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Comparison of Different Laboratory Methods in the Detection of Anti-dsDNA Antibodies and Their Diagnostic Utility

Anti-dsDNA Antikorlarının Varlığının ve Tanısal Değerinin Belirlenmesinde Farklı Laboratuvar Tanı Metotlarının Karşılaştırılması

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Abstract

Introduction: We aimed to analyze the positivity of Crithidia luciliae immunofluorescence tests (CLIFT), to compare CLIFT with ANA-IFA (antinuclear antibody–immunofluorescence assay), ANA-IB (immunoblot), and ELISA (enzyme-linked immunoassays), and to determine the relevant method to test anti-dsDNA in systemic lupus erythematosus (SLE).

Materials and Methods: We conducted a retrospective, cross-sectional study between January 1st, 2015 and January 1st, 2016. We focused on the positive CLIFT results firstly, then, we compared the ANA-IFA, ELISA, and ANA-IB results to diagnose SLE. Demographic features were obtained from the hospital records.

Results: To analyse CLIFT, 3242 seras were tested, and 72 (2.2%) were positive. Among CLIFT positivity [n=64; 57 female, 7 male (mean, range; 41.96, 11–82)]; 73% (n=47) had SLE. Out of 61 patients were analyzed by ANA-IFA, 36 had peripheral (n=1) and homogenous (n=35) patterns; 83% (n=30) had SLE. Out of 46 patients were analyzed by ANA-IB, 30 had dsDNA; 73% (n=22) had SLE. Out of 25 patients who were analyzed by ELISA, 18 had dsDNA; 83% (n=15) had SLE. In the two-sided correlations, CLIFT positivity (≥grade 2) was found to be statistically significantly associated with having SLE (p=0.005, r [64]=0.92); CLIFT positivity was also statistically significantly associated with ANA-IFA (p=0.003, r=0.85). In order to exclude SLE diagnosis, CLIFT positivity was statistically significantly correlated with ANA-IB (p=0.002, r=0.90).

Conclusion: CLIFT can not be used instead of ELISA and ANA-IB, but it can reduce their usage. We recommend to use CLIFT and ANA-IFA for first-line screening; and ANA-IB and ELISA for confirmation and identification of dsDNA.

Keywords: Anti-dsDNA, laboratory methods, systemic lupus erythematosus

Öz

Giriş: CLIFT (Crithidia luciliae immünofloresan test) pozitifliğinin sıklığını belirlemeyi, CLIFT'i ANA-IFA (anti-nükleer antikor-immünfloresan analizi), ANA-IB (immünblot) ve ELISA (enzim-bağlantılı immün analiz) ile kıyaslamayı ve SLE'de (sistemik lupus eritematozus) anti-dsDNA'yi saptamak için uygun yöntemi belirlemeyi amaçladık.

Gereç ve Yöntemler: 1 Ocak 2015 ile 1 Ocak 2016 arasında geriye dönük kesitsel bir çalışma gerçekleştirilmiştir. Öncelikle, CLIFT pozitif sonuçlara odaklandık, sonrasında CLIFT pozitif hastaların ANA-IFA, ELISA ve ANA-IB sonuçları incelendi ve laboratuvar yöntemleri SLE tanısı ile karşılaştırıldı. Hastaların demografik özellikleri hastane kayıtlarından elde edilmiştir.

Bulgular: CLIFT analizi için, toplam 3242 serum çalışıldı ve bunların 72'si (%2,2) pozitifti. CLIFT pozitiflikleri (n=64; 57 kadın / 7 erkek) arasında ortalama yaş 41,96 (aralık, 11–82) ve %73'ü (n=47) SLE tanılıydı. ANA-IFA 61 olguda çalışıldı; periferik (n=1) ve homojen (n=35) örüntü 36'sında mevcuttu ve bu olguların %83'ü (n=30) SLE tanılıydı. Kırkaltı hastada ANA-IB çalışıldı; ve 30'unda dsDNA saptandı. Bunların %73'ü (n=22) SLE tanılı idi. Hastaların 25'inde ELISA metodu kullanıldı ve bunların 18'inde pozitiflik saptandı. %83'ü (n=15) SLE tanılıydı. İki yönlü korelasyonlarda, CLIFT pozitifliği (≥2. derece), SLE tanısı ile güçlü korelasyon gösterdi (p=0.005, r [64]=0,92); SLE tanısını doğrulamada; CLIFT testi ANA-IFA ile ileri derecede ilişkili bulundu (p=0.003, r=0,85). Ayrıca SLE tanısını dışlamada; CLIFT ile ANA-IB güçlü korelasyon gösterdi (p=0.002, r=0,90).

Sonuç: CLIFT, ELISA ve immünoblot tetkiklerinin yerini alamaz, fakat onların kullanımını azaltabilir. Sonuçta, CLIFT ve ANA-IFA'nın ilk basamak görüntülemede; ANA-IB ve ELISA'nın ise doğrulama ve tanımlamada kullanımını önermekteyiz.

Anahtar Sözcükler: Anti-dsDNA, laboratuvar metotları, sistemik lupus eritematozus

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Introduction

Systemic Lupus Erythematosus (SLE) is a chronic, multisystem autoimmune disorder. Anti-dsDNA, which was discovered in 1957, is one of the primary autoantibodies present in patients with SLE.[1–3] They are excellent indicators of SLE disease activity[4,5] and their elevated levels usually precede exacerbation of disease.[6] Several immunological methods are used to determine dsDNA antibodies. Anti-dsDNA antibodies are generally detected and quantified by commercially available kits for ELISA (enzyme-linked immunoassays) and radio-immunoassay (RIA) methods developed according to the Farr technique (FARR-RIA).

Crithidia luciliae immunofluorescence assay (CLIFT) uses *Crithidia luciliae* which are hemoflagellates nonpathogenic for man and easy to culture, and they have a giant mitochondrion in which the mitochondria1 DNA is concentrated in a single large network, the kinetoplast.[7]

Different combinations of these methods are used in diagnostic laboratories worldwide without a consensus on exclusive methods.[8,9] ELISA detects antibodies of both low and high avidity, whereas CLIFT and FARR-RIA assays predominantly detect antibodies of high avidity.[10] Therefore, the practical approach has been done to find an assay that detects both high and low avidity anti-dsDNA antibodies as a primary screen.[11] The assay of choice could be either CLIFT[12] or ELISA.[13] The problem with ELISA is that they often give false-positive results due to the binding of immune complexes (with negatively charged moieties) to the pre-coat intermediates.[13] They are observed not only in patients with SLE but also in other connective tissue diseases, such as primary systemic sclerosis (PSS) and myositis.[10] Even though the presence of the dsDNA antibody could be suspected by ANA-IFA (antinuclear antibody-immunofluorescence), CLIFT has been considered as first-line screening because of its high sensitivity and low cost. ELISA and ANA immunoblot (ANA-IB) tests are also used for verification and quantitation.

In our study, we aimed to analyze the value of using CLIF, to compare CLIFT with ANA-IFA, ANA-IB, and ELISA, and to determine the better method to test anti-dsDNA in SLE.

Materials and Methods

We conducted a retrospective, cross-sectional study between Jan 1st, 2015, and Jan 1st, 2016. During this period, 3242 sera were analyzed with CLIFT for the diagnosis the suspected autoimmune disease (AID) at the Laboratory of Allergy and Immunology, Department of Internal Medicine, Division of Allergy and Clinical Immunology, Izmir.

We focused on the positive CLIFT (IMMCO Diagnostics) results at first, then we evaluated the ANA-IFA (IMMCO Diagnostics), ELISA (EUROIMMUNE), and ANA-IB (EUROIMMUNE) results which were already analyzed to diagnose SLE among the CLIFT positive patients, and compared the four laboratory test results to diagnose SLE. Demographic features (age, sex, and diagnosis) were obtained from the hospital records. All ANA-IFA studies were performed by the 25-year-old experienced immunology laboratory technician.

The study was approved by the hospital's ethics committee. Written informed consent was obtained. The reference range of assays was determined by the manufacturers.

Normal Range and Cut-off Values for Anti-dsDNA Assays

ANA detection

ANA was detected by IFA with a HEp-2 ANA Complete Kit (IMMCO Diagnostics®, Buffalo, New York, USA). For all samples, the starting serum dilution was 1:160. Samples that showed fluorescence underwent serial double dilution and re-testing until fluorescence disappeared. The results were recorded as the highest dilution of serum that produced positive findings. Samples with a positive fluorescence titer higher than 1:5120 were recorded as 1:5120 (Table 1).

CLIFT detection

CLIFT was performed by IFA with *Crithidia luciliae* Double-Stranded DNA Antibody (dsDNA) IgG Kit (IMMCO Diagnostics®, Buffalo, New York, USA). For all samples, the cut-off value was <1:10 titer. Positive samples at a 1:10 screening dilution are tittered to an endpoint at an additional charge (Table 1).

ANA IMMUNOBLOT (EUROLINE: ANA Profile 3)

ANA immunoblot test strips can be automatically incubated and evaluated using the systems EUROBlotOne,

Table 1. Characteristics of the four anti-double-stranded DNA (dsDNA) assays

Assay	Manufacturer	Method	Isotype detection	dsDNA origin	Threshold value between negative and positive
ANA	IMMCO Diagnostics	Microplate/ manual	IgG/human epithelial cell	Human epithelial cell type-2	1:160 dilution
CLIFT	IMMCO Diagnostics	Microplate/Manual	IgG/*Crithidia luciliae*	Kinetoplast DNA	Presence of fluorescence at kinetoplast for a serum dilution at 1/10
ANA Immunoblot	EUROIMMUNE	Manual/ Semi-quantitative	IgG	Human serum/ plasma	1:101 dilution
ELISA	EUROIMMUNE	Manual/quantitative	IgG	Human serum/ plasma	100 IU/mL (1:201 dilution)

ANA, antinuclear antibody; ELISA, enzyme-linked immunoassays; CLIFT, *Crithidia luciliae* immunofluorescence test; IgG, immunoglobulin G; IU, international units.

EUROBlotMaster, and EUROLineScan. Differentiation of antibodies against cell nuclei (ANA) was performed (EUROIMMUNE, Perkin Elmer Germany Diagnostics GmbH, Lübeck, Germany) to detect anti-dsDNA antibodies. For all samples, conjugate class anti-human IgG with AP-labelled was used and the cut-off value was 1:101 dilution. With the EUROLINE ANA Profile 3, fifteen autoantibodies can be determined: antibodies against nRNP/Sm, Sm, SS-A, Ro-52, SS-B, Scl-70, PM-Scl, Jo-1, centromere protein B, PCNA, dsDNA, nucleosomes, histones, ribosomal P-proteins, AMA M2 (Table 1).

ELISA

The ELISA method (EUROIMMUNE®, PerkinElmer Germany Diagnostics GmbH, Lübeck, Germany) is a quantitative monospecific detection of antibodies against dsDNA. Due to good sensitivity and specificity, the anti-dsDNA-NcX ELISA stands out by high diagnostic efficiency. Double-stranded DNA complexed with nucleosomes (NcX) is used as an antigen. High concentrations of autoantibodies against dsDNA in the ELISA are considered to be a reliable marker for the diagnosis or prognosis of SLE. Individual changes in the dsDNA antibody concentration correlate with the activity of the disease and can be used for monitoring the development of the disease in SLE patients. Under immunosuppressive therapy or in clinical remission dsDNA antibodies often cannot be detected with ELISA anymore. Antibodies against dsDNA can be determined quantitatively in IU/ml. For all samples, conjugate class anti-human IgG with POD (peroxidase)-labelled was used and the cut-off value was 1:201 dilution (100 IU/mL) (Table 1).

Statistical Analyses

In descriptive statistics, percentage (%), frequency (number and percentage), mean (range) values were used for categorical variants as appropriate, and the chi-square and t-tests were used for comparisons of categorical variables. The non-parametric tests; Mann-Whitney U and Kruskal-Wallis H were used to compare numerical variables, where the numbers were <30. The two-sided Spearman's correlation coefficient was used to determine the relationship between variables. Statistical analyses were performed using the SPSS software package, version 23 (SPSS Inc., Chicago, IL, USA). Results with $p<0.05$ were evaluated as statistically significant.

Results

Among 3242 seras, 72 (2.2%) samples that belonged to 64 patients were positive for anti-dsDNA antibodies by CLIFT. Fifty-seven were female and 7 were male with a mean age of 41.96 (range, 11–82 years).

All of those 64 patients had AID and 62 of them were diagnosed with autoimmune rheumatic diseases, including SLE (73%, n=47), Sjogren's Syndrome (SS) (n=8), mix connective tissue diseases (MCTD) (n=5), Progressive Systemic Sclerosis (PSS) (n=2), Primary Biliary Cirrhosis (PBC) (n=1), and peripherical polyneuropathy (n=1).

The sera of 61 patients were studied with ANA-IFA and all of them were found positive (100%). The identified staining patterns were homogenous in 35, granular in 22, nucleolar in 2, peripherical in 1, and nuclear dots in 1 patient (Figure 1, Table 2). Peripheral and homogenous patterns were the strongly suggestive staining patterns for the presence of anti-dsDNA antibody which were detected in the seras of 36 patients. Of those, 30 patients were diagnosed (83%) as SLE. The diagnosis of the remaining 6 patients were SS (n=3), both SS and MCTD (n=2), and MCTD (n=1) (Figure 1, Table 2).

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Forty-six of 64 patients with positive CLIFT results were analyzed with anti-ANA-IB. Anti-dsDNA antibody positivity was found in 30 (65%), and 22 (73%) of those who had SLE diagnosis. The diagnosis of the remaining 8 patients were MCTD (n=4), SS (n=2), PBC (n=1), and PSS (n=1) (Figure 1, Table 2).

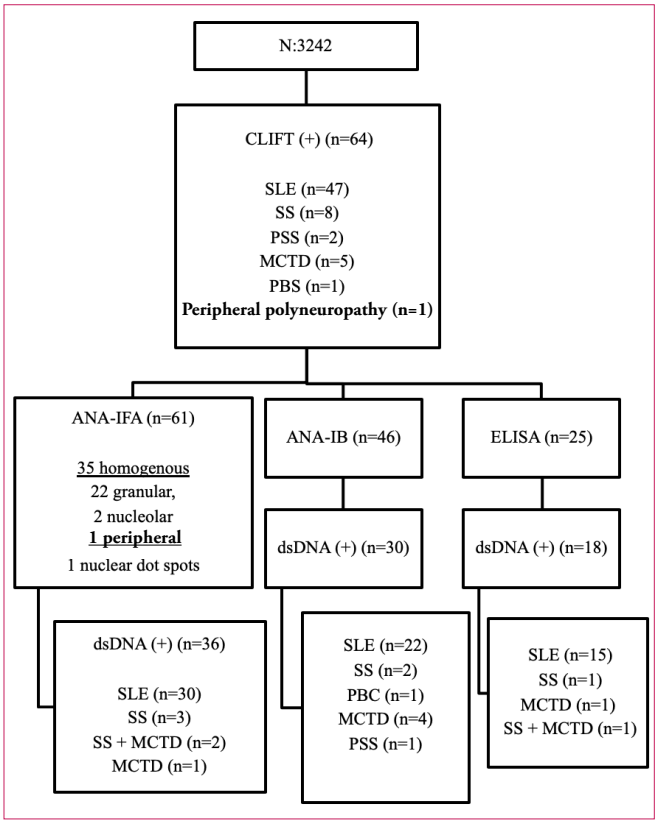

Figure 1. The comparison of the four methods which were used to detect anti-dsDNA antibodies, and the final clinical diagnosis of the patients with positive anti-dsDNA antibodies (CLIFT, *Crithidia luciliae* immunofluorescence tests; ANA-IFA, anti-nuclear antibody–immunofluorescence; ANA, anti-nuclear antibody; ELISA, enzyme-linked immunoassays; dsDNA, double-stranded DNA; Hep2, human epithelial type 2; SLE, systemic lupus erythematosus; PSS, progressive systemic sclerosis; SS, Sjogren's syndrome; PBC, primary biliary cirrhosis; MCTD, mix connective tissue diseases).

Table 2. Results with four different assays

	CLIFT	ANA-IFA	ANA-IB	ELISA
Seras detected for dsDNA (n)	3242	61	46	25
Positive results (n)	64	36	30	18
SLE diagnosis (n)	47	30	22	15
SLE diagnosis (%)	73	83	73	83

ANA-IFA, anti-nuclear antibody–immunofluorescence; ELISA, enzyme-linked immunoassays; CLIFT, *Crithidia luciliae* immunofluorescence test; ANA-IB, anti-nuclear antibody–immunoblot; SLE, systemic lupus erythematosus.

Table 3. The correlation analysis between SLE diagnosis/exclusion and the results of the four laboratory methods

Correlation	r value	p-value
SLE vs CLIFT grade (≥2)	0.92	0.005
CLIFT vs ELISA	0	0.82
CLIFT vs ANA-IFA	0.85	0.003
CLIFT vs ANA-IB	0	0.65
To exclude SLE		
CLIFT vs ELISA	0.42	0.048
CLIFT vs ANA-IFA	0.35	0.05
CLIFT vs ANA-IB	0.90	0.002

ANA-IFA, anti-nuclear antibody–immunofluorescence; ELISA, enzyme-linked immunoassays; CLIFT, *Crithidia luciliae* immunofluorescence test; ANA-IB, anti-nuclear antibody–immunoblot; SLE, systemic lupus erythematosus.

Seras from 25 of the 64 patients were analyzed with ELISA. Anti-dsDNA antibody was positive in 18 (72%), and 15 (83%) with dsDNA positivity had SLE diagnosis. The diagnosis of the remaining 3 patients were SS (n=1), MCTD (n=1), and both SS and MCTD (n=1) (Figure 1, Table 2).

As a result, 73% of the patients with positive CLIFT, 83% of those with peripherical and homogenous ANA-IFA patterns, 73% of those with anti-dsDNA antibody positivity by ANA-IB, and% 83 of those with anti-dsDNA antibody positivity by ELISA had SLE diagnosis.

In our study group, we classified CLIFT results according to their strength of flourescence as CLIFT grade 0 (suspicious) (n=11, 17%), grade 1 (mild) (n=10, 16%), grade 2 (moderate) (n=19; 29%), grade 3 (strong) (n=19; 29%), and grade 4 (very strong) (n=5, 7%).

While 21 patients with grades 0 and 1 (+) CLIFT results did not have SLE diagnosis, the rest 43 patients with CLIFT results greater than grade 2 had SLE diagnosis. As a result, to have positive CLIFT results more than grade 2 is statically significant and had very strong Spearman's Rank correlation coefficient to have SLE diagnosis (p=0.005, r [64]=0.92) (Table 3).

Out of 43 patients with SLE we 38 patients with SLE had positive ANA-IFA, 21 patients had positive ANA-IB, and 10 patients with SLE had positive ELISA test. CLIFT and ANA-IFA were found to have positive correlation. (p=0.003, r=0.85) (Table 3).

When we compare the results of other laboratory methods with CLIFT negativity (n=21) to exclude SLE, we detected

that, 20 patients (95%) had negative ANA-IB results, 17 patients (81%) had negative ELISA, and 15 patients (71%) had negative ANA-IFA. To exclude SLE diagnosis, the results of CLIFT and ANA-IB had strong Spearman's Rank correlation coefficient (p=0.002, r=0.90). ANA-IFA and ELISA were also correlated with CLIFT results to exclude SLE, however, weaker correlation coefficients were detected (Table 3).

Discussion

We determined that CLIFT was the most suitable method for the first line anti-dsDNA antibody detection in combination with ANA-IFA. However, CLIFT as a qualitative assay cannot substitute ELISA but the test can reduce its use, since the results of 3242 consecutive sera showed that only 2.2% of patients were tested positive for anti-dsDNA antibody by CLIFT and needed quantitative confirmation by ELISA.

Having compared CLIFT with ELISA and ANA-IFA; for ELISA, as a quantitative assay; they concluded that greater sensitivity did not guarantee a larger number of truly positive samples. The major problem of anti-dsDNA-ELISA is the nonspecific binding to the plastic surfaces.[14, 15] However, their availability, ease of use, and quantitative output have kept ELISA as the method of choice in many laboratories.[15]

Antico et al. reported on new-generation immunoassays as an effective alternative to CLIFT.[16] They examined 5 different tests; chemiluminescent immunoassay, fluorometric enzyme immunoassay, two classical ELISAs, CLIFT, and FARR and they found that, CLIFT could be used as a confirmatory test in enzyme immunoassay positive sera.[16]

Their result was quite different than ours. The use of anti-dsDNA tests to analyze only positive patients increases specificity and positive predictive value in the diagnosis of SLE especially in patients with a homogeneous, speckled and peripheral patterns of ANA staining. This strategy is also somewhat less sensitive, but more affordable.[17-19] Sensitivity could be increased by using a combination of methods. The CLIFT+ELISA combination is more sensitive than CLIFT alone and could potentially be used. The most efficient screening strategy is limiting the use of CLIFT+ELISA only to patients with a homogeneous, speckled, and peripheral patterns of ANA staining. In the present study, positive predictive value increased from 73% to 83% when only one type of anti-dsDNA test was positive in patients with positive results for two tests. In addition, determining anti-dsDNA by the CLIFT+ELISA combination only in patients with homogeneous, speckled, and peripheral ANA staining yielded the highest positive percentage, which was significantly greater than for any of the four methods separately (CLIFT, ANA-IFA, ANA immunoblot or ELISA). Panels of tests are commonly used to increase sensitivity and specificity or, when used sequentially, to decrease costs.[17]

The sequential use of two methods has been recommended to detect anti-dsDNA antibodies.[14,20] We have shown here that the simultaneous use of several methods to determine anti-dsDNA increased diagnostic performance. Although the anti-dsDNA antibodies that were detected by any available method can be used to classify patients with SLE[21], the choice of the assay to use is decisive to detect anti-dsDNA antibodies of clinical importance.[22] Homogeneous and speckled patterns are known to be the most prevalent types of ANA staining in patients with SLE.[23] It also has been associated with certain clinical features.[24] There is a difference in the specificity and selectivity of anti-dsDNA assays. They have shown to be highly heterogeneous.[25]

As summarized by Ghirardello et al.[26], the choice of DNA source and anti– DNA assay preparation is crucial. According to their publication, the best performance was obtained with human genomic DNA or pure dsDNA of adequate length. Anti-dsDNA antibody pathogenicity is strongly related to affinity maturation, IgG class or IgG/IgM ratio, complement activation, and cross-reactivity with glomerular basement membrane components.[27-32] Assay value depends on a standardized way to recognize and measure pathogenic autoantibodies.[26,33]

In another study by Yang JY et al.,[34] a total of 142 sera of patients with systemic rheumatic diseases had been tested by 6 different assays using different antigenic sources of DNA; CLIFT, salmon testes (IB), human (ELISA I), salmon testes with nucleosome linker (ELISA II), plasmid (ELISA III), and synthetic oligonucleotides (chemi-luminescence immunoassay, CLIA); and they concluded that ELISA I had a greater sensitivity than the other five assays, and the specificities of ELISA II, ELISA III, CLIA, and CLIFT were higher than those of ELISA I and IB.[34]

Tong DW et al. published a study in 2009 about the detection of anti-dsDNA antibodies.[35] The authors analyzed 200 serum samples including 120 serum samples of SLE, 20 serum samples of rheumatoid arthritis, 20 serum samples of MCTD, 20 serum samples of SS, 20 serum samples of PSS, and 50 serum samples of healthy measured by IFA, Farr, and ELISA. In patients diagnosed with SLE, detection of the anti-dsDNA antibody of the serum samples with the methods of IIF, ELISA, and Farr resulted in 25%, 32%, and 32%.[35] Their results were compatible with ours that ELISA showed the highest positive predictive value. Similarly to our study, they stated that the detection of the anti-dsDNA antibody using two methods at the same time, namely with CLIFT/ANA-IFA and ELISA, increased the positive predictive rate than that of single method in.[35] Likely, we found a strong correlation between CLIFT and ANA-IFA to confirm the SLE diagnosis.

Limitations of our study are our retrospective design and non-availability to evaluate all of the four laboratory methods at the same time.

We could only evaluate the patients with positive CLIFT results, due to the retrospective design, so we did not have detailed information about the CLIFT negative patients. So, we could not be able to analyze the sensitivities and specificities of these laboratory methods.

In conclusion, anti-dsDNA antibody detection should only be assessed in patients with CLIFT positivity and ANA-IFA staining patterns. This approach maximizes availability and positivity. A combination of two quantitative methods was more efficient than any single method.

Ethics Committee Approval: The study was approved by the Hospital's Ethics Committee.

Informed consent: Written informed consent was obtained.

Conflict of interest: The authors declared that there were no conflicts of interest.

Contribution of authors: Concept: CTD, AZS; Design: CTD, AZS, ENMG; Data Collection and Processing: CTD, AZS, ENMG; Analysis or Interpretation: CTD, AZS, ENMG; Literature Search: CTD, AZS; Writing: CTD, AZS; Critical Review: CTD, AZS.

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