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# ORIGINAL ARTICLE

## Requesting pattern of antinuclear antibodies (ANA) and anti-extractable nuclear antigens (ENA) simultaneously by clinicians in a tertiary care hospital in Malaysia

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*Abstract*

*Introduction:* International guidelines recommend having a positive anti-nuclear antibody (ANA) and clinical suspicion of systemic autoimmune rheumatic diseases (SARD) when requesting ANA subserologies. Compliance with these guidelines by physicians has been questioned in different parts of the world. Objective: To analyse the requesting pattern of ANA and anti-extractable nuclear antigens (ENA) simultaneously in the University of Malaya Medical Centre (UMMC). *Materials and Methods:* This is a retrospective descriptive study involving 1529 adult patients who had their ANA and ANA subserologies requested simultaneously by clinicians. The ANA, anti-ENA screening (ENASc) and anti-ENA specific (ENASp) results were retrieved. Their case records on their relevant diagnosis and follow-up tests were reviewed. *Results:* Among the 1,529 samples, 536 (35%) patients were positive, and 993 (65%) patients were negative for ANA by indirect immunofluorescence assay (IIF). In the ANA positive group, 109 (20%) were positive and 46 (9%) were borderline for ENASc. Of those ENASc positive patients, only 47 patients were requested for ENASp. Forty-one (87.2%) were positive for ENASp, In the ANA negative group, 111 (11%) were positive and 66 (7%) were borderline for ENASc. The majority of the ENASc positive (86, 77%) or borderline (63, 95%) had not been requested for ENASp in this group. Of those who had ENASp tests done (28), 19 (76%) were positive and 3 were borderline positive for ENASp. A total of 223 patients were diagnosed with SARD, out of which 147 had SARD in the ANA positive group (66%), with systemic lupus erythematosus being identified as the commonest SARD. A total of 76 patients were diagnosed with SARD in the ANA negative group (34%), with rheumatoid arthritis being identified as the commonest SARD. *Conclusion:* A large number of ENASc negative results are obtained concurrently with ANA negative results, suggesting clinicians do not comply with international guidelines when requesting ENA tests. This survey strongly suggests implementing measures in hospitals to comply with international recommendations on ENA testing.

*Keywords:* requesting pattern, ANA, ENASc, ENASp

### INTRODUCTION

Diagnosis of systemic autoimmune rheumatic diseases (SARDs) can be challenging at times based on history, physical examination, and baseline laboratory investigations. Anti-nuclear antibody (ANA) testing by indirect immunofluorescence (IIF) using HEp-2 cells is considered the "gold standard" initial diagnostic evaluation tool for most of the SARDs as recommended by the American College of Rheumatology (ACR) ANA Task Force in 2009. However, it is useful when interpreted with clinical suspicion of SARDs. It can be positive in a wide range of non-rheumatic diseases (for example, infections, malignancies, and drugs) and SARDs, including systemic lupus erythematosus (SLE), Sjogren's syndrome (SjS), systemic sclerosis (SSc), mixed connective tissue diseases (MCTD) and idiopathic inflammatory

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myopathies (IIM). ANA is usually reported in variable titres where 1:80 and above are considered positive in most of the laboratories.[1] ANA can appear in different patterns attributable to different SARDs with high sensitivity ranging from 75 to 95% but low specificity in diagnosis of SARDs.[2,3] ANA testing by IIF using Hep2 cells can give rise to 30 different patterns which can be categorised into 4 major groups: negative, nuclear, cytoplasmic, and mitotic patterns.[4] Further testing with ANA subserologies or anti-extractable nuclear antigens (ENA) may be necessary to confirm the diagnosis. Some of the ENAs are highly specific for certain SARDs. For example, anti-dsDNA for SLE (90%), anti-Scl-70 for SSc (99%), anti-Jo-1for anti-synthetase syndrome (98%) and anti-RNP for MCTD (98%).[2]

Most of ANA subserologies are positive in patients with positive ANA, though majority of the positive ANA are still negative for subserologies. However, it could be positive in a small percentage of patients with negative ANA. It is important to understand that ANA can be positive in 13.2% to 13.8% of healthy individuals in the general population, usually in low titres with frequency higher in females than males without any clinical evidence of disease.[5,6] Some of these patients may evolve into a specific SARD or undifferentiated connective tissue disease (UCTD) over time as antibodies could appear years before the onset of clinical symptoms.[7] ANA and its subserologies are usually negative in some of the SARDs such as rheumatoid arthritis, psoriatic arthritis, spondyloarthritis and vasculitis. ANA is not considered a diagnostic criterion in this group of diseases, but a negative ANA test could be of value in reaching a specific clinical diagnosis.

Clinicians should understand the appropriate applications and limitations of ANA and ANA subserologies when ordering these tests. Premature ordering for ANA subserologies without adequate clinical suspicion can lead to waste of resources and funding. ACR recommends against testing ANA subserologies without a positive ANA and clinical suspicion of immune mediated disease.[8] We are not sure how much our clinicians adhere to such guideline recommendations in clinical practice. Identifying this pattern will help suggest to clinicians that to adopt the ACR recommendations and other international guidelines on ANA subserology testing. This might help to reduce financial constraints on patients, institutions, and governments by avoiding unnecessary investigations without compromising patient care.[9] In addition, this might reduce the burden on laboratory staff and allow them to focus on more productive tasks, particularly in resource limited settings. In University Malaya Medical Centre (UMMC), algorithm of ENA testing is not routinely practiced and this test can be requested by doctors from any department and subspecialties. In previous years, ANA and simultaneous ENA testing were done, with request for either ENASc or ENASp. However, with recent changes in the local laboratory practice, ENASp would be done if specifically requested for with an indication provided or if ENASc was tested positive. Hence, we undertook a study to analyse the requesting pattern of ANA and ENA simultaneously in UMMC.

## MATERIALS AND METHODS

*Methodology*

This retrospective descriptive study was conducted at UMMC, a multidisciplinary tertiary referral hospital and was approved by the Medical Research Ethics Committee (MREC): N20181115953. As this was a retrospective chart audit there was no consent obtained from the patients. Patient identity, test results and medical records of these patients were kept confidential throughout the study. We identified 1529 reports of samples processed for ANA together with ANA subserologies at UMMC Laboratory over a period of four years (Jan 2014 to Dec 2017) and their results were traced from the laboratory Information System (LIS). All samples taken for ANA and ENA testing simultaneously from adult patients above the age of 18 years were included in the study. Results of samples ordered from paediatric age group were excluded from the study. Their case records reviewed regarding the relevant diagnosis and follow-up tests. Follow-up tests included repeat ENA screen and ENA specifics.

*Antibody testing systems*

Our ANA test system is an IIF antibody test setup for the semi-quantitative detection of ANA in human serum. The kit system used for ANA test was Immunoconcepts (Immuno Concepts, NA Ltd, Sacramento, CA 95827). This test system uses HEp-2 cells which has been transfected with multiple copies of the specific DNA sequence that carries the information for the SSA/Ro autoantigen where autoantibodies to SSA/Ro may show a distinctive staining pattern on the

transfected cells. In this test system, the patient samples are incubated with antigen substrate to allow specific binding of autoantibodies to cell nuclei. If ANAs are present, a stable antigen-antibody complex is formed. After washing to remove non-specifically bound antibodies, the substrate is incubated with an anti-human antibody conjugated to fluorescein. When results are positive, there is formation of a stable three-part complex consisting of fluorescent antibody bound to human ANA, which is bound to nuclear antigen. This complex can be visualized with the aid of a fluorescent microscope. In positive samples, the cell nuclei will show an apple-green fluorescence with a staining pattern characteristic of the nuclear antigen distribution within the cells. Detection of antibodies at dilutions of 1:80 and above were considered positive. The results of ANA are reported in the sequence: positive/negative—pattern—titre by the laboratory. For the positive results, the nomenclature for ANA patterns is reported as per the consensus reached in the first ICAP workshop.[10] Nuclear, cytoplasmic and mitotic patterns are reported by the laboratory.

