

Diffuse fine speckled (DFS70) antibody in ANA testing: identification and proposed management in a New Zealand tertiary hospital setting

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ABSTRACT

Objectives: DFS70 antibody gives an ANA Immunofluorescent (IIF) staining pattern (homogeneous / speckled), frequently at high titre that is often seen in AARD patients although DFS70 antibody is rarely associated with systemic autoimmune – based rheumatic disease. The purposes of this study were to (a) identify if DFS70 antibody was present in our test population (b) determine if DFS70 patterns could be identified with first-round IIF ANA testing (c) establish the relationships between IIF, CMIA (Chemiluminescence) and LIA (Line Immunoassay) methodologies for identification of DFS70 antibody and (d) propose a testing algorithm for implementation at LabPLUS that would allow the identification of DFS70 antibody. This is the first published study on a NZ patient cohort.

Methods: Patients referred for routine ANA testing were selected as presumptive DFS70 antibody positive and were then subsequently tested by both CMIA (INOVA Diagnostics BIOFLASH™) and LIA (Euroimmun EUROLINE Profile 3 Plus DFS70™) methodologies.

Results: DFS70 antibody was proven to occur in NZ patients referred for ANA testing. IIF methodology in the hands of experienced operators correctly identified approximately 80% of DFS70 antibody positive patients. As second round confirmatory assays, both the CMIA and LIA methodologies were effective in the role.

Conclusions: On the basis of the results of the study a testing algorithm was designed to allow the identification and reporting of DFS70 antibody. It is believed that when testing is implemented there will be significant fiscal benefits from reduced inappropriate testing as well as improved rheumatology outpatient clinic patient triaging over time. We further believe that implementation of DFS70 antibody testing and reporting will, for a proportion of patients reduce their anxiety and greatly reduce if not completely eliminate the opportunity for inappropriate treatment based on apparently significant ANA results.

Keywords: AARD, IIF, CMIA, LIA, DFS70 antibody, NZ patient cohort, testing algorithm.

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INTRODUCTION

The detection of antinuclear antibodies (ANA), directed against intracellular antigens is a key serological feature of ANA associated rheumatic diseases (AARD) (1-3). Although the acronym 'SARD' (systemic autoimmune rheumatic diseases) is often used synonymously with AARD, the latter also encompasses RA, AAV and APS diseases (1) which is why AARD was adopted for this study. The most commonly used method for the detection of ANA is indirect immunofluorescence (IIF) using Human Epithelial Polyoma type-2 (Hep-2) cells (4,5) and this has been the screening test of choice for ANA for many years (5). A recent review by the American College of Rheumatology indicated that this should remain the gold standard for ANA testing despite new techniques and technologies being available (6).

Although high in assay sensitivity, the main drawback of ANA testing is the limited specificity for SARD (2,4,7), as the presence of ANA directed against intracellular antigens is associated with a wide range of disorders (8). Studies have also shown that ANA can return positive in up to 20% of apparently healthy individuals (HI) (2,4,8) with the majority of these positives resulting from antibodies against the dense fine speckled 70 antigen (anti-DFS70) (2,8).

Anti-DFS70 was first identified in 1994 in a patient with interstitial cystitis (3,4) but has since been found in sera of patients with a variety of conditions (2) including healthy individuals and those with no evidence of AARD

(2,9-10). Due to the high prevalence of anti-DFS70 antibodies in ANA positive healthy individuals (1), it has been postulated that the presence of anti-DFS70, particularly when this occurs in isolation from other clinically relevant autoantibodies (2,9), could be used to help exclude the diagnosis of AARD (2,10). This is important, as the distinction between ANA positive HI's and those with early/undiagnosed AARD is vital in appropriate triage and referral of patients to tertiary services (3).

Although multiple studies have been performed worldwide and evidence has been provided to suggest that anti-DFS70 antibodies are not associated with AARD, there is no literature or study that is specific to New Zealand. As a tertiary hospital – based referral laboratory, LabPLUS processes ANA screening tests from patients with high and low pre-test probabilities of having AARD as well as those patients with identified AARD's who are under treatment.

