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Original Article

Microbiology Section

Prevalence of Anti-Dense Fine Speckled 70 Antibodies in Systemic Autoimmune Rheumatic Diseases and Healthy Controls: A Cross-sectional Study

MVNL RAM MOHAN[1], SUKANYA SUDHAHARAN[2], LIZA RAJASEKHAR[3], VD TEJA[4], SUDHA TALASILA[5]

ABSTRACT

Introduction: Recognition of Dense Fine Speckled (DFS) Antinuclear Antibody (ANA) pattern is challenging as it is frequently confused with speckled or homogeneous patterns. Identification and confirmation of DFS pattern is important as it is more often seen in healthy individuals, routine laboratory referrals with relatively lesser prevalence in Systemic Autoimmune Rheumatic Diseases (SARD). As there is limited data on the prevalence of DFS pattern in Indian population, present study determined the prevalence and clinical significance of DFS pattern followed by anti-DFS 70 Enzyme Linked Immunosorbent Assay (ELISA) for confirmation.

Aim: To estimate the prevalence of anti-DFS 70 antibodies in ANA screening tests, SARD, and healthy individuals by Indirect Immunofluorescence (IIF) screening and confirmation by anti-DFS 70 ELISA.

Materials and Methods: A cross-sectional study was conducted over a period of 15 months from January 2017 to April 2018 at Nizams Institute of Medical Sciences, a tertiary care institute in Hyderabad, Telangana, India. A total of 7,550 serum samples were tested by IIF during the study duration. Sera displaying DFS pattern was tested for anti-DFS antibodies and anti-Extractable Nuclear Antigen (ENA) antibodies by ELISA. A total of 49 patients from rheumatology department satisfying SARD criteria and 184 healthy patients were also tested for anti-DFS antibodies.

Results: Anti-DFS 70 antibodies were seen in 0.47%, 16.3%, 39.6% among routine ANA screening referrals, SARD patients and healthy individuals, respectively. DFS 70 antibodies were positive in 60% of DFS IIF positive samples with none of them showing anti-ENA positivity or clinical evidence of SARD. Anti-DFS 70 positivity was more commonly seen in younger patients. Anti-DFS antibodies were significantly more common in healthy individuals compared to SARD patients (p -value=0.0022).

Conclusion: It is important to confirm DFS IIF pattern with a specific ELISA as isolated DFS antibodies are frequently associated with non SARD conditions.

Keywords: Antinuclear antibody, Systemic autoimmune rheumatic diseases, Indirect immunofluorescence

INTRODUCTION

The ANA are a hallmark of SARD. IIF using Human Epithelial cell (HEp-2) substrate is a widely used and recommended method of screening for ANA. A positive ANA test is followed by search for disease associated antibodies. DFS ANA pattern is a positive ANA pattern caused by autoantibodies targeted against DFS 70 antigen.

DFS pattern was first described in a patient with interstitial cystitis but later was found to be associated with wide spectrum of clinical conditions like chronic inflammation, atopic dermatitis, autoimmune thyroiditis, various cancers and even in healthy individuals [1,2]. This pattern has uncertain clinical significance and many studies have concluded that isolated anti-DFS antibodies are not associated with ANA associated SARD like Systemic Lupus Erythematosus (SLE), Sjogren's syndrome, Scleroderma, Dermatomyositis [3,4].

According to the International Consensus on ANA Patterns (ICAP), the DFS IIF pattern (AC-02) is characterised by three morphologic features: fine speckles distributed throughout the interphase nucleus with characteristic heterogeneity in their size, brightness, and distribution; denser and looser areas of speckles throughout the interphase nucleus and strong speckled pattern in the metaphase plate [5].

DFS IIF pattern is difficult to distinguish from common disease associated homogenous and speckled patterns where uniformly stained metaphase plate with homogeneous nucleoplasm and speckled nucleoplasm with unstained metaphase plate is seen, respectively. Hence, accurate identification and confirmation of DFS IIF pattern is necessary to avoid repeated follow-up testing, possible misdiagnosis, and unwarranted immunosuppressive therapy.

As there is limited data on the prevalence of DFS IIF pattern in Indian population [6], the present study was initiated to estimate the prevalence and clinical relevance of DFS pattern in routine clinical laboratory ANA screening, SARD patients and healthy individuals.

MATERIALS AND METHODS

This observational cross-sectional study was conducted over a period of 15 months from January 2017 to April 2018 at Nizam's Institute of Medical Sciences, a tertiary care institute in Hyderabad, Telangana, India.

Sample size calculation: A total of 7,550 samples were received for ANA screening during the study period. Considering the prevalence of anti-DFS 70 antibodies in SARD patients as 3% [7], 49 consecutive patients with confirmed SARD were selected in SARD group from the total 7,550 patients. A total of 184 patients with no rheumatic diseases, infections, and inflammation from Rheumatology department during the same period were selected as healthy controls considering the prevalence in healthy individuals to be around 20% [8].

Inclusion criteria: Serum samples displaying DFS IIF pattern and available for ELISA testing (n=25) from 7,550 ANA samples tested were included in the study group. Also, 49 consecutive patients from the 7,550 patients with confirmed SARD [9-13] and a positive ANA IIF pattern were included in the SARD group.

One hundred and eighty-four healthy patients with no previous rheumatic diseases from Rheumatology department formed the healthy control group.

Exclusion criteria: All ANA negative samples and ANA positive samples showing other than DFS IIF pattern were excluded except 49 patients who were selected in the SARD group.

Study procedure: Sera were tested for ANA by IIF at 1:100 dilution using Euroimmun Mosaic HEp-20-10 and primate liver cell substrate (Euroimmun AG, Germany, Lübeck) as per the manufacturer's instructions. Interpretation of the IIF patterns was done on fluorescent microscope by two independent microbiologists blinded to clinical diagnosis. The fluorescence intensity was recorded at ×400 from 1+ to 4+ relative to the intensity of the positive (4+) and negative controls.

Sera with DFS IIF pattern were subjected to anti-DFS 70 ELISA (Euroimmun, Lübeck, Germany) at 1:200 dilution as per manufacturer's instructions. All the patients in SARD group were tested by anti-DFS 70 ELISA. Cut-off values {sample Optical Density (OD)/calibrator OD} > 1 were considered anti-DFS antibody positive. DFS IIF positive sera were also tested for anti-Sm (anti-Smith), anti-Sm/RNP (Ribonucleoprotein), anti-Ro, anti-La, and anti-ds DNA antibodies (double stranded DNA) by ELISA were done to determine the proportion of monospecific anti DFS antibodies.

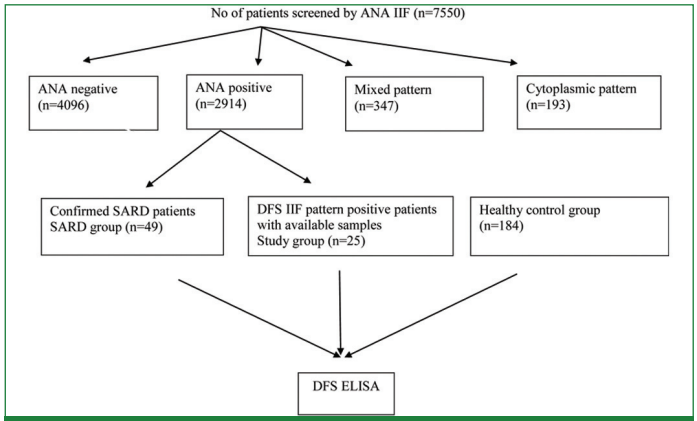
STATISTICAL ANALYSIS

Statistical analysis was performed using the Graph pad prism statistical software Version 9.5.0 (730); categorical variables were compared using Fisher's exact test. The p-value <0.05 were considered significant.

RESULTS

Of the 7,550 samples tested, 54.2% (4096/7550) of samples were ANA negative. Nuclear pattern was observed in 38.5% (2914/7550) of the patients; Cytoplasmic pattern in 2.5% (193/7550) patients and mixed pattern (cytoplasmic and nuclear) in 4.6% (347/7550) of them. Male to female ratio was 1:1.7.

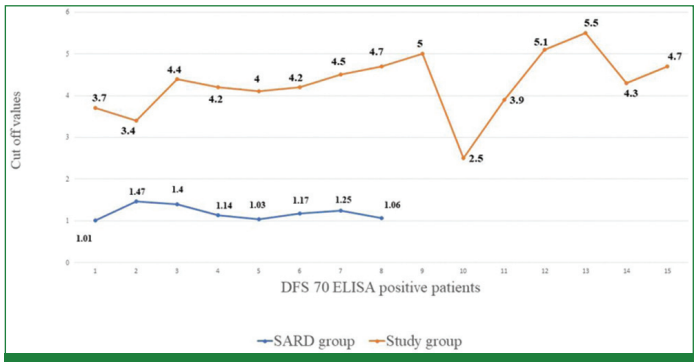
DFS IIF pattern was suspected in 0.47% (36/7550) of the total ANA tests received during the study period. Of the 36 samples with suspected DFS pattern on IIF, 25 samples were available for further testing by anti-DFS 70 ELISA and formed the study group; 15 of the 25 samples (60%) tested positive for anti-DFS antibodies by ELISA. Eight of the 49 patients in SARD group (16.3%), had anti-DFS antibodies. 73/184 (39.6%) healthy controls were anti-DFS 70 antibody positive. Selection of samples for anti-DFS ELISA is shown in [Table/Fig-1].



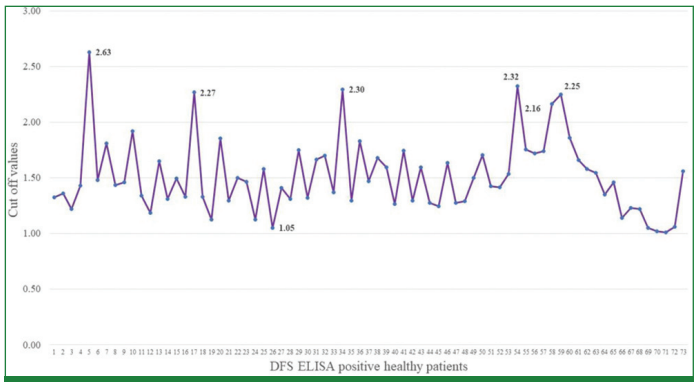
[Table/Fig-1]: Selection of samples for DFS ELISA.

Mean age of DFS positive patients (years) in the study group, SARD patients and healthy patients was 35.5 years (range -7 to 75), 32.4 years (range- 11 to 66 years) and 31.1 years (range-18 to 56 years), respectively. Eighty percent (80%, 12/15) of anti-DFS antibody positive patients in the study group were females. In SARD group, anti-DFS antibodies were detected in 1/4 males and 7/45 females. Proportion of DFS positivity among the healthy control group was similar in both genders; females- 36/73 (49.3%) and males- 37/73 (50.6%).

Amongst DFS ELISA positive patients, higher levels of DFS antibodies were observed in the study group (mean cut-off: 4.28) compared to the SARD group (mean cut-off: 1.19) as shown in [Table/Fig-2]. In the healthy control group, optical OD of DFS ELISA positive patients varied was from 1.01 to 2.63 (median OD:1.46) as shown in [Table/Fig-3]. Anti-DFS antibodies were significantly more common in healthy individuals compared to SARD patients (p=0.0022).



[Table/Fig-2]: Anti-DFS antibody levels in SARD group and study group.
*Cut-off = Sample OD/Calibrator OD



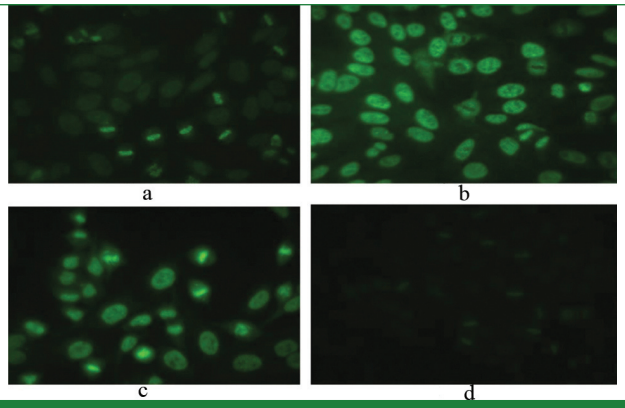
[Table/Fig-3]: Anti-DFS antibody levels in healthy control group.

Four of the fifteen (26.7%) anti-DFS antibody positive patients in the study group were diagnosed with Rheumatoid Arthritis (RA) [Table/Fig-4]. None of the 15 patients with anti-DFS antibodies in the study group had disease associated antibodies like anti-Sm, anti-Sm/RNP, anti Ro, anti La, anti ds DNA. Bright speckled metaphase plate, relatively dull nucleoplasm with characteristic bright speckles and lack of nuclear staining in the primate liver section were the characteristic features in identifying isolated DFS IIF pattern [Table/Fig-5a].

DFS ELISA positive (n=15)		DFS ELISA negative (n=10)	
Diagnosis	**Number (%)**	**Diagnosis**	**Number (%)**
Rheumatoid arthritis	4 (26.7)	Chronic kidney disease	3 (30)
Arthralgia	4 (26.7)	Sjogren's syndrome	2 (20)
Hypothyroidism	2 (13.3)	Systemic lupus erythematosus	2 (20)
Erythema nodosum	2 (13.3)	Multiple myeloma	1 (10)
Cardiorenal syndrome	1 (6.7)	Stroke	1(10)
Neuropathy	1 (6.7)	Bronchiectasis	1 (10)
Vascular disease	1 (6.7)		

[Table/Fig-4]: Referring diagnoses of DFS ELISA positive and negative patients in study group.

Forty percent (10/25) of the patients with suspected DFS IIF pattern in the study group were negative for anti-DFS antibodies by ELISA. Four of these 10 patients tested positive for one of the anti-ENA antibodies by ELISA and were diagnosed as SLE and Sjogren's syndrome (2 patients each). Isolated nonspecific metaphase plate staining with speckled nucleoplasm mimicked DFS pattern (4 of 10 DFS IIF positive, ELISA negative) [Table/Fig-5b]. Further testing of the remaining 6 DFS ELISA negative, IIF positive patients did not reveal any ENA antibodies.



[Table/Fig-5]: Indirect immunofluorescence images of suspected DFS IIF positive sera. a) Dense fine speckled pattern; b) Speckled pattern with metaphase staining; c) Mixed Homogenous and speckled pattern; d) Homogeneous pattern 2+ intensity with speckled metaphase plate.

Four of the ten ELISA negative samples could be mixed homogeneous and speckled pattern [Table/Fig-5c]. A new name "Pseudo DFS pattern" has been suggested by Infantino M et al., for a more homogeneous distribution and more uniform brightness of the nuclear speckles than in the typical DFS pattern was found in present study [14]. The remaining two samples had 2+ fluorescence intensity with speckled metaphase plate and homogeneous nucleoplasm [Table/Fig-5d].

In SARD group, anti-DFS antibodies were mostly seen among SLE cases (7/31; 22.5%) and one patient with Undifferentiated Connective Tissue Disease (UCTD) (1/11; 9.09%) and in none of the patients with systemic sclerosis, dermatomyositis, Sjogren's syndrome and RA.

DISCUSSION

ANA targeting the DFS antigen, DFS70 are gaining importance due to their low frequency in systemic rheumatic diseases, increased incidence in routine laboratory referrals and their recognition as a pattern seen in healthy individuals.

DFS70/LEDGFp75 is a stress response transcription coactivator that protects mammalian cells against diverse environmental stressors [15]. Overexpression of this oncoprotein in cancer cells promotes tumour aggressive properties such as increased clonogenicity, migration, invasion, chemotherapy resistance, stress survival, angiogenesis, and tumour growth [15,16]. In addition, it has an essential role in the integration of Human Immunodeficiency Virus-1 (HIV-1) [17].

The reported prevalence of DFS antibodies in routine ANA testing varies from 0.8-12.3% [2,7,18-20]. Various factors influence the prevalence of DFS antibodies like gender, ethnicity, geographical location, environmental exposures of the tested populations; the assay used and laboratory expertise in DFS pattern identification [7,15]. Data from India is limited with a brief report from North India reporting a prevalence of 1.6% from routine ANA screening patients [6]. The strongest associations found for these antibodies are young age and female gender [7]; 80% of DFS positivity in the study group was observed in females although similar prevalence was seen in both genders in SARD group.

Anti-DFS antibodies were detected in 39.6% of the healthy control group in the present study compared to the reported prevalence of 0 to 21.6 % in various studies. A study in Brazil in 2011 to differentiate ANA positive healthy individuals and patients with SARD found that ANA was present in 12.9% of healthy individuals and 90.2% of SARD patients and DFS pattern was seen only in healthy individuals.

In a study by Mahler M et al., from USA, the frequency of anti-DFS antibodies by IIF was 1.62%. The prevalence of anti-DFS70 antibodies was 8.9% in healthy individuals, 2.8% in SLE, 2.6% in RA, 4.0% in asthma, 5.0% in interstitial cystitis, 1.7% in Graves' disease, and 6.0% in Hashimoto's thyroiditis. The prevalence of anti-DFS70 was significantly higher in healthy individuals compared to patients with SARD (p=0.00085). A 2016 Mexican study to determine the frequency of anti-DFS70 autoantibodies detected relatively low frequencies of anti-DFS70 antibodies in patients with dermatomyositis (1.4%), RA (4.3%), and obese individuals (6.6%), and elevated frequency (17.4%) in healthy individuals. The prevalence of monospecific anti-DFS70 antibodies (10.9% vs. 1.9%, p=0.02) and antibody levels (p=0.01) were significantly higher in healthy subjects than in patients with SARD's in a study from Israel in 2018 [2,7,21-27].

A critical literature review of articles related to DFS70/LEDGF protein concluded that higher titres of anti-DFS antibodies are observed in apparently healthy individuals who did not develop any SARD after a follow-up period of 5 years [18]; similar results were seen in present study where the mean OD of DFS positive patients in the study group was markedly higher compared to those of the SARD group (Mean OD: 4.28 vs 1.19).

Reported prevalence of anti-DFS antibodies in RA varies from 0 [1,23] to 16.9% [28]; it was 16% (4/25) in present study group. The reported prevalence rates of DFS IIF pattern in Arthralgia are 5% and 19.8% and it was 16% (4/25) in present study [3,20]. Eight percent (2/25) of the DFS IIF positive patients were hypothyroid; although antithyroid antibodies were not tested for these two patients, the prevalence of DFS IIF pattern in Hashimoto's thyroiditis has been reported to be 6% and 16% in two studies [2,20]. Two of the twenty-five DFS ELISA positive patients had erythema nodosum; both were females and oral contraceptive pills were the suspected cause in one of them. Although direct association of DFS reactivity with erythema nodosum could not be found in the literature, erythema nodosum like skin lesions are reported in 50% of the Behcet's disease [29]; Yamada K et al., reported a strong association (34.4%, 11/32) between Behcet's disease and DFS positivity [30].

Twenty four percent (6/25) of the anti-DFS antibody positive patients in the SARD group were SLE patients. Frequency of anti-DFS antibodies in SARD is variable with rates reported upto 16% in SLE and RA. Seven of the 251 samples from SLE patients were positive for anti-DFS antibodies with anti-ENA antibodies seen in all except one of these patients by Mahler M et al., in 2012 [2]. Only one SLE patient in SARD cohort of 51 patients had anti-DFS70 antibodies and they were accompanied with anti-Sm and anti-dsDNA antibodies in a study by Shovman O et al., in 2018 [22]. None of the systemic sclerosis patients had anti-DFS antibodies in four studies from Italy, America, Japan and South Korea similar to present study findings [Table/Fig-6] [1,2,22,24,28,31].

Reference study	SLE* (%)	RA† (%)	SSc‡ (%)	MCTD§ (%)
Bizzaro N et al., [31]	0	11.1	0	0
Mahler M et al., [2]	2.8	2.6	0	||
Shovman O et al., [22]	3	||	||	0
Watanabe A et al., [1]	2	0	0	||
Va´zquez-Del Mercado M et al., [24]	||	4.3	||	||
Kang SY and Lee WI [28]	15.4	16.9	0	||
Present study	22.5	0	0	||

[Table/Fig-6]: Reported prevalence of anti-DFS antibodies in Systemic Autoimmune Rheumatic Diseases (SARD) [1,2,22,24,28,31].
*Systemic lupus erythematosus; †Rheumatoid arthritis; ‡Systemic sclerosis
§Mixed connective tissue disease; ||not tested

The accurate identification of the DFS pattern is challenging and has been classified as the competency level recognition pattern by ICAP [5,7]. it may sometimes result in misinterpretation, especially when discriminating between DFS and mixed homogeneous and speckled patterns [22,32].