The anti-extractable nuclear antigen screen (ENASc) and anti-extractable nuclear antigen specific (ENASp) tests are qualitative indirect enzyme immunoassay (EIA) test system. In ENASc test system, blends of stabilised affinity-purified ENAs (Sm, RNP, SSA/Ro, SSB/La, Scl-70, and Jo-1) were coated onto the surface of the microwell and served as an antigenic substrate. Incubation of patient samples will allow specific antibodies (if any) in the sample to react with the antigens on the solid phase. Excess unbound antibodies and other serum proteins were then washed before the wells were incubated with goat anti-human antibody which are specific to human IgG light and heavy chain and labelled with horseradish peroxidase. A stable three-part complex is formed which consists of horseradish peroxidase-conjugated anti-human antibody bound to human ANA (bound to the antigen stabilized on the plastic surface) if the result is positive. After another washing step, this complex is detected by adding a solution of tetramethylbenzidine (TMB) and $H_2O_2$ as a chromogenic substrate. The degree of colour development is proportional to the concentration of anti-ENA in the serum sample. Each microwell is read using a spectrophotometer at 450 nm.

In the ENASp test system, each individual stabilized preparations of affinity-purified ENAs of Sm, RNP, SSA/Ro, SSB/La, Scl-70, and Jo-1 were coated onto the surface of the individual microwell. Similar processes of incubation, washing steps as well as the detection process of the formed complex as the ENASc test system described previously were involved in this ENASp test system. The kit system used for ENASc test was Immunoconcepts (Immuno Concepts, NA Ltd, Sacramento, CA 95827), whereas for ENASp test was Euroline Anti ANA profilePlus 1 (IgG) (EUROIMMUN Medizinische Labordiagnostika AG Seekamp 31, 23560 Lübeck).

*Statistical analysis*

Statistical analysis was done using SPSS 25.0. The descriptive data is expressed in frequencies and percentages. Chi-square test is performed to test for the association between two categorical variables. Results with *p*-value less than 0.05 is considered statistically significant.

## RESULTS

Among the 1,529 samples requested for ANA and ENA simultaneously from different departments, 536 (35%) patients were positive, and 993 (65%) patients were negative for ANA by IIF (Figure 1). In the ANA positive group, most of the ENA screen results were negative (381, 71%) while the rest of them were either positive 109 (20%) or borderline 46 (9%) for ENASc (Figure 1). Of these ENASc positive patients, only 47 patients were requested for ENASp and 41 (87%) patients were positive for ENASp. Of the 46 patients, ENASc borderline patients only 8 were requested for ENASp and 3 of them (38%) were positive for ENASp. A total of 62 (57%) of the ENASc positive and 38 (83%) of ENASc borderline, were not requested for further evaluation with ENASp in the ANA positive group.

In the ANA negative group, 816 (82%) were negative for ENASc, while the rest of them were either positive (111, 11%) or borderline positive (66, 7%) for ENASc (Figure 1). Further evaluation with ENASp was requested in only 28 out of 177 ENASc either positive or borderline patients. Of these, 19 (76%) patients were positive for ENASp and 3 patients turned out to be borderline positive (Figure 1). ENASp was not requested in 86 (77%) patients of the ENASc positive and 63 (95%) of the ENASc borderline patients among the ANA negative group. The number of ENASp antibodies identified in the ANA positive and ANA negative group are tabulated in Table 1.

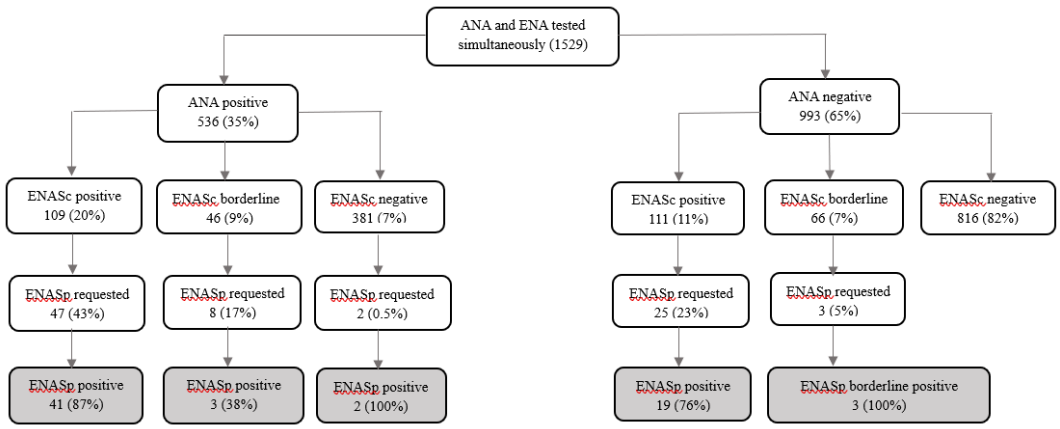


Figure 1: Flow chart summary of the anti-nuclear antigen (ANA), anti-extractable nuclear antigen screening (ENASc) and anti-extractable nuclear antigen specific (ENASp) results. Total number of patients grouped into two: ANA positive and negative. Subsequent percentage calculations have been derived from each group separately out of the 536 ANA positive and 993 ANA negative patients.

A total of 147 patients were diagnosed with SARD with positive ANA and positive ENASc including borderline positive ENASc, while 14 patients were diagnosed with an SARD in those with negative ANA and positive ENASc, including borderline positive ENASc (Table 2).

The available ENASp results in patients diagnosed with SARD varies depending upon the diagnosis. The most common ENASp antibodies in our Sjogren patients are SSA, SSB and Ro52, either alone or in combination. These antibodies are specific for Sjogren's. Two patients with Sjogren had antibodies of RNP and anti-Smith each. In SLE patients, antibodies for SSA, SSB and anti-Smith, either alone or in combination were seen frequently, which are specific for lupus. 5 lupus patients had anti-RNP, while 3 had anti-Jo-1 and one had borderline anti-ScL70 antibody which could possibly be a random finding. In inflammatory myositis conditions such as polymyositis and dermatomyositis, anti-Jo-1 was observed which is specific to the condition. Only one patient had SSA antibody. SSA antibody was also seen in a systemic sclerosis patient. In MCTD patients, anti-RNP and anti-Ro52 antibodies were frequently observed. Antibodies Jo-1, ScL70 and Ro52 were observed in 2 UCTD patients.

Table 3 reveals that a higher percentage of those with positive ANA results (29%) will have positive ENASc as opposed to that observed for those with negative ANA (18%). Thus, the ANA and ENASc results are not independent (test: p-value < 0.001). Since the results are dependent, it follows that knowing the results for ANA will give a good indication of the expected results for ENASc.

**Table 1: Frequency and percentage of anti-extractable nuclear antigen specific (ENASp) in anti-nuclear antibody (ANA) positive and negative patients**

| ENA | ANA positive | ANA negative |
|---|---|---|
| SSA/Ro & SSB/La | 12 (28%) | 2 (11%) |
| SSA | 7 (16%) | 4 (21%) |
| Sm & SSA/Ro | 5 (11%) | 2 (11%) |
| SSB/La | 5 (11%) | 1 (5%) |
| RNP | 4 (9%) | 0 |
| RNP & SSA/Ro | 1 (2%) | 0 |
| Jo-1 | 1 (2%) | 4 (21%) |
| ScL-70 | 1 (2%) | 1 (5%) |
| SSA/Ro & Jo-1 | 0 | 2 (11%) |

ANA: anti-nuclear antibody; ENASp: anti-extractable nuclear antigen specific; Sm: Smith; RNP (ribonucleoprotein)

**Table 2: Frequency and percentage of systemic autoimmune rheumatic diseases (SARD) diagnosis in anti-nuclear antibody (ANA) positive and negative patients**

| SARD | ANA positive | | | ANA negative | | |
|---|---|---|---|---|---|---|
| | ANA positive (n) | ENASc positive (n) | ENASc negative (n) | ANA negative (n) | ENASc positive (n) | ENASc negative (n) |
| Rheumatoid arthritis | 24 | 10 | 20 | 21 | 3 | 17 |
| Systemic lupus erythematosus | 59 | 39 | 19 | 8 | 0 | 6 |
| Sjogren's syndrome | 10 | 13 | 6 | 5 | 3 | 0 |
| Systemic sclerosis | 10 | 6 | 4 | 1 | 0 | 1 |
| Mixed CTD | 9 | 3 | 0 | 2 | 0 | 0 |
| Undifferentiated CTD | 5 | 4 | 3 | 3 | 2 | 0 |
| Inflammatory myositis | 5 | 3 | 2 | 6 | 2 | 5 |
| Ankylosing spondylosis | 3 | 0 | 2 | 1 | 0 | 2 |
| Psoriatic arthritis/psoriasis | 4 | 0 | 2 | 13 | 3 | 7 |
| Systemic vasculitis | 1 | 1 | 1 | 6 | 1 | 5 |
| Primary APLS | 8 | 3 | 5 | 4 | 0 | 4 |
| Others | 9 | 2 | 7 | 6 | 0 | 6 |
| Total | 147 | 84 | 71 | 76 | 14 | 53 |

SARD: systemic autoimmune rheumatic diseases; ANA: anti-nuclear antibody; ENASc: extractable nuclear antigen screen; SLE: systemic lupus erythematosus; CTD: connective tissue diseases; # ENASc positive includes borderline positive; * Overlap: Rheumatoid arthritis/Sjogren's (n=4), SLE/Sjogren's (n=4), SLE/Rheumatoid arthritis (n=2), Mixed CTD/ systemic sclerosis (n=1); ** Others: Behcet, palindromic rheumatism, adult-onset Still's disease, polymyalgia rheumatica, Kikuchi, lupus profundus, reactive arthritis, juvenile idiopathic arthritis, scleromyxedema, SAPHO syndrome, inflammatory bowel disease

## DISCUSSION

International guidelines on testing ANA subserology recommends having clinical suspicion of immune-mediated disease in addition to a positive ANA serology.[4] If ANA is negative no further ANA testing is recommended unless there is a significant change in clinical features.[1] There is no evidence to support serial testing for ANA and ENA to follow immunologic disease activity or response to treatment.[8,11] There may be exceptions where ENA could be useful in the presence of a negative ANA. For example, anti-Jo-1 may be positive in a subset of inflammatory myopathy (anti-synthetase syndrome). A positive ENA test in a negative ANA sample should be interpreted with caution especially in the absence of supporting clinical features.[12] Evaluation of a positive ANA or ENA also depends on sensitivity, specificity, positive predictive value, and negative predictive value of the test used. Specific ANAs can be detected by several techniques including immunodiffusion, counter-immunoelectrophoresis, ELISA, line-immunoblot, immunodot, or Western blot.[13-15] Understanding by clinicians on its applications, limitations and relevant guidelines can avoid unnecessary investigations.

**Table 3: Crosstabulation of the results of anti-nuclear antibody (ANA) versus anti-extractable nuclear antigen specific (ENASp)**

| | | ANA Positive | Negative |
|---|---|---|---|
| ENASc | Positive | 155 (29%) | 177 (18%) |
| | Negative | 381 (71%) | 816 (82%) |
| | Total | 536 | 993 |

Lack of adherence by clinicians to these guidelines is evident in this study where 1529 patients were requested simultaneously to check for ANA and its subserology in a single centre over a period of 4 years. A similar study in Riyadh observed that 141 (47%) out of the 300 ANA tests included ANA subserology

simultaneously and most of them were requested by non-rheumatology departments (253, 84.7%).[16] Another study by Davis L.A, *et al.* (2015) involving 3,221 samples reported 211(6.6%) of the ANA tests were ordered simultaneously with ANA subserologies, with the most common concurrent subserologies were dsDNA (21.8%), SSA (20.8%) and SSB (19.7%).[17] Predictors of concurrent ordering of ANA and subserologies include that the labs were ordered from subspecialty care or from emergency/urgent care or from inpatient care. This indicates that providers at the institution typically do not overuse ANA subserology testing but wait for the ANA to return before initiating subserology testing. This ordering pattern indicated by the author is due to their hospital setting, a safety net hospital with a systematic effort toward efficient use of resources when cost is a daily concern. Overuse of these tests can lead to unnecessary referrals, patient anxiety and financial burden on health care systems. ANA is a test with low positive predictive value in the general population and is commonly performed by the general practitioners. However, ENA is a test with high positive predictive value usually requested by specialists. A Canadian study by Ahrari *et al.* evaluated a cohort of 638 rheumatology referrals and found that 20% of them had ANA testing done with no clinical indication. The majority (73.8%) of the ANA was ordered together with ANA subserology.[18] Measures to minimise unnecessary testing is already in place in most of the laboratories. Educating health care professionals on testing ANA and ENA can have a significant impact on the frequency of test requests.[19]

It is highly likely that a negative ANA indicate a negative ENA with rare exceptions.[12] As high as 82% of the ANA negative patients in this study tested negative for ENASc. In this study, almost one third (35%) of the patients were ANA positive and two third 65% were ANA negative. Much lower rates (23%) of ANA positivity have been reported in other studies involving large number of patients.[20] Detection of ENASp is expensive and time consuming and requires expertise. ENASp further refines ANA subserology tests. In our survey, the rate of ENASp being positive is 41 (87%) out of 47 patients requested from the ENASc positive group in the ANA positive group; whereas in the ANA negative group, 19 out of 25 patients requested (76%) were tested to have ENASp positive from the ENASc positive group. A previous study in a small cohort of Malay ethnicity has shown ENA positivity of 9.6% among SLE patients with positive ANA.[21] Similar ENASp rate of 6.6% was reported when ANA and ENA subserology were performed concurrently.[17] These findings further validate the ACR recommendations on ENA testing.[8]

As mentioned, in the ANA positive group, a total of 44 patients were positive for ENASp in this group, with the commonest ENASp identified was a combination of SSA/Ro and SSB/La (12, 28%) followed by SSA/Ro (7,16%) (Table 1). A much higher rate of positive ENASp (21.1%) was reported in a large cohort study conducted by Peene *et al*., involving 10,550 patients with positive ANA.[20] Failure to request for ENASp in some of the ENASc positive patients could be a reason for the lower rates of ENASp in our study. An alternative method of testing also could have contributed to this difference.[22] Second generation ANA and ENA analysis with Digital fluorescence Cytobead could be an alternative for some of the problems associated with the conventional two-tier testing by IIF and ELISA.[23]

Several combinations of ENASp were identified in both ANA positive and negative groups in contrast to other studies in which no combination ENASp was detected.[22] It is unclear if this translates into a meaningful clinical disease outcome. However, Sánchez-Guerrero *et al*. have shown that anti-ENA, especially SSA/Ro to be a useful predictor for the diagnosis of SLE in those who are positive for ANA and negative for anti-dsDNA.[24] Importantly, we observed that ANA and ENASc results are not independent (Table 3). Since the results are dependent, it follows that knowing the results for ANA will give a good indication of the expected results for ENASc. In other words, for example, a negative result for ANA points to a high chance that the ENASc will be negative. Requesting for ENASc after knowing ANA as internationally recommended will help in reducing wastage due to unnecessary request.

The limitations to be considered in this study which includes, not looking into relationship of the ENASp with that of the clinical phenotypes, as the results are heterogenous, however the majority of the SARD diagnosis fits with the ENASp results. The frequency of ENASp would have been different, had we performed ENASp in all those patients who are ENASc positive or borderline in both groups.

## CONCLUSION

A large number of ENASc negative results is obtained concurrently with ANA negative results indicating the probability of detecting specific ENA antibodies is low in samples with negative ANA. When ENASc tests are requested, it is important to follow up with ENASp tests. Strengthening the communication between the laboratory and clinical staff is necessary to improve the follow-up with autoimmune test results. Educating clinicians on testing ANA and ENA and compliance to international guidelines when requesting ENA is crucial to avoid unnecessary testing. This will help to reduce the financial burden on patients and minimise workload on laboratory staff. This survey strongly suggests implementing measures in the hospitals to comply with ACR recommendations on ENA testing.

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