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REVIEW ARTICLE

The recognition of anti-nuclear antibody's dense fine speckled pattern and the detection of anti-DFS70 antibodies in the laboratory practice: Its prevalence and clinical significance

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Abstract

Anti-nuclear antibody test (ANA) is the test commonly requested for the working diagnosis of systemic autoimmune rheumatic diseases (SARDs) particularly in ANA-associated rheumatic diseases (AARDs) such as SLE, systemic sclerosis, Sjogren syndrome, mixed connective tissue diseases, and dermatomyositis. Dense fine speckled (DFS) pattern is an ANA fluorescence pattern that is commonly encountered in laboratories. This pattern is largely detected among the healthy population and in non-SARDs patients. Although this pattern is still can be observed among SARDs patients, the low prevalence of monospecific or isolated anti-DFS70 antibodies makes it useful for ruling out AARDs diagnosis. Thus, the inclusion of anti-DFS70 antibodies is perhaps logical for the exclusion of SARDs/AARDs. This review provides evidence of the prevalence of anti-DFS70 antibodies in different populations including healthy individuals, patients with SARDs and non-SARDs. The algorithm that includes the detection of anti-DFS70 antibodies during ANA screening is also suggested.

Keywords: anti-nuclear antibody, anti-DFS70 antibodies, dense fine speckled

INTRODUCTION

Anti-nuclear antibody (ANA) is a common laboratory test requested for the screening of an autoimmune disease. This test is particularly important in the diagnosis of systemic autoimmune rheumatic diseases (SARDs) such as systemic lupus erythematosus (SLE). There are two types of ANA detection which can be categorised as indirect immunofluorescence (IIF) and solid-phase assays. There are many methods of ANA detection by the solid-phase assays that include ELISA and immunoblot. In some laboratories, the ELISA is used as the initial screening method for ANA and subsequently, the positive samples will be further tested for ANA using the IIF method. The ELISA was shown to perform well with better sensitivity and good specificity compared to IIF when performed on the known SLE sera.[1] In another study, it was observed if the HEp-2 cell extracts were included in the enzyme immunoassay method, the performance of this solid-phase assay was comparable to that of IIF.[2] The immunoblot is commonly used for the detection of the specific autoantibodies associated with certain SARDs or collectively known as the anti-extractable nuclear antigen (ENA). This method is commonly applied after the ANA-IIF test is positive which allows the clinician to categorise the patient or help them to make the diagnosis based on the presence of the specific autoantibodies. The immunoblot has the advantage of multiplex whereby several autoantibodies can be detected in a single run.

Anti-nuclear antibody detection by indirect immunofluorescence method is considered the gold standard for ANA testing. This is because the antigen used allows for the detection of a wide range of nuclear antigens. The sensitivity of ANA-IIF differs from one condition to another. The sensitivity is high achieving more than 90% in the cases of SLE and systemic sclerosis but not that high in other conditions such as polymyositis and Sjogren syndrome. The

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substrate of choice for ANA-IIF testing is the HEp-2 cells. The laboratories that perform ANA-IIF usually provide the report that includes the observed fluorescence pattern and end titration of positive well in any positive sample.[3] The titration was shown to play important role in predicting the underlying SARDs. Studies have shown that higher ANA titration is significantly associated with SARDs diagnosis.[4,5] However, it is important not to underestimate ANA with low titration because in some cases ANA titration can be low, but the clinical features are suggestive of a specific diagnosis. Thus, it is always important to correlate with the clinical features of the patients.

The ANA patterns are categorised into nuclear, cytoplasmic, and mitotic patterns. The report of the nuclear staining pattern is considered standard practice. The common nuclear patterns are homogeneous, speckled, nucleolar, and centromere. The reports of the cytoplasmic and mitotic patterns are still not standardised. Some of the laboratories routinely report these patterns but some are not. The majority of SLE patients were noted to have a homogeneous pattern but other patterns such as speckled and homogeneous-speckled were also seen in this condition.[6] Recently, the International Consensus on Antinuclear Antibody Patterns (ICAP) classified the patterns into several levels that include competent-level and expert-level. Most of the nuclear staining patterns are categorised as the competent level whereby most of the laboratories that perform ANA-IIF should be able to recognise and report all these patterns.[7] Dense fine speckled (DFS) is one of the important ANA fluorescence patterns that is categorised as competent level recognition. The DFS pattern is commonly found at high titre among healthy individuals and in those without SARDs.[8] It was previously described that 93.9% of those with these autoantibodies did not have AARDs.[9] Although it is important to recognise the DFS pattern under the microscope this is not an easy task.[10] In one study that involved 230 IIF technologists, only about 50% of the participants can correctly identify this pattern and this was worse in the mixed nuclear patterns samples which only less than 10% of the participants could identify the DFS pattern.[11] The prevalence of DFS pattern was also varied among different populations even within the same country.[12] Anti-DFS70 antibody is the autoantibody commonly associated with this pattern. This antibody can be detected by various laboratory techniques such as IIF, ELISA and chemiluminescence assay. The significance of this autoantibody has been the subject of research interest since the past 10 years as this autoantibody was shown to be an important marker to rule out AARDs. Thus, this review was conducted to highlight on the challenges in detection of DFS pattern and the clinical significance of anti-DFS70 antibodies.

Dense fine speckled pattern and anti-DFS70 antibodies

The (DFS) pattern can be difficult to be recognised because the pattern can be masked by other ANA patterns. It is also important to differentiate this pattern from homogeneous and speckled patterns. The DFS pattern is described as unique dense and heterogeneous staining of both nucleoplasm of interphase cells and the metaphase chromosomal plates.[7,8] This pattern is shown in Figure 1. It is important to recognise this pattern because it is rarely associated with underlying SARDs. However, this association is only important if the DFS pattern is confirmed to be an anti-DFS70 antibody and if the DFS pattern is monospecific to anti-DFS70 antibodies.[13] The DFS pattern was first identified in patients with interstitial cystitis and subsequently among patients with atopic dermatitis. The autoantibody that was responsible for this pattern was identified to have activity against the 70-75 kD protein by using Western blot analysis thus it is called anti-DFS70 antibody.[14] The antigen was recognised as the growth factor derived from the lens epithelium or known as LEDGF and/or DNA transcriptional coactivator p75.[15] This protein was shown to serve as a cofactor for human immunodeficiency virus (HIV) replication through the interaction with viral integrase.[16] It was suggested that this could be an option for the treatment of HIV-1 infection.[17,18]

Detection of the dense fine speckled pattern and anti-DFS70 antibodies

Several laboratory methods can be used to detect anti-DFS70 antibodies. These methods include indirect immunofluorescence with immunoadsorption, chemiluminescence immunoassay (CLIA), enzyme-linked immunosorbent assay (ELISA), and immunoblot.

For most laboratories, the DFS is detected by observing the fluorescence pattern in HEp-2 cells. As this pattern can be mistakenly identified, another laboratory method is commonly used to confirm the pattern. In a previous study, the accuracy of ANA IIF reading for the detection

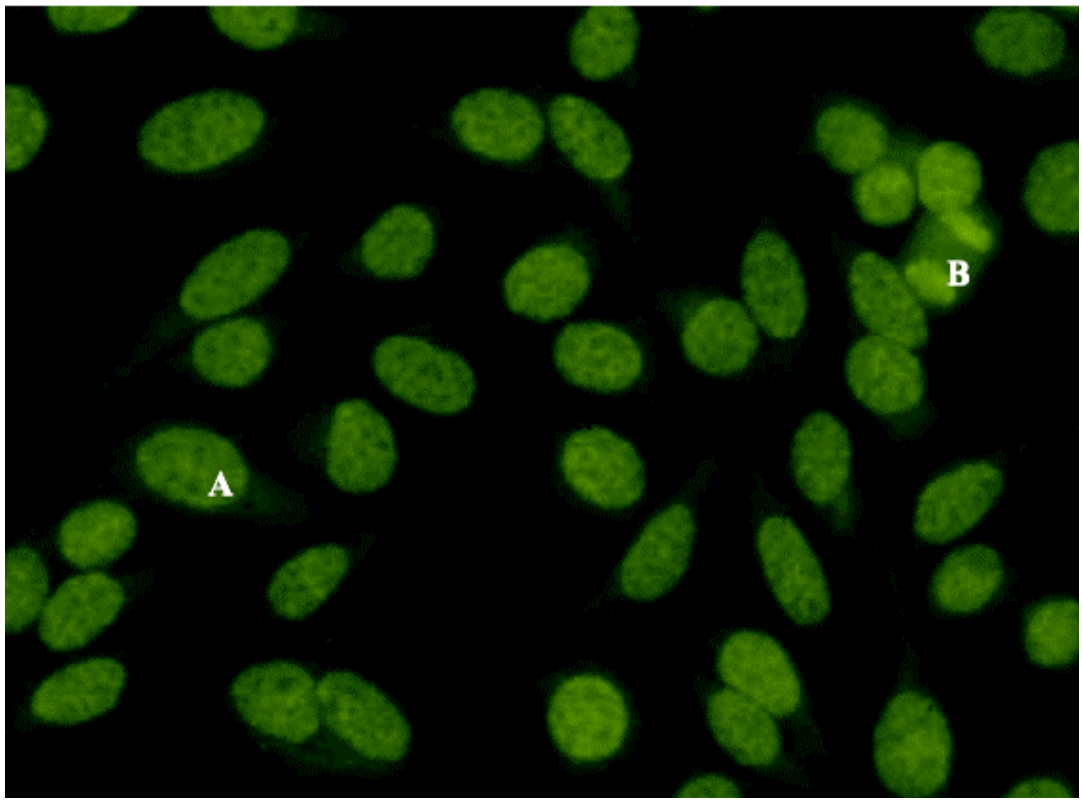

FIG 1: Indirect immunofluorescence on HEp-2 substrate with dense fine speckled pattern. The characteristic heterogeneous fluorescence staining can be observed in both nucleoplasm of interphase cells (A) and metaphase chromosomal plate (B).

of anti-DFS70 antibodies was only at 68.8%.[19] The immunoadsorption method is one of the methods that can be used for confirmation. At the moment the only commercially available kit is the NOVA LITE HEp-2 Select kit with DAPI (Inova Diagnostics, San Diego, CA, USA). This kit includes the DFS70 antigen-containing diluent. The patient's serum is first mixed in this DFS70 antigen-containing diluent. The DFS70 antigen in the diluent removes anti-DFS70 antibodies in samples by immunoadsorption. Thus, the anti-DFS70 antibodies in the patient's serum will be removed, and subsequently, the usual step of ANA-IIF test is followed. Thus, the DFS pattern will be inhibited if it is not mixed with any other patterns. The data from a previous study showed the NOVA LITE HEp-2 Select kit was effectively inhibiting anti-DFS70 antibody binding to its nuclear target.[20] However, the inhibition was unlikely to be demonstrated from the sera of AARDs patients.[21] This is because, in the AARDs, it is likely these patients have concomitant AARDs-related autoantibodies. The IgG was demonstrated as the predominant antibodies isotype in the samples with DFS ANA-IIF pattern.[22]

The chemiluminescence immunoassay (CLIA) is another laboratory method for the detection of anti-DFS70 antibodies. In this assay, the antigen used consists of recombinant DFS70 fragment expressed in *Escherichia coli*, spanning from amino acid 349 to 435.[23] The antigen is coated onto paramagnetic beads. This test is performed on the automated chemiluminescence instrument. The result is expressed in relative light units (RLUs). The RLUs are proportionate to the amount of isoluminol conjugate that is bound to the anti-human IgG which is also proportionate to the amount of anti-DFS70 antibodies bound to the antigen on the beads.[24] Subsequently, the RLU value is converted into calculated units (CU) based on the standard curve. This anti-DFS70 antibody CLIA kit is manufactured by Inova Diagnostics and the cut-off for the test was set at 20 CU by the manufacturer.[21]

Enzyme-linked immunosorbent assay (ELISA) has also been used for the detection of anti-DFS70 antibodies.[19,25-31] There are many commercially available ELISA kits for this purpose. In ELISA, the DFS70 antigen is

pre-coated into the well. Subsequently, patient sera are added and incubated. The anti-human IgG is then added after the washing step. The concentration of the anti-DFS70 antibodies is determined accordingly. The reference value is varied between the commercially available kits. There was also a report by Hayashi *et al*., whereby the immunoadsorption was first used before the ELISA test was performed.[25]

The immunoblot assays can also be used for the detection of the anti-DFS70 antibodies.[32-35] Several commercially available kits also included DFS70 antigen in their panels. These include dot blot ANA plus DFS70 IgG manufactured by Alphadia (Wavre, Belgium) and the line blot Euroline ANA Profile 3 plus DFS70 IgG (Euroimmun, Luebeck, Germany). Both kits used recombinant antigens even though with different amino acids length. The DFS70 antigen used in Alphadia method is a 349-433 amino acid recombinant protein expressed in *Escherichia coli* while the Euroimmun kit method used a full-length recombinant protein (amino acid 1-530). Both assays have different cut off based on the intensity of the dots or lines.

No method is suggested as a gold standard for the detection of anti-DFS70 antibodies for now. Observing the DFS fluorescence pattern alone may not be adequate as the pattern can mask or be masked if there is more than one patterns present in the sample.[36] Previously, it was shown that only 50% of the DFS fluorescence pattern were positive by at least one of the anti-DFS70 antibodies tests.[35] This finding highlighted the DFS70-like pattern can be caused by antibodies other than anti-DFS70 antibodies. The commercially available kits use either full-length or truncated DFS70 protein for the detection of anti-DFS70 antibodies.[30] The ELISA and line immunoassay kits manufactured by Euroimmun incorporated full-length DFS70 protein while the CLIA kit produced by INOVA Diagnostics uses truncated DFS70 antigen.[30] However, it was previously shown that no difference in overall diagnostic accuracy between the methods that used either full length or truncated DFS70 antigen. These three methods were previously compared to ANA IIF titration. Carbone *et al*., showed an excellent correlation between DFS70 IIF titration and AU of immunoblot assay with regression analysis of R^2=0.99 to confirm the relationship between the two methods.[32] In another study comparing the performance of ELISA anti-DFS70 antibodies with sera that were positive for anti-DFS70 antibodies both by ANA IIF and immunoblot assay, the sensitivity and specificity were 89% and 95% respectively.[37] The anti-DFS70 antibodies concentration by ELISA was also shown to correlate with ANA IIF titration among healthy individuals.[25] Similarly, the CLIA assay showed an excellent correlation with the DFS fluorescence pattern. The previous study also described an excellent agreement between the CLIA QUANTA Flash assay (Inova Diagnostics, San Diego, USA) and the DFS IIF fluorescence pattern with kappa between 0.88 to 0.97.[21,38] CLIA method and immunoblot were also shown to achieve an excellent agreement of 94%.[39] The excellent correlation was also shown between the ELISA and CLIA methods with Spearman correlation co-efficient of 0.91.[40] When these three methods (ELISA, blot, and CLIA) were evaluated together, the overall agreement between the methods was good with a kappa value of 0.749.[30]

The prevalence of anti-DFS70 antibodies in healthy population

The prevalence of the anti-DFS70 antibodies was thought to be more common in conditions not related to (SARDs) or AARDs. The prevalence among the healthy population ranged from 0.78% to 16.4%.[21,24,25,37,40] It was also seen in 2.1% of healthy children.[41] This pattern was thought to be exclusively found in the healthy population.[4] However, it is important to highlight that the prevalence is may be influenced by the detection methods being used. The prevalence is even lower if the isolated anti-DFS70 antibodies are applied, for example, Hiyashi *et al*., recorded an overall prevalence of 16.4% (41/250) but if the isolated anti-DFS70 antibodies were taken into account the number of positive cases reduced to 37.[25] The prevalence of anti-DFS70 antibodies was also varied between different countries. Albesa *et al*. demonstrated that the prevalence of anti-DFS70 antibodies was 1.2% in Italy and 8.5% in the USA among the blood donor.[24] The same study also showed that the anti-DFS70 antibodies were more common among females and in younger individuals.[24] The concentration of anti-DFS70 antibodies was also found to be significantly higher in healthy individuals than in patients with AARDs.[21]

The prevalence of anti-DFS70 antibodies in conditions other than systemic autoimmune rheumatic diseases (SARDs)/ ANA-associated rheumatic diseases (AARDs)

The anti-DFS70 antibodies were also detected

in conditions other than SARDs. Dense fine speckled pattern was first detected in patients with underlying interstitial cystitis. Subsequently, it was detected in many other conditions. Atopic dermatitis (AD) was among the conditions in which anti-DFS70 antibodies were demonstrated to may have a role in the pathogenesis. The study in AD showed the DFS70 antigen is predominantly located in the nucleus of the basal epidermal cells and then during differentiation, is translocated into the cytoplasm in which it accumulates in the keratohyalin granules.[42] The presence of serum IgE and IgG4 anti-DFS70 antibodies may contribute to the severity of AD.[14]

In alopecia, twenty percent of the patients had positive anti-DFS70 antibodies whereby the IgG subclasses of IgG1 and IgG2 anti-DFS70 antibodies were dominant.[31] Further analysis demonstrated the DFS70 antigen was localized predominantly in the outer root sheath cells.[31] Taking together the findings in the alopecia population, the antibodies against the DFS70 antigen may be related to the aetiology in a certain population of alopecia areata.[31]

A previous study showed the anti-DFS70 antibodies were detected among patients with idiopathic interstitial lung disease (ILD) but the occurrence was lower than in healthy population and not different from those with connective tissue disease-related ILD.[29] Another interesting observation was the association between anti-DFS70 antibodies with thrombosis. It was shown the patients with anti-DFS antibodies were unexpectedly had a high prevalence of thrombosis or maternal complications related to thrombosis.[43] The odd ratio of getting thrombosis for unexplained thrombophilia with positive anti-DFS antibodies was 4.04.[43] The healthy individuals with positive anti-DFS70 antibodies were noticed to display a low aPTT ratio and they were possibly at risk of suffering from thrombotic events due to this hypercoagulable state.[43] However, a recent report suggested there was no clear association between higher prevalence of anti-DFS70 antibodies with arterial, venous, or recurrent pregnancy loss when the specific CLIA and LIA assays were used for the anti-DFS70 antibodies testing.[44]

Among the other autoimmune diseases, the anti-DFS70 antibodies were detected most commonly in autoimmune thyroiditis.[22,40] These antibodies were also detected among the oncologic or neoplastic patients,[13,45] multiple sclerosis,[43] infections,[21] non-specific musculoskeletal complaints, and other miscellaneous conditions.[22]

The prevalence of anti-DFS70 antibodies in systemic autoimmune rheumatic diseases (SARDs)/ ANA-associated rheumatic diseases (AARDs)

Peker *et al*. described that there was no significant difference observed between the blood donor population and SARDs patients.[27] However, most other studies described the frequency of an isolated anti-DFS70 antibody as lower in AARDs than in a healthy population.[25,30,33] Because of its lower prevalence in SARDs, the presence of these anti-DFS70 antibodies particularly the monospecific or isolated anti-DFS70 antibodies in routine ANA screening samples was perhaps able to rule out this diagnosis. The studies that described the prevalence of the anti-DFS70 antibodies among SARDs/AARDs were as shown in Table 1. The prevalence of anti-DFS70 antibodies was ranging from as low as 0% to as high as 17%.[4,25] Additionally, the prevalence of isolated anti-DFS70 antibodies was ranging from 0.4% to 6.5%.[25,40] Choi *et al*., showed the distribution of anti-DFS70 antibodies among the AARDs patients varied in different countries.[46] Similarly, another study by Bonroy *et al*., also indicated the variation in the prevalence among the different laboratories in Belgium.[30]

Table 1: Previous studies of anti-DFS70 antibodies which demonstrated the prevalence in SARDs/AARDs.

Reference	Country	DFS/Anti-DFS70 antibodies detection	Number of samples	Findings
Mariz *et al*. 2011[4]	Brazil	-ANA IIF -anti-ENA antibodies by double diffusion -Western blot analysis of nuclear fine speckled and nuclear dense fine speckled sera	-918 healthy individuals -153 ARDs sera (87 SLE, 45 systemic sclerosis, 11 Sjogren syndrome, 10 idiopathic inflammatory myopathy	-nuclear DFS pattern was observed in 39 (33.1%) of ANA positive subjects. -None of ARDs patients had nuclear DFS pattern.

Infantino *et al*. 2019[13]	Italy	-Anti-DFS70 antibodies detection by CLIA method by QUANTA Flash DFS70 kit (Inova Diagnostics, San Diego, USA) -Anti-ENA and dsDNA were detected by the QUANTA Flash anti-ENA and dsDNA respectively (Inova Diagnostics, San Diego, USA)	-Three groups of patients included: -333 AARD patients -384 non-AARD patients -51 UCTD patients -Total of 768 patients included	-7/333 (2.1%) of AARDs patients had positive anti-DFS70 antibodies; Sjogren syndrome=3, SLE=4. -None had isolated anti-DFS70 antibodies. -The distribution of monospecific was more prevalent among the UCTD group; 0/7 (0%) in AARDs versus 2/9(22%) in non-AARDs versus 3/3(100%) in UCTD, p=0.007.
Shovman *et al*. 2018[21]	Israel	-ANA IIF and Immunoadsorption by NOVA Lite HEp-2 ANA Select (Inova Diagnostics, San Diego, USA) -Anti-DFS70 antibodies detected by CLIA method, QUANTA Flash kit (Inova Diagnostics, San Diego, USA) -Detection of specific ANAs by BioPlex 2200 ANA Screen system -Monospecificity of anti-DFS70 antibodies was defined by successful and complete inhibition of ANA reactivity by the DFS70 antigen in the HEp-2 Select buffer	-Patients were divided into 3 groups: -51 diagnosed with AARDs (SLE=33, Sjogren=10, DM/PM=6, MCTD=2) -85 routine ANA cohort -92 healthy individuals	-Anti-DFS70 antibodies by CLIA were positive at higher frequency in healthy population than in patients with AARD (10.9% versus 1.9%, p=0.02). -At the same time, the anti-DFS70 antibodies level was higher in healthy population than in patients with AARDs. -Only one patient with AARD had positive anti-DFS70 antibodies. This patient was diagnosed with SLE and had concomitant other autoantibodies detected. -The ANA fluorescence pattern was not inhibited by ANA Select kit.
Hayashi *et al*. 2021[25]	Japan	-ANA-IIF, HEp-20-10 (Euroimmun, Luebeck, Germany) -ELISA (MBL, Nagoya, Japan) Immunoadsorption followed by ELISA for the confirmation of anti-DFS70 antibodies -ELISA for the detection of other CTD-related autoantibodies	276 AARD patients 250 healthy individuals	-17% (47/276) AARD had positive anti-DFS70 antibodies. -18 (6.5%) had an isolated AARD. -Prevalence of isolated antiDFS70 antibodies was lower among AARD compared to the healthy population.

Koo *et al*. 2019[26]	Korea	-ANA IIF was performed using the FLUORO HEPANA TEST kit (MBL, Nagoya, Japan). The screening dilution was performed at 1:80 and up to 1:5120 by successive twofold dilution -In samples with ANA positive, ANA IIF was performed again on Mosaic HEp-20-10 (Euroimmun, Luebeck, Germany) -ELISA kit by Euroimmun was used for the detection of anti-DFS70 antibodies. -Other autoantibodies were detected using different laboratory methods	-Routine ANA sera that include 5509 patients -Patients with DFS pattern in ANA IIF were included in this study -Patients were categorised into AARD and non AARD	-125 patients showed DFS pattern in ANA IIF. -From these 125 patients, 59 were diagnosed with SARD and subsequent analysis showed 22/59 SARD had AARDs. -From ELISA test, 75/125 of the patients with DFS pattern had positive anti-DFS70 antibodies. -26/75 were diagnosed with SARDs and only 10 patients (10/26) were diagnosed with AARDs. -From these 10 patients, eight were diagnosed with SLE and two with Sjogren syndrome. -Thus, the prevalence of anti-DFS70 antibodies was higher in the non-rheumatic diseases versus other rheumatic diseases and AARDs (74.2% versus 43.2% versus 45.5%). -Receiver operating curve (ROC) analysis showed AUC of 0.642 (p=0.032) discriminated between non-AARD and AARDs. -Anti-dsDNA and anti-ENAs were detected in all patients with AARDs, thus there was no patient who had an isolated anti-DFS70 antibodies.

Peker *et al*. 2019[27]	Turkey	-ANA IIF used HEp-20-10/liver biochip kit (Euroimmun, Luebeck, Germany), screened at 1:100 dilution -Anti-DFS70 antibodies confirmation by the ELISA kit (CusaBio Biotech, Wuhan, China) -Anti-ENAs were identified by line immunoassay kit, ANA Profile (Euroimmun, Luebeck, Germany)	-3224 SARDs that include AARDs, non-AARDs and UCTD -507 healthy blood donor	-The prevalence of DFS fluorescence pattern among the SARDs patients was 1.35% compared to 0.78% among the healthy blood donors. -Further analysis by ELISA, no significant difference was noted between the prevalence of anti-DFS70 antibodies among SARDs and healthy blood donor.
Bonroy *et al*. 2018[30]	Belgium	-ANA IIF used HEp-2 and HEp-2000 kits. ANA cut off was standardised at 1:160 -Detection of anti-DFS70 antibodies: -ELISA DFS 70 (Euroimmun, Luebeck, Germany) -Line Blot (EROLINE ANA profile 3 plus DFS70, Euroimmun) -Chemiluminescence assay (QUANTA Flash, Inova Diagnostics, USA)	-Four Belgian clinical routine laboratories in different care settings -Algemeen Medisch Laboratorium Antwerp (AML): primary care. -OLV Hospital Aalst (OLVA) and GZA Hospitals Antwerp (GZA): secondary care -University Hospital Ghent (UGZ): tertiary care	-The distribution of anti-DFS70 antibodies varied between the centers when taken either one of the methods was positive. -The highest frequency of anti-DFS70 antibodies among AARDs was found at UZG (10/13,77%) followed by GZA (5/20, 25%) and OLVA (1/16,6%). -In isolated anti-DFS70 antibodies, defined by HEp-2 select analysis, the association of AARDs was lower in UZG (2/4, 50%) and absent in the secondary centers.
Yumuk *et al*. 2020[34]	Turkey	-ANA IIF HEp 20-10 (Euroimmun, Luebeck, Germany). Screening dilution at 1:100. -Line immunoassay (LIA) was used for the confirmation of the anti-DFS70 antibodies. The LIA used was EUROLINE ANA Profile 3 plus DFS70 (Euroimmun, Luebeck, Germany)	3432 sera from routine ANA screening. From this, 390 patients were diagnosed with SARD	-5.1% (20/390) had DFS IIF pattern. - 42/390 (10.8%) of SARD patients positive anti-DFS70 antibodies by LIA. -Overall, 25 (6.4%) had isolated anti-DFS70 antibodies - 7/25 had typical DFS IIF pattern.

Miyara *et al*. 2013[38]	France	-ANA-IIF HEp-2000 (ImmunoConcept) -Chemiluminescence immunoassay (CLIA) using the QUANTA Flash DFS70 kit (Inova Diagnostics, San Diego, SA, USA) for the detection of anti-DFS70 antibodies -ANA ELISA using QUANTA Lite (Inova Diagnostic, San Diego, SA, USA) was used for the detection of other antibodies	-100 consecutive ANA sera with DFS pattern on ANA IIF -100 non DFS pattern as comparison group	-12% SARD patients had DFS IIF pattern. -Only five patients with SARDs had mono-specific anti-DFS70 antibodies. -The anti-DFS70 antibodies detection by CLIA was shown able to discriminate between SARD and non SARD with area under the curve (AUC) of 0.73.
Mahler *et al*. 2012[40]	Canada	-ANA-IIF, HEp-20-10/liver(monkey) (Euroimmun, Luebeck, Germany) -ELISA (MBL International) -CLIA using QUANTA Flash (Inova Diagnostics, USA) -Recombinant DFS70/ LEDGF and Western blot -Affinity purification of anti-DFS70 anti-bodies -Detection of other autoantibodies	-53 sera from 3263 sequential routine ANA cohort -Clinically defined samples of healthy individuals and various disease conditions	-2.8% (7/251) of SLE patients were positive anti-DFS70 antibodies. -Only one patient had isolated anti-DFS70 antibodies. -No significant different among SLE patients (anti-DFS70 positive versus anti-DFS70 negative) in term of: - mean age - gender (female/male ratio) - clinical features -Most of anti-DFS positive SLE presented with arthritis (100%) and photosensitivity (71.4%). -Hemolytic anemia was higher in anti-DFS positive group while SLEDAI score was higher in anti-DFS70 negative.

Choi *et al*. 2017[46]	Multi-center, 32 centers in 11 coun-tries	-ANA IIF HEp-2000 kit (ImmunoConcepts, Sacramento, CA, USA), screening at 1:160 dilution HEp-2 Select Kit (Inova Diagnostics, San Diego, USA) for DFS70 confirmation -Anti-DFS70 antibodies by CLIA (Inova Diagnostics, San Diego, USA) -Detection of other related autoantibodies -Monospecific anti-DFS70 antibodies was defined as only anti-DFS70 antibodies positive and negative for other autoantibodies	-SLICC inception cohort -Enrolled patients fulfilled ACR criteria for definite SLE within 15 months of diagnosis -Total patients included were 1137	-Anti-DFS70 antibodies positive by CLIA was 7.1% (81/1137), but monospecific only in 13 patients or 1.1% (13/1137) overall. -In term of ANA IIF, DFS pattern was observed in 10 patients with positive anti-DFS70 antibodies versus 7 patients with negative anti-DFS70 antibodies (10/81,12.3% versus 7/1056,0.7%). -From the 10 patients with positive anti-DFS70 antibodies, only one patient had DFS pattern inhibited. -Other patterns were observed in the remaining patients. -Interestingly, from 13 patients with monospecific anti-DFS70 antibodies, only two patients had typical DFS fluorescence pattern. Multivariate analysis showed: -Patients from Canada and Europe less likely to have anti-DFS70 antibodies. -Patients with anti-ß2 glycoprotein antibodies were more likely to have positive anti-DFS70 antibodies. -Those with anti-SSB/La were less likely to have anti-DFS70 antibodies.

Muro *et al*. 2008[47]	Japan	-ANA-IIF using commercial HEp-2 cells -Immunoblot method was used for the confirmation of anti-DFS70 antibodies -ELISA method was used for the detection of other autoantibodies	500 ARD patients	-22/500 (4.4%) of the patients were positive for anti-DFS70 antibodies on both ANA IIF and immunoblot. -Majority of them 19/22 (86%) had simultaneous disease-related autoantibodies. -Only three patients (0.6%) had isolated anti-DFS70 antibodies.
Aragon *et al*. 2020[48]	Cali, Columbia	-ANA IIF was performed and the sera with DFS pattern were confirmed by the immunoadsorption kit NOVA LITE HEp-2 Select with DAPI (Inova Diagnostics, San Diego, USA). Screening dilution at 1:160.	-127 patients, aged 18 and above -64 patients were diagnosed with SLE -63 patients were not diagnosed with any autoimmune diseases	-The IIF with immunoadsorption were performed on all 127 samples. -The immunoadsorption showed positive in 21 individuals without the autoimmune diseases (33%), and 8 (12%) in SLE group (p=0.005). -Among the SLE patients, two of them had an isolated anti-DFS70 antibodies. -Among all SLE patients; -anti-dsDNA was positively associated with anti-DFS70 antibodies negative patients. -SLEDAI was higher in anti-DFS70 antibodies negative than positive patients. -Majority of the anti-DFS70 antibodies positive patients had kidney involvement but no statistically significant showed between positive and negative anti-DFS70 antibodies with the development of lupus nephritis.

Tan *et al*. 2020[49]	Singapore	-ANA IIF was detected by the HEp-2010 kit (Euroimmun, Luebeck, Germany) -Anti-DFS70 antibodies were detected by ELISA kit (Euroimmun, Luebeck, Germany) -The anti-DFS70 antibodies were performed on all positive ANA IIF sera	-Routine ANA cohort that included 929 patients. -592 positive ANA sera were further tested for anti-DFS70 antibodies detection	-Anti-DFS70 antibodies were positive in 59 (10%) of ANA positive sera. -Only eight out of the 59 patients were diagnosed with SARDs. (SLE=4, Sjogren syndrome=2, SLE/ Systemic sclerosis=1 and systemic vasculitis=1). -Overall, positive anti-DFS70 antibodies was not associated with the absence of SARDs. -The odd ratio of having SARDs was not different when the anti-DFS antibodies were either positive or negative.
Sener *et al*. 2015[50]	Turkey	-ANA IIF HEp-2010 kit (Euroimmun, Luebeck, Germany). The screening dilution was at 1:100. Sera with DFS pattern re-evaluated at 1:320 and 1:1000 titers. -Anti-ENAs were checked by line immunoassay. This was performed on all samples with DFS pattern. The line immunoassay was performed on the commercial kit (Euroimmun, Luebeck, Germany)	-5800 sera from routine ANA cohort	-ANA IIF was positive in 1320 sera -DFS pattern was noted in 16 of ANA positive sera (1.2%) -Only one patient had SARD diagnosis. (0.08%) -All the DFS pattern patients did not have associated anti-ENAs.
Lucas *et al*. 2018[51]	New Zealand	-ANA IIF HEp-2 -Anti-DFS70 antibodies, QUANTA Flash (Inova Diagnostics, San Diego, USA) -Anti-ENA (Autoimmune EIA ANA profile test)	-211 positive ANA (102 with SARDs and 109 without SARDs)	-Anti-DFS70 antibodies were detected in 7% (7/109) of non SARDs sera and 0.98% (1/102) of SARDs sera. -5/8 including the only SARDs with anti-DFS70 antibodies had isolated anti-DFS70 antibodies.

The prevalence of primary Sjogren syndrome was high among the patients with DFS fluorescence pattern observed in a study by Conticini *et al*. They found that seven out of nine patients with DFS fluorescence pattern were diagnosed with the disease.[52] The prevalence was also high in the undifferentiated connective tissue diseases (UCTD) population with 13.3% of them testing positive for anti-DFS70 antibodies.[53] Among the paediatric conditions, the highest prevalence of anti-DFS70 antibodies was detected in juvenile dermatomyositis (2/11, 18.2%) followed by juvenile localised scleroderma (4/29, 13.8%) and childhood SLE (19/331, 5.7%).[41]

Inclusion of anti-DFS70 antibodies detection in the routine ANA testing and reporting

Although the DFS pattern can be recognised by ANA fluorescence pattern, the pattern does not necessarily mean anti-DFS70 antibodies. Previous studies already proved that not all DFS pattern samples were positive for anti-DFS70 antibodies when detected by other laboratory methods such as CLIA, ELISA, or immunoblot. Furthermore, the pattern can be wrongly interpreted by the inexperienced observer. The pattern can also be masked by other ANA fluorescence patterns particularly the homogeneous pattern but in this case, the detection of DFS pattern may not be that important.

The most common reason for performing ANA test is to help in the diagnosis of SARD particularly AARD. Knowing the fact that anti-DFS70 antibodies are present in low frequency among the SARD, perhaps it is important to include these antibodies in the laboratory's diagnostic algorithm for SARD. Previous studies had successfully shown that anti-DFS70 antibodies were able to discriminate between SARDs and non SARDs.[26,38] Kiefer *et al*., also showed had high specificity of 97.6% and a positive predictive value of 89.5% in detecting the absence of connective tissue diseases[54] Overall, the pooled sensitivity and specificity of anti-DFS70 antibodies in exclusion of SARDs were 0.19 (95% CI: 0.12-0.28) and 0.93 (95% CI: 0.88-0.96) respectively.[55] The recognition of the isolated anti-DFS70 antibodies is more important to rule out the AARD.[13] Thus, through these observations, performing a multiplex assay that include the detection of anti-DFS70 antibodies is more cost-effective. The currently available multiplex assay using the line immunoassay technique that includes the DFS70 is useful. The performance of this line immunoassay was shown to be correlated with the CLIA.[35]

Different algorithms for the inclusion of the anti-DFS70 antibodies detection have been suggested. Mahler *et al*. introduced the algorithm whereby the confirmation of anti-DFS70 antibodies and ANA (ELISA) were used in the samples producing DFS fluorescence patterns.[56,57] The other algorithm suggested determining anti-DFS70 antibodies if the ANA IIF showed DFS pattern. In this algorithm, the ANA determination by ELISA was not included.[58] The third algorithm used a different approach. The detection of specific antibodies was performed after positive ANA IIF and if the specific antibodies detection was negative, an anti-DFS70 antibodies test is performed.[10] Hissaria *et al*., suggested an algorithm that if the DFS fluorescence pattern was observed, then the interpretation would be SARD is unlikely.[59] Taking into account the previous algorithms, the algorithm as outlined in Figure 2 is perhaps can be used for the screening purpose of SARDs.

The inclusion of the anti-DFS70 antibodies in the routine ANA screening was proven to be cost-effective.[60] The algorithm used by Gundin *et al*., was shown to reduce the cost of subsequent laboratory tests during follow up and more importantly it significantly reduced the number of clinic visits thus reducing the cost for clinic time and staff.[60] Another study among the undifferentiated connective tissue disease patients also showed effective cost saving when the anti-DFS70 antibodies test was introduced in the algorithm and the cost saving is greater when the prevalence of anti-DFS70 antibodies is higher.[61] It is also important if the family physicians can request this test especially in patients who are ANA positive but ENA negative to reduce the risk of inappropriate treatment for AARDs and reduce the need to refer to specialist clinics.[62]

The conundrum of anti-DFS70 antibodies role

The DFS70/LEDGFp75 is involved in several important cellular functions that include apoptosis signalling, stress survival, the inflammatory response through implicating in the activation of IL-6/STAT3 pathway, and protein-protein interaction.[63] The DFS70/LEDGFp75 has two major splice variants namely p75 and p52. The C-terminal region that contains the autoepitope (aa 347-429) is recognised by the autoantibodies and consistently recognised as

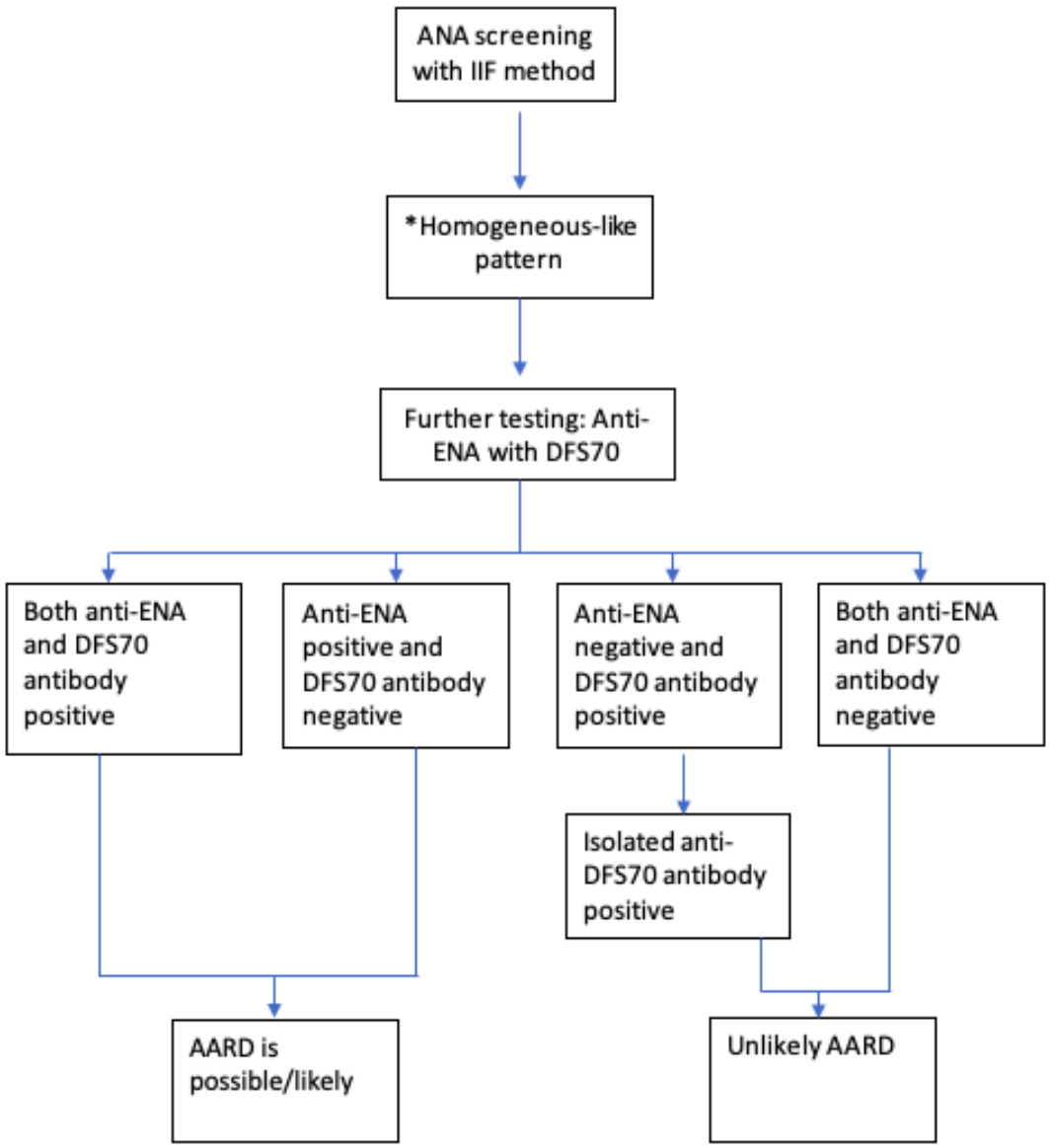

FIG. 2: The suggested algorithm for the inclusion of anti-DFS70 antibodies in ANA screening. *A homogeneous-like pattern is a pattern that looks similar to homogeneous but it is atypical whereby the nucleoplasm of the interphase cell will show heterogeneous fluorescence staining rather than homogeneous. This pattern can either be quasi homogeneous or DFS pattern.

a single band of 70-75 kD in the immunoblot of cell lysate.[64] Interestingly, this immunogenic region is shared with the HIV-1 integrase binding domain (IBD, residues 347-429) where the HIV-1 integrase interacts.[64] Thus, the role of the presence of anti-DFS70 antibodies in the HIV-1 infection remains unclear. It is not known if the autoantibodies do have a protective role towards this infection.

Based on the current evidence, anti-DFS70 antibodies may have protective and pathogenic roles in different conditions. The fact that it is more frequently encountered among the healthy population and the IgG is the predominant isotype might indicate that this is a natural antibody.[63,64] They might involve in removing the DFS70/LEDGFp75 and its cleavage from the cellular debris generated following non-inflammatory apoptotic cell death.[63] Thus, their presence among the healthy population might act as a sensor of undetected chronic inflammation. These autoantibodies were previously shown to

demonstrate the cytotoxic effect in vitro against the lens epithelial cell (LEC) and the binding of the antibodies to DFS70/LEDGFp75 preventing the uptake by the neighbouring cells contributing their pathogenic role in atopic dermatitis.[65]

CONCLUSION

The anti-DFS70 antibodies have a lower prevalence among SARDs than non-SARDs patients, particularly the monospecific or isolated anti-DFS70 antibodies. Including the detection of anti-DFS70 antibodies into the ANA screening algorithm can help to reduce subsequent clinic and laboratory costs and may provide reassurance for those with an isolated anti-DFS70 antibody that they are unlikely to develop SARDs. However, it is important to highlight that the detection of DFS pattern in ANA HEp-2 IIF may not always be reliable thus the need for further analysis by other laboratory methods for the confirmation of anti-DFS70 antibodies.

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