In present study, 60 % (15/25) of the samples with suspected DFS IIF pattern were found to be positive for anti-DFS antibodies which underscore the utility of IIF in picking up suspected isolated DFS pattern.

The concordance rates between DFS IIF and specific anti-DFS70 assays like Chemiluminescence Immunoassay (CLIA), ELISA, Western blot range between 14% [33] and >90% [2,20,34]. A study in Italy in 2007 showed that 86% of sera exhibiting DFS IIF pattern were negative for DFS antigen when tested by ELISA or CLIA [19] whereas another study reported that all samples with DFS IIF pattern were DFS antigen positive [2].

In present study, the six DFS IIF suspected, ELISA negative samples with no anti-ENA positivity might be a misinterpretation of DFS pattern. On the contrary, heterogeneity among the anti-DFS antibodies might result in a typical DFS IIF pattern but a negative confirmatory test as all these antibodies do not react with antigenic substrate in confirmatory assays. In addition, antibodies against methyl CpG-binding protein 2 (MeCP2) which colocalises along with DFS 70 antigen in nucleus might produce a DFS-like Hep-2 staining pattern [35]. Periodic follow-up testing of these six patients might reveal additional information.

None of the DFS positive patients in the study group had any disease associated antibodies. Conrad K et al., highlighted the utility of isolated DFS ANA pattern in ruling out systemic autoimmune disease except RA [7]. A meta-analysis of five studies including 1243 SARD patients established that solitary anti-DFS70 is very rare (0.7±0.9%, mean deviation 0.45%) in SARD patients [18]. A study in Canada to evaluate the clinical and serological profile of patients referred through a central triage system due to a positive ANA result found that the Likelihood Ratio (LR+) for the absence of SARD with anti-DFS antibodies was 5.4 and approaches a significant value of 10.9 in patients with exclusive anti-DFS antibodies [3]. Isolated DFS positivity can be useful as a negative predictive biomarker to exclude SARD [1,3,7,18,22,23,36-38].

Absence of disease specific antibodies on a positive ANA IIF background would lead the clinician to periodically order repeat ANA testing at each outpatient visit further adding to the financial burden. A new ANA work-up algorithm including confirmatory anti-DFS70 antibody tests to clinically discriminate SARD from non-SARD patients in ANA-IIF positive individuals in Spain proved to be very cost-effective in terms of both laboratory costs and outpatient visits [39]. Routine testing with ANA line immunoassays with DFS 70 antigen would reveal the true prevalence of isolated DFS antibodies with a shorter turnaround time. This is likely to prevent additional tests for SARD diagnosis, resulting in cost-effective patient management as suggested in various studies [31,32,35,40,41].

Limitation(s)

DFS 70 line immunoassay was not performed in the present study. Another study with both DFS 70 line immunoassay and ELISA is planned based on the present study findings.

CONCLUSION(S)

All patients with suspected DFS IIF pattern and negative antibody results need to be tested by specific confirmatory anti-DFS assays. Exploring anti-DFS antibodies in ANA IIF positive patients with no anti-ENA specificity will guide the clinicians to discriminate SARD from non SARD conditions.

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PARTICULARS OF CONTRIBUTORS:
1. Associate Professor, Department of Microbiology, Nizam's Institute of Medical Sciences, Hyderabad, Telangana, India.
2. Associate Professor, Department of Microbiology, Nizam's Institute of Medical Sciences, Hyderabad, Telangana, India.
3. Professor, Department of Rheumatology, Nizam's Institute of Medical Sciences, Hyderabad, Telangana, India.
4. Professor, Department of Microbiology, Nizam's Institute of Medical Sciences, Hyderabad, Telangana, India.
5. Lab Investigator, Department of Microbiology, Nizam's Institute of Medical Sciences, Hyderabad, Telangana, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Liza Rajasekhar,
Professor, Department of Rheumatology, Nizam's Institute of Medical Sciences, Hyderabad-50082, Telangana, India.
E-mail: mylavarapu.24@gmail.com; lizarajasekhar@gmail.com

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