Usporedba testa *AtheNA Multi-Lyte ANA* za određivanje autoantitijela s indirektnom imunofluorescencijom i testom ELISA

Comparative analysis of multiplex AtheNA Multi-Lyte ANA test system and conventional laboratory methods to detect autoantibodies

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Sažetak

Uvod: Nove tehnologije temeljene na istovremenoj višestrukoj detekciji specifičnih antinuklearnih antitijela (engl. *multiplexed bead base immunoassay*) poboljšavaju dijagnosticiranje i praćenje autoimunih bolesti.

Cilj našeg ispitivanja bio je utvrditi karakteristike nove metode mnogostrukog određivanja autoantitijela (engl. *AtheNA Multi-Lyte ANA test system*) u odnosu na dosad korištene metode indirektne imunofluorescencije (IIF ANA) i enzimimunološke metode (ELISA).

Materijali i metode: Obradili smo 897 uzoraka seruma. Svi uzorci ispitani su testom *AtheNA Multi-Lyte ANA* na 9 specifičnih Ag: SSA, SSB, Sm, RNP, Scl-70, Jo-1, ds DNA, centromere B i histone; i imunofluorescencijom. Pozitivni su uzorci analizirani metodom ELISA na specifična autoantitijela protiv SSA, SSB, Sm, RNP, Scl-70, Jo-1, ds DNA, centromera B, histona i rezultati uspoređeni testom *AtheNA Multi-Lyte ANA*.

Rezultati: Podudarnost kvalitativno izraženih rezultata testa *AtheNA Multi-Lyte ANA* i indirektne imunofluorescencije bila je 92,3% (70% negativnih i 22.3% pozitivnih rezultata). Analitička osjetljivost za specifična autoantitijela kretala se od 80% (Scl-70) do 100% (SSA), a specifičnost od 92,3% (dsDNA) do 98,3% (Sm) između metode *AtheNA Multi-Lyte ANA* i ELISA. Pozitivni uzorci (200) analizirani su metodom ELISA, a usporedba s testom *AtheNA Multi-Lyte ANA* za 9 pojedinačnih autoantitijela pokazala je značajnu korelaciju ($P < 0,001$), kao i procjena slaganja dviju metoda izražena kapa- koeficijentom ($P < 0,001$).

Zaključak: Naši su rezultati pokazali da za određivanje specifičnih autoantitijela u imunološkom laboratoriju *AtheNA Multi-Lyte ANA* može zamijeniti metodu ELISA, ali ne u potpunosti metodu IIF ANA.

Ključne riječi: Antinuklearna antitijela, test *AtheNA Multi-Lyte ANA*, ELISA, autoimune bolesti

Abstract

Introduction: New technologies that employ multiplexed bead based immunoassays have contributed to the improvement in the diagnosis and monitoring of autoimmune diseases.

The aim of this study was to determine the performance of multiplexed bead based immunoassay (AtheNA Multi-Lyte ANA test system) relative to established indirect immunofluorescent analysis (IIF ANA) and enzyme-linked immunosorbent assay (ELISA) currently used in our laboratory.

Materials and methods: 897 serum specimens were tested with AtheNA Multi-Lyte ANA test system for nine analytes (SSA, SSB, Sm, RNP, Scl-70, Jo-1, dsDNA, Centromere B and Histone), and with IIF ANA. Only positive specimens were tested with single-antigen ELISA and compared with AtheNA Multi-Lyte ANA.

Results: There was a complete concordance between multiplexed bead based immunoassay and IIF ANA in 92.3% cases (70% of total cases were negative and 22.3% positive). For specific autoantibodies, sensitivity ranged from 80.0% (Scl-70) to 100% (SSA), and specificity from 92.3% (ds DNA) to 98.3% (Sm) for AtheNA Multi-Lyte ANA and ELISA. All positive sera (200) were tested by single antigen ELISA test and compared with results obtained with AtheNA Multi-Lyte ANA test system. The correlation established for all autoantibodies was significant ($P < 0.001$), as well as the kappa value ($P < 0.001$).

Conclusion: Our results showed that the AtheNA Multi-Lyte test system could replace single antigen ELISA test for the measurement of specific autoantibodies, but not completely the IIF ANA method in an immunology laboratory.

Key words: antinuclear antibodies, AtheNA Multi-Lyte ANA test system, ELISA, autoimmune diseases

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Uvod

U dijagnostici sistemskih i organospecifičnih bolesti detekcija autoantitijela je jedan od osnovnih imunoloških dijagnostičkih postupaka. Uz kliničke simptome, detekcija specifičnih autoantitijela na jezgrine autoantigene te citoplazmatskih antitijela jest ključna za postavljanje dijagnoze (1). Svaku sistemsku autoimunