Frequencies and results of anti-nuclear, anti-dsDNA and anti-ENA in a tertiary-care hospital in Karachi, Pakistan
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Abstract
Objectives: To analyse frequencies and results of anti-nuclear antibodies, anti-double stranded deoxyribonucleic acid and anti-extractable nuclear antigens tests ordered in a tertiary-care hospital.
Method: The retrospective study was conducted at a tertiary care hospital in Karachi, and comprised all tests ordered for anti-nuclear antibodies, anti-double stranded deoxyribonucleic acid and anti-extractable nuclear antigens from March 2017 to January 2018. Data was retrieved from the institutional electronic database. The frequencies and results of the tests were determined. Anti-nuclear antibodies test was determined by indirect immunofluorescence, while the other two tests were determined by enzyme-linked immunosorbent assay. Patterns emerging from anti-nuclear antibodies tests were also analysed.
Results: Of the 1053 cases studied, 1000(95%) were tested for for anti-nuclear antibodies. The test was positive in 260(26%) patients, and was repeated in 8(3%) of the positive and 9(1.2%) of the negative patients. Anti-double stranded deoxyribonucleic acid test was ordered in 300(40.5%) and anti-extractable nuclear antigens test in 125(17%) patients who had tested negative for anti-nuclear antibodies. Among those who tested positive for anti-nuclear antibodies, the commonly observed patterns were homogenous 109(41.9%) and speckled 103(39.6%). Rod and ring pattern was seen in 10(3.8%) patients, and none of them were on anti-viral treatment.
Conclusion: There was injudicious and unjustified ordering of auto-antibodies testing, indicating the need for greater physician education and cost-effective protocols.
Keywords: Auto-antibodies, Homogenous, Speckled, Rod and ring pattern, Hepatitis C, Cost-effective.

Introduction
Antinuclear antibodies (ANA) are antibodies targeting the body’s own cells. ANA positivity indicates the presence of autoimmune diseases. These antibodies are also found in other inflammatory conditions, chronic infectious diseases, malignancies, drug-induced conditions and even in some healthy individuals. Therefore, correct interpretation of ANA results in association with clinical features is important for further laboratory evaluation of a patient’s condition.

Presence of ANA has been determined by immunoassays, mainly enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescent assay (IFA). IFA is considered the gold standard to date for the diagnosis of ANA. Apart from the greater analytical superiority, the determination of the pattern gives an indication to the type of antigen against which the ANA is directed. There are a number of patterns, each of which is associated with discrete or overlapping group of diseases. The presence of particular patterns, in association with clinical findings and further analysis of contributing antigens by anti-double stranded deoxyribonucleic acid (anti-dsDNA) and anti-extractable nuclear antigens (anti-ENA) helps in reaching the diagnosis. The most common pattern observed is homogenous in which the entire nucleus is stained. Antibodies producing this pattern include those directed against histones, DNA and DNA-histone complexes. Another common pattern observed is speckled in which the nucleus is stained in a sparse dotted pattern. The antibodies producing the speckled pattern include those against nuclear antigens, such as uridine ribonucleoprotein (U1-RNP), Smith (Sm), Sjogren’s antigen types A and B (SSA/Ro and SSB/La), and Smith and topoisomerase I (Scl-70). Nucleolar pattern, in which the nucleoli stain either homogenously or in speckles, is produced by auto-antibodies directed against ribonucleic acid (RNA) polymerase, fibrillarin, polymyositis-scleroderma (PM-Scl) and RNA helicase.

Instead of using a stepwise testing algorithm, physicians at times order ANA along with anti-dsDNA and anti-ENA testing for the diagnosis of autoimmune diseases. This practice imposes undue costs in terms of material and human resources, and should be curbed. This is especially important in the context of hospitals located in countries with financial constraints, like Pakistan.

Laboratory audits are often undertaken to identify, monitor and improve quality of processes. The current study was planned to analyse frequencies and results of
ANA, anti-dsDNA and anti-ENA tests ordered in a tertiary-care hospital in order to identify if there was any over utilisation of these testing resources.

Materials and Methods
The retrospective study was conducted at a tertiary care hospital in Karachi, and comprised all tests ordered for ANA, anti-dsDNA and anti-ENA from March 2017 to January 2018. After exemption from the institutional ethics review board, data was obtained from the hospital information system related to all patients tested for ANA, anti-dsDNA and anti-ENA during the study’s specific timeframe. All previous or subsequent tests done on these patients were excluded. As per the laboratory policy, all the ordered tests had been entertained. Total number of patients was initially categorized into two groups depending on whether or not ANA was performed on them during the study period. The ANA group was then further divided into ANA-positive and ANA-negative groups. Each group was subsequently subdivided into those on whom the further requests of repeat ANA, anti-dsDNA or anti-ENA were carried out. Positivity rates of these tests were also determined. Frequency of various ANA patterns was determined.

ANA was determined by IFA technique on slides (Euroimmun, Germany). IFA involved incubating dilutions of patient’s sera with a monolayer of fixed cells belonging to human epithelial type 2 (Hep2) cell line on a slide. Antibodies adhered to the cell monolayer, and then reacted with an anti-human immunoglobulin reagent conjugated to a fluorescent tag. The distinct patterns could be visualised under a fluorescent microscope. Anti-dsDNA and anti-ENA were detected on ELISA (Euro Diagnostica and Biosystems respectively).

Results
Of the 1053 cases studied, 1000(95%) were tested for ANA. It was positive in 260(26%) patients, and was repeated in 8(3%) of the positive and 9(1.2%) of the negative patients. Anti-dsDNA was ordered in 300(40.5%) and anti-ENA in 125(17%) ANA-negative patients and none of them tested positive (Figure-1).

The most commonly observed ANA patterns were homogenous 109(41.9%), speckled 103 (39.6%) and rod and ring pattern 10(3.8%) (Figure-2). All patients with rod and ring pattern had presented with varied conditions and none had been on any anti-viral treatment.

Amongst the ANA-positive group, there were 60(23%) cases who were also anti-ENA-positive. On ANA staining,
30(50%) of them demonstrated speckled pattern and 27(45%) had homogenous pattern (Table). Anti-Ro antibodies were the most frequent anti-ENA antibodies detected in 24 (40%) of these patients.

Discussion
The American Society of Rheumatology recommends avoiding testing for sub-serologies unless positive ANA is detected. The current study observed that on testing only 26% cases were found ANA-positive. Among ANA-negative cases, single or multiple testing of anti-dsDNA was done on 41% and anti-ENA on 17%, and all of them tested negative. This is an eye-opener and caution is mandated in ordering these tests. Unnecessary testing of sub-serologies when primary ANA is negative, or in the absence of relevant clinical indications, results in an increased cost of material and manpower. The testing requires expertise and at least three hours of a dedicated technologist’s time per batch. It is estimated that at least Pak Rupee (PKR) 150,000 to 200,000 would have been saved over the 11 months of the study by judicious and selective ordering. A possible reason for this ordering tendency could be an effort by the clinician to save time as these are -intensive tests and are usually performed batch-wise. Waiting for the results of the primary test before ordering the second antibody could result in wasting of precious time. A possible way to get around this problem is to implement reflex testing protocols, and to make available the required test if there is clinical and laboratory indication. The results of the current study call for intensive physician education and laboratory’s role in restricting test ordering, promoting cancellation of unjustified tests, reflex or smart testing and greater coordination with clinical teams, as recommended in literature. We have recently started to implement some of these measures at the centre as a step toward bridging the gap between laboratory and clinic for better

Table: Nuclear patterns associated with Positive Anti-ENA (n=60).

<table>
<thead>
<tr>
<th>ANA pattern</th>
<th>Frequency (%)</th>
</tr>
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<tbody>
<tr>
<td>Speckled</td>
<td>30 (50)</td>
</tr>
<tr>
<td>Homogenous</td>
<td>27 (45)</td>
</tr>
<tr>
<td>Mixed Homogenous and Speckled</td>
<td>1 (1.6)</td>
</tr>
<tr>
<td>Mixed Homogenous and rod and ring</td>
<td>1 (1.6)</td>
</tr>
<tr>
<td>Other (MSA)</td>
<td>1 (1.6)</td>
</tr>
</tbody>
</table>

ANA: Anti-nuclear antibodies; Anti-ENA: Anti-extractable nuclear antigens, MSA: Myositis specific autoantibodies.
utilisation and cost-effectiveness of the tests. The impact is yet to be determined. An algorithm was developed in a Canadian health region proposing cancellation of anti-ENA and anti-dsDNA if ANA was negative, and cancellation of ANA request if it was repeated within a year. This simulated a cost saving of 30% and can potentially prove to be very useful strategy in setting.

Our results showed that the most frequently associated ANA pattern with detectable anti-ENA was speckled, as is shown in other international and local studies. Also, amongst the anti-ENA antibodies, anti-Ro was the commonest, detected in 40% of positive anti-ENA patients. This finding also concurs with literature. This is not surprising as anti-Ro are the most frequently found antibodies in various autoimmune conditions although they are typically considered hallmark antibodies for diagnosis of systemic lupus erythematosus (SLE) and Sjogren’s syndrome (SS).

There was a sub-group of patients in the current study who were not tested for ANA, but were tested for anti-dsDNA and anti-ENA one or more times. It is possible that these patients were either investigated for ANA before the onset of the study period or were tested elsewhere before coming to our institute.

The most common patterns observed in our study were homogenous and speckled, which is consistent with literature. Different ANA patterns are found in a variety of autoimmune diseases, connective tissue disorders, malignancies, non-specific conditions and even in some healthy individuals. This mandates careful interpretation of ANA tests by clinicians. Based on the pattern, further delineation of the antigen targeted can be assessed. Commonly tested ANAs are anti-dsDNA, anti-SSA/Ro, anti-SSB/La, anti-U1 RNP, anti-Sm and scl 70, collectively lumped as anti-ENA. A consensus has been developed as to the terminology to be used for the different patterns seen during IFA for ANA detection. Our reporting terminologies were in accordance with it.

Rod and ring pattern was seen in 3.8% of the positive cases. A look at the electronic medical records of these patients showed that none of them were on anti-viral treatment for hepatitis C and had varied unrelated presentations. Rod and ring is a recently discovered pattern with the earliest case reported only a decade ago. Rod and ring demonstrates a unique florescent pattern within the cytoplasm. It consist of 3-10um rods, 2-5um diameter rings in cytoplasm. They are protein assemblies composed of cytidine triphosphate synthetase type 1 (CTP51) and inosine monophosphate dehydrogenase type 2 (IMPDH2), key enzymes in cytosine triphosphate (CTP) and guanosine triphosphate (GTP biosynthesis). They are a metabolic response to diminished intracellular GTP and/or CTP pools, and form when exposed to IMPDH inhibitors or when there is a deficiency of nutrients required for guanosine monophosphate (GMP) synthesis. These antibodies are overwhelmingly seen in patients on antiviral treatment for Hepatitis C, and have been proposed as drug-induced antibodies.

A longitudinal study on patients with Hepatitis C has shown that rod and ring antibodies develop with the initiation of treatment with antiviral agents interferon alpha and ribavirin, and remit after discontinuation. However, there are rare reports of subjects in whom rods and ring patterns have been identified in the absence of interferon treatment. In the large National Health and Nutrition Examination Survey (NHNES) conducted in the United States, it was documented for the first time that rod and ring pattern can develop in patients with no hepatitis C virus (HCV) infection. Furthermore, another finding of this survey was that this pattern occurred in individuals using poly-pharmacy, most common drugs being anti-hypertensives, albuterol, synthetic hormones and anti-clotting medications. Poly pharmacy is a very common practice in our country with medicines generally being sold over the counter. It remains to be seen if the frequency of rod and ring patterns seen locally without concomitant anti-viral treatment is related to any drug commonly used by our patients. A multi-centre study is proposed for a detailed analysis on this phenomenon.

The current study has limitations due to its retrospective nature and short study duration. We did not do clinical correlation. It will be interesting to know the disease profile of patients testing positive for these antibodies. The number of patients with positive rod and ring pattern were few but important. It would be worth undertaking a follow-up study on patients exhibiting rod and ring pattern without HCV infection and to look into their drug history to find potential linkages.

Conclusion
There was injudicious and unjustified ordering of auto-antibodies testing, indicating the need for greater physician education and cost-effective protocols. Homogenous and speckled patterns were the most common antinuclear antibody patterns seen. The aetiology of positive rod and ring pattern without co-existent hepatitis C anti-viral treatment should be studied further.

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References