The aims of this study were to (a) identify if DFS70 antibody was present in our test population; (b) determine if DFS70 patterns could be identified with first-round IIF ANA testing; (c) establish the relationships between IIF, CMIA (Chemiluminescence) and LIA (Line Immunoassay) methodologies for identification of DFS70 antibody; and (d) propose a testing algorithm for implementation at LabPLUS that would allow the identification of DFS70 antibody.

METHODS AND MATERIALS

Patient cohort

Over the period November 2014 – November 2015, sera from 57 patients tested for ANA by IIF were retained at -80°C . The cohort was selected on the basis that all sera had homogeneous and speckled patterns that implied (by comparing with literature – based images) a high pre-test probability for the presence of DFS 70 antibody.

Five patients had >1 bleed giving a total of 63 sera. The cohort comprised 13 males (median age 51 years; range 6 – 83) and 44 females (median age 52 years; range (17-95). Patient diagnosis (of AARD or otherwise) was determined by clinical chart review of medical records. Patient identity was not disclosed and data was used anonymously. As this was a retrospective study with no modification on clinical decision making or individual follow up, patient consent was not required.

All 63 sera were tested by both CMIA and LIA methodologies. Sera tested by CMIA and LIA underwent a single freeze-thaw cycle. Patients were identified as having DFS70 antibody if both CMIA and LIA methodologies were reactive. Equally, if both methodologies were non-reactive, patients were identified as not possessing DFS70 antibody. Patients demonstrating discordant CMIA / LIA results were identified as having an undetermined DFS70 antibody status.

Diagnostic assays

Indirect Immunofluorescence (IIF)

Patient sera were initially screened at a 1:80 dilution and those giving an initially reactive screen result had testing repeated at dilutions of 1:80, 1:320 and 1:1280. The assay used was supplied by Immunoconcepts™ (USA) and the cell line was their SSA transfected Hep-2000™ product. Testing was performed in accordance with the manufacturer's instructions. Serum dilutions, incubations and wash steps were performed using a Therdiag CARIS® 4 – probe robotic pipetting system. Resultant fluorescence was viewed independently by two senior medical laboratory scientists with a Zeiss LED AXIO Lab.A1 microscope. Images were captured with a Zeiss Axio Cam MRC® digital camera and ZEN® image processing software.

Chemiluminescence (CMIA)

All specimens were processed in accordance with manufacturer's instructions on the QUANTA Flash® benchtop CMIA analyser (Werfen group / INOVA Diagnostics, USA). Briefly, sera is pre-diluted by the instrument and then mixed with paramagnetic beads coated with recombinant DFS 70 antigen. Following 37°C incubation, beads are magnetised and washed. This is followed by second 37°C incubation with an isoluminol derivative labelled anti-human IgG conjugate. Again, beads are re-magnetised and washed. Triggers (high pH, hydrogen peroxide and an unstated catalyst) are added to the reaction cuvette which initiates the light reaction, recorded and interpreted by the instrument as Relative Light Units (RLU's). The RLU produced is directly proportional to the concentration of DFS70 IgG in the original serum specimen. The assay cut-off for reactivity is 20 RLU.

Line immunoassay (LIA)

All specimens were processed in accordance with manufacturer's instructions using the EUROIMMUN™ EUROLINE ANA Profile 3 plus DFS70 LIA® and the EUROlineScan® band interpretation software. Briefly, 1:100 diluted sera are incubated with re-hydrated strips containing recombinant DFS70 antigen. After a 30 minute ambient incubation and wash step strips are re-incubated (30 minutes) with an alkaline phosphatase labelled anti-human IgG reagent. Strips are re-washed and incubated for 10 minutes with a substrate (NBT-BCIP) reagent. Strips are then dried, mounted, scanned and digitally evaluated using the EUROlineScan® software. Line intensity readings of >11 units are considered specific.

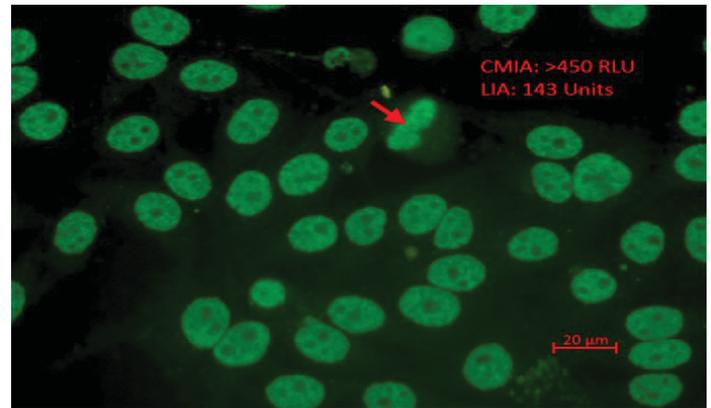
Statistical analyses

Statistical analyses were performed using GraphPad PRISM 7™ software (La Jolla, California, USA).

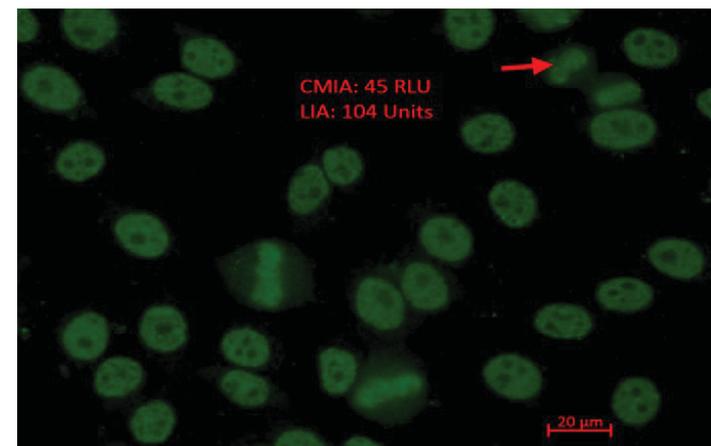
RESULTS

IIF

The success rate of identifying DFS70 antibody in the selected patient cohort was 75% (47/63 sera). Patients with multiple bleeds had the same pattern and end-point titres on all occasions. The staining pattern for DFS70 antibody (homogeneous / speckled with discrete moderate sized speckles visible in both interphase and mitotic cells) was consistent irrespective of assay strength as determined by CMIA and LIA methodology (Figure 1).



(a)



(b)

Figure 1. DFS70 antibody IIF staining pattern on Hep-2000 cell substrate. Micrographs of DFS 70 antibody staining on Hep-2000 cell line X400 magnification where antibody is present in both (a) high and (b) low levels. Discrete speckling is visible in both interphase and mitotic (arrow) cells.

CMIA and LIA

A single patient did not have sufficient specimen to allow LIA testing. The qualitative CMIA result for the patient was 'not detected' with a RLU value of <3.0 . All sera (N=10/ 10) with a CMIA RLU value of <3.0 were negative for DFS70 antibody by LIA, and on this basis, this patient serum was characterised as DFS70 antibody negative.

Method agreement was 94% (10 dual methodology DFS70 antibody negative; 48 dual methodology DFS 70 antibody positive). The two methods demonstrated a moderate to strong correlation ($r = 0.811$) when \log_{10} transformed sample to cut-off (S/CO) ratios from DFS 70 antibody positive sera were subjected to LR analysis (Figure 2). The five patients with >1 specimen, all of whom were DFS70 antibody positive effectively had stable antibody levels over time (Table 1).

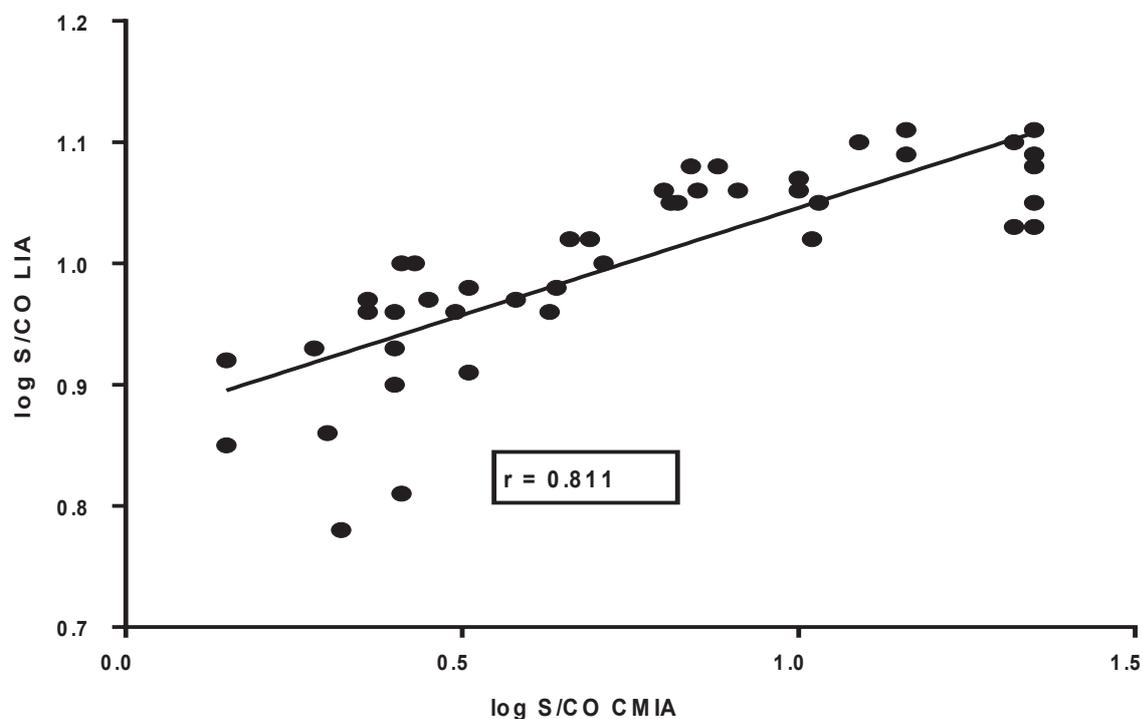


Figure 2. Methodology (CMIA / LIA) correlation in a cohort of patients demonstrating dual method reactivity for DFS70 antibody.

Table 1. Stability of DFS70 antibody levels in five patients.

Patient	Blood	CMIA (RLU)	LIA (Units)
1	Initial	128	122
	+40 days	138	132
	+55 days	139	126
2	Initial	55	110
	+50 days	52	110
3	Initial	243	140
	+37 days	213	124
4	Initial	>451	132
	+35 days	>451	119
5	Initial	56	102
	+16 days	76	102

Table 2. Patients with discordant methodology results for DFS70 antibody.

Patient	CMIA RLU Cut-off: >20	LIA Units Cut-off: >11
1	12	68
2	12	48
3	18	74
4	11	46

There were 4 patients with discordant methodology outcomes, all of whom were reactive by LIA and negative by CMIA (Table 2). Interestingly, the mean \pm SE CMIA RLU (13.25 ± 1.601) from this group was significantly different ($P < 0.05$; Student's t -test) from both the dual methodology DFS70 antibody positive group (155.9 ± 20.1) and the dual methodology DFS70 antibody negative group ($< 3.0 \pm 0.0$), implying the presence of low level specific DFS70 antibody. None of the four patients were reactive for either dsDNA or ENA antibodies.

Patient cohort

Due to difficulties in accessing clinical notes, 51 of the original 57 patients were included and analysed with respect to their pathology status. For both males and females there was an inverse relationship between numbers of patients tested and frequency of DFS70 antibody per age class as patient age increased. Over the age range of 30-54 years there was a 1:1 ratio of patients tested: patients DFS70 antibody positive for both sexes (Figure 3).

Two DFS70 antibody positive patients had low titre (7-12 IU/mL [< 7]) dsDNA antibody (RIA) results without detectable ENA antibodies. Both patients did not have clinical features of a CTD. A further DFS70 antibody positive patient with a history of SLE was negative for dsDNA antibody (RIA) but had isolated Ro52 antibody detected on ENA antibody panel testing.

A single DFS70 antibody negative patient with stage 4 kidney disease due to lupus nephritis had high titre (> 45 IU/mL [< 7]) dsDNA antibody (RIA) results with isolated Ro52 antibody detected on ENA antibody panel testing.

Within our selected patient cohort we identified a high rate ($> 75\%$) of non-specific ANA requesting (with respect to clinical features of a possible AARD) which applied in the settings of DFS70 antibody being present or absent (Figure 4).

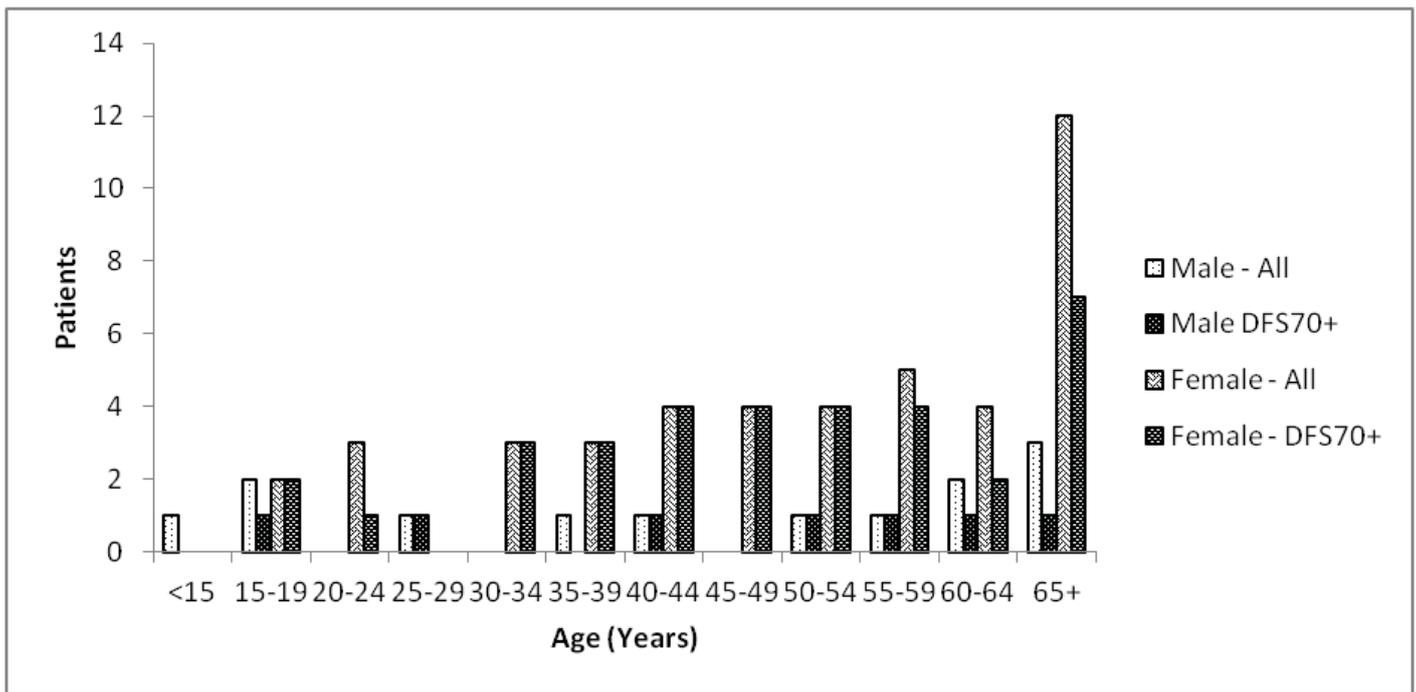


Figure 3. Frequency of DFS70 antibody in a selected patient cohort.

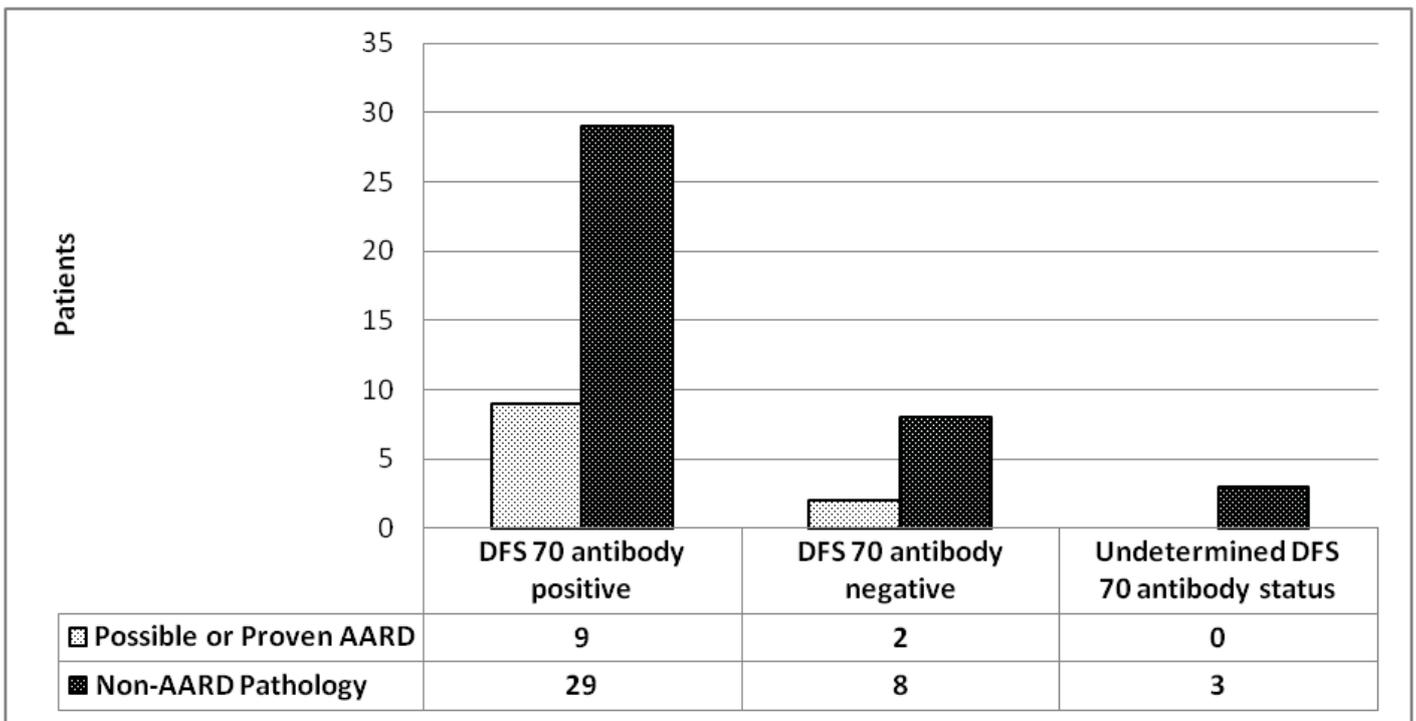


Figure 4. Relationship of the frequency of DFS70 antibody to AARD pathology in a selected patient cohort.

DISCUSSION

This study proved that a proportion of patients who are referred for ANA testing at LabPLUS have DFS70 antibody and, the titres and patterns (range 1:320 - >1:1280; homogeneous - speckled) reported are also seen in patients with AARD's. We believe this is the first published study in a NZ patient cohort. This study further demonstrated that the DFS70 antibody, when present, remains at a consistent titre over time. Published studies to date have used characterised disease and non-disease single bleed patients as opposed to a longitudinal series of specimens from single patients (2,11-13). Although our patient numbers with multiple bleeds were small (N=5), they all demonstrated detection stability of DFS70 antibody over time using multiple methodologies, which we consider a valuable finding with respect to implementation of testing protocols.

A key question that had to be answered was which methodology should be used for DFS70 antibody identification. Using IIF methodology, backed up by experienced microscopists the standard DFS70 antibody pattern was recognised in approximately 8 out of 10 patients, a frequency which is consistent with that seen in other studies (10-11). This study is not a method comparison; however, in our hands, after reviewing results from the INOVA Diagnostics BIOFLASH™ CMIA system and the EUROIMMUN EUROLINE Profile 3 plus DFS70™ LIA we found very similar levels of performance which is in agreement with a review article by Karsten *et al.* in 2016 who concluded that the method of detection for DFS70 antibody was less relevant than that for ANA testing (1). As both methods use recombinant DFS 70 antigen and independent studies have demonstrated DFS70 antigen homology (14-15) good method agreement is not an unexpected finding.

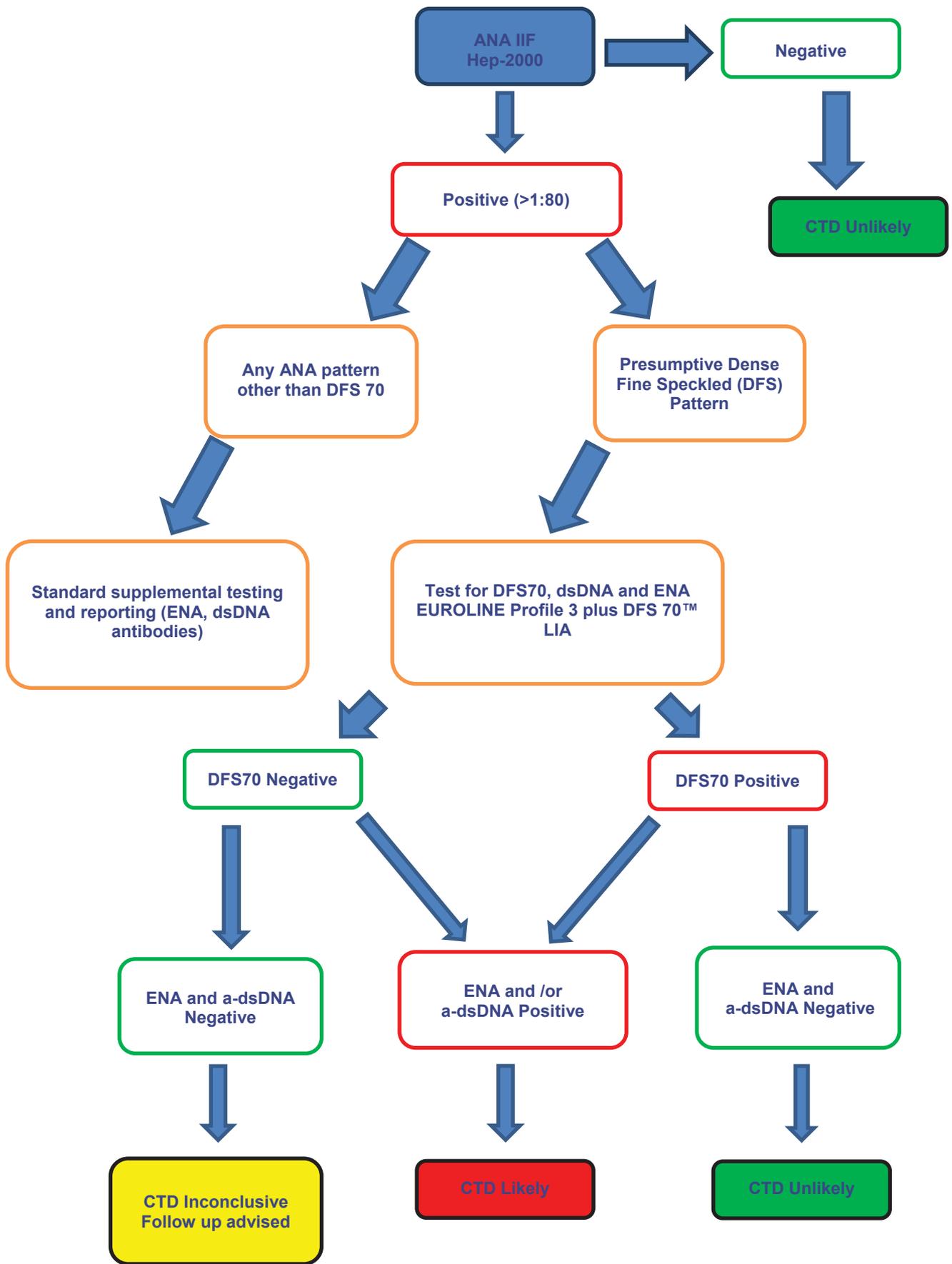


Figure 5. Proposed modified ANA testing algorithm for use at LabPLUS to identify and report DFS70 antibody.

Our study implied that the EUROLINE Profile 3 plus DFS70™ LIA may have slightly superior assay sensitivity compared to the CMIA assay. This was an unexpected finding, given that CMIA methodology has both high assay sensitivity and wide analytical measuring ranges (16). At the time of submission of this paper, we were unable to find any publications comparing the performance of CMIA and LIA methodologies, although Mutlu *et al's* study in 2016 suggested that LIA (different manufacturer: Immco Diagnostics) had lower sensitivity than ELISA (11). Mahler *et al's* finding of good correlation between ELISA and CMIA in 2012 (12) was reflected in our study which showed good correlation between CMIA RLU's and LIA densitometry values. A particular strength of our study design was subjecting all presumptive IIF DFS70 antibody positive sera to both specific techniques. This allowed confidence in choosing an assay that not only would detect DFS70 antibody when it was present but equally important for our purposes would give a negative result when the antibody was absent. For a second-round (post-IIF) confirmatory procedure, we consider either method to be suitable; but when we add the parameters of result TAT and expected low test numbers, LIA methodology was identified as the best fit for our testing environment and patient matrix.

As stated earlier, unexpected high titre ANA results frequently causes patients to be referred to Rheumatology services in the Auckland region. Complete reliance on clinical presentation for exclusion of AARD is not suitable in all cases as autoantibodies can develop and be detected years before clinical manifestations are seen (16). Furthermore, we have seen evidence (unpublished) that different preparation techniques of Hep-2 cell substrates by manufacturers may impact on DFS70 antibody detection. In our own setting, we have experienced two separate cases where DFS70 antibody was present giving a high ANA titre (1280) on a SSA transfected Hep-2 (Hep-2000™) cell line but gave low titre (80) on an un-transfected Hep-2 cell line implying the transfection process may inadvertently make the cell line more permissive to DFS70 antibody binding. To refute or validate this observation, testing of higher numbers of characterised DFS70 antibody positive sera on both transfected and un-transfected Hep-2 cell lines will be required. On limited evidence it appears as though DFS70 antibody may play a part in the reduced standardisation of ANA results across the Auckland region by those laboratories using IIF methodology highlighting the urgency for both identification and reporting. Implementation of DFS70 antibody testing and reporting has shown to have significant fiscal benefits (reduced testing costs for both ENA and ds DNA antibodies) as well as gains in Rheumatology out-patient clinic triaging efficiency (4).

In our setting we noted a high rate of non-specific ANA requesting. While requestor education will address this issue, it is expected this will be a mid to long-term project. In conjunction with the education initiative we propose the immediate implementation of a testing algorithm using IIF as the first-round screen and then LIA to confirm presumptive IIF DFS70 antibody positive sera (Figure 5). This proposed algorithm is similar to others in the literature (1,4) but it is uniquely adapted to our institution, our patient matrix, and the findings from this study. The proposed algorithm has the benefit of fitting seamlessly into our established testing procedures while retaining the safety of not excluding patients from ENA and dsDNA antibody testing based upon DFS70 antibody testing outcomes. We believe that benefits will accrue from (a) reductions in unnecessary follow up testing, (b) reduced rheumatology outpatient clinic appointments, and (c) enhanced clinical understanding for a proportion of patients where there are significant differences in ANA reported results from laboratories across the Auckland region using IIF methodology.

Beyond the likely fiscal benefits and system efficiency gains that will likely accrue from DFS70 antibody testing and reporting we must also consider the implications for patients. In New Zealand, medical laboratory practitioners (scientists and technicians) are governed by a code of ethics (17) of which points 4 and 5 combined require that our core values must be that of beneficence (to do good) and non-maleficence (to do no harm) whilst ensuring that the clinical information provided be both valued and precise. Having identified that DFS70 antibody is present in our test population and knowing that standard ANA reporting may lead to increased patient anxiety and potentially inappropriate treatment, we believe that for ethical code compliance it is mandatory that our testing strategy be modified to identify and report DFS70 antibody when present. We encourage all laboratories using IIF methodology to adopt this position.

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