >45 years of age. Autoantibodies in females are due to increase in their hormonal Alterations per menstrual cycle (oestrogen, progesterone, and prolactin) during puberty, pregnancy, menopause. Use of oral contraceptives are considered to be the probable reasons for the preponderance of autoantibodies in females [5] [36]. With respect to the common screening tests, the positive cases were observed more frequently in females by IFA (40.50%) as compared to males (27.36%). Also in the common confirmatory tests the positive cases were observed more frequently in females by Blot (37.23%) as compared to males (16.79%).

II) Prevalence of autoantigens and IFA patterns:

The presence of circulating autoantibodies is deleterious as they hamper vital cellular functions. Jo-1 antibody is directed against antihistidyl-tRNA synthetase involved in charging tRNA with amino acids, Sm antibody is directed against small nuclear riboproteins, Mi-2 attacks NuRD complex involved in transcription regulation, PCNA is directed against auxiliary proteins of DNA polymerase involved in DNA replication. Histone and Nucleosome antibody are against histones and nucleosome complex which play a major role in DNA packaging. Ribosomal-P-Protein antibody targets epitopes of ribosomal protein P0, P1 and P2. The target of dsDNA antibody is double stranded DNA, PM-Scl-100 antibody is directed against exosome PM/Scl. Centromere and CENP-A/B antigens attack centromere and centromere protein A/B which keeps a pair of sister chromatid linked together. AMA-M2 antigens attack 2-oxacid dehydrogenase complex present on inner mitochondrial membrane. In our study population, we have observed antibodies against SSA (10.6%) and Ro-52 (8.74%) antigens to be the most prevalent ones. Anti-SSA antibody are invasive and are produced in response to small ribonucleic acid proteins which is closely related to cell mitosis and protein synthesis. They are usually found in patients suffering from Sjogren Syndrome. Anti-Ro-52 antibody is usually detected along with Anti-SSA antibody, which aides in diagnosis of various autoimmune disorders. Similar findings have been reported by Xiaoyan Li et al [15] in Chinese population, Sodani et al [31] in population of central Madhya Pradesh, Hayashi et al [23] in Japanese population, Guo et al [6] in Japanese population and Banhuk et al [4] in Brazilian population. The other two frequently detected autoantibodies in our study are Anti-Ku antibody (6.56%) and Anti-RNP/Sm (IgG) antibody (6.16%). Anti-Ku antibody is generated against Ku protein, a dimeric protein complex involved in DNA repair. It is commonly found in patients suffering from Myositis and interstitial lung disease. Anti-RNP/Sm (IgG) antibody attacks proteins associated with U1 RNA which plays a major role in splicing of pre-mRNA. Banhuk et al [4] reported Anti-RNP/Sm (IgG) antibody as the subsequently prevalent one in their study as well. Satoh et al [20] reported Anti-RNP/Sm (IgG) antibody to be seen most frequently in ANA positive patients of United States. Prevalence of Anti-Ku antibody was not observed even by them. The most prevalent ANA-IFA patterns observed in our study population are Nuclear patterns - Speckled pattern (44.35%), Nucleolar pattern (12.85%), and Homogenous pattern (9.19%) followed by Cytoplasmic speckled pattern (9.08%). We observed mixed patterns in 8.68% cases. Madhavi et al [16], observed mixed patterns as most prevalent pattern in their study followed by Speckled, homogenous, nucleolar, mitotic, nucleus dotted and cytoplasmic pattern. High frequency of mixed pattern in their study was because most of the IFA tests were done only in 1:100 dilution and further dilutions was not done to find out the most prominent pattern.

Biswas et al [2] reported Speckled as the most frequent IFA pattern, Homogenous, Cytoplasmic and Nucleolar being the subsequent ones in the population of West Bengal. Mariz et al [11] also reported Speckled pattern as the most frequently occurring IFA pattern. Speckled pattern also found to be prevalent in ANA positive patients of central Madhya Pradesh [31]. Mengeloglu Z et al [19] evaluated 3127 patients from Turkey using Hep 20-20, EUROIMMUN system and reported Speckled and Nucleolar as frequently occurring patterns in them. Speckled pattern being most common one correlates with our finding of SSA/Ro-52 antigens occurring most frequently amongst our population.

III) Total number of screening and confirmatory tests opted by patients:

The American College of Rheumatology has identified IFA as "gold standard for ANA testing" primarily based on its high sensitivity (>95%) for the diagnosis of SLE [18]. Hep-20-10/liver (Monkey) cells used as a substrate allows detection of autoantibodies against more than 100 nuclear and cytoplasmic antigens. The Hep-20-20/Primate liver substrate is a source of authentic antigens and negligible amount of antigen loss from substrate preparation to processing of the samples is the highlighting feature of IFA. In this study, the presence of ANA in samples was detected using two screening tests (ANA-IFA and ANA-ELISA) and two confirmatory tests (ANA-ELISA Profile and ANA-Blot). ANA-IFA was opted in majority (88.73%) of cases for ANA screening. Thus, being highly sensitive and of low cost, ANA-IFA is the choice of screening test by most of the patients.

IV) Total number of positive and negative cases using screening tests and confirmatory tests:

We observed 36.48% positive cases by ANA by IFA. Biswas et al [2] reported the positive cases of IFA as 50.37% in their study and 43.08% IFA positivity rate was observed in the study of Sodani et al [31]. The positive cases by ANA by ELISA screening test was 11.46% in our study. Out of the two confirmatory tests, we observed 23.36% cases positive for ELISA Profile and 31.90% cases positive by ANA- Blot in our study. Biswas et al [2] and Sodani et al [31]reported the positivity rate for ANA-Blot as 33.46% and ELISA 10.62% respectively.

V) Comparison of ANA-IFA with ELISA Profile and ANA-Blot:

ANA-ELISA, ANA-ELISA Profile and ANA-Blot tests detects autoantibodies only against those antigens that are coated onto the wells. The antigens used for coating are either purified proteins derived from native cells/tissues or are produced synthetically. Therefore, another drawback with these tests is, they show variations in their sensitivity to some extent. Also, sometimes these antigens might bind to other non-specific targets in the patient's, resulting in a masking effect [18][24][21][26]. Our study demonstrates the results of 1966 (29.81%) patients who were ANA positive by ANA-IFA but negative by ELISA Profile test. Similarly, 3318 (32.33%) patients were positive by ANA-IFA but negative by ANA-Blot test and the results of 159 (11.18%) patients who were ANA by ELISA positive and ELISA profile negative similarly for confirmation by Blot, 105(10.81%) patients were positive by ELISA but negative by BLOT. This is comparable with findings of Petchiappan V et al [25], Tayde A et al [34], Minz R.W et al [21], and Beronaite R et al [1]. Kolahi S et al [32] have also reported IFA as more sensitive than ANA-ELISA and ANA-Blot. Contrary to this, Copple et al [33] report ELISA as a better choice of ANA detection technique in SLE patients. They demonstrated higher sensitivity and specificity (90% to 97%) for ELISA as compared to 80% sensitivity for IFA. This discordance in their study was attributed to destruction of HEp substrate while preparation. In the study by Gniewek et al, ANA-ELISA was reported to have equivalent sensitivity, higher specificity, higher PPV and NPV than ANA-IFA. In our study, we also observed that 914 (13.86%) cases were reported positive by ANA-ELISA Profile test but negative by ANA-IFA. 908(8.85%) cases were reported positive by ANA-Blot but negative by ANA-IFA. Similar, findings were also reported by Petchiappan V et al [25] and Minz R.W et al [21], which they attributed to the failure of standardizing the manufacturing of ANA-IFA assay and the fact that IFA requires skilled readers to report the patterns.

VI) Prevalence of clinical characteristics in ANA positive cases:

The attacking autoantibodies causes swelling and inflammation at the diseased site. We observed that pain (27%) was the majorly reported clinical feature in ANA positive patients. Fever/cold (11%), dryness of mouth (8%), cough (6%), weakness (6%), swelling (6%) and itching (6%) were other prevalent clinical features in them. McGhee et al [17] also mentioned pain, fever, swelling and fatigue as prevalent symptoms in their study population.

Limitation:

Limitation of this study is that comparison between the two screening tests could not be done as no patient ordered for both the screening tests together. Thus, a more in-depth study is required on these lines.

CONCLUSION:

Our study emphasizes the importance of ANA testing in developing countries. It provides an overview of total number and distribution of different tests used in the diagnosis of anti-nuclear antibody amongst Indian population. In accordance with global guidelines, ANA IFA remains the initial screening test for detecting autoantibodies while ELISA Profile or ANA blot is done as a confirmatory test to detect specific autoantibodies against antigens causing autoimmunity. When used in combination, ANA-IFA along with ANA-Blot was found to be better in diagnosis, sub-syndrome categorization, prognosis, and further help in planning of clinical follow-up and therapeutic strategies in various autoimmune diseases.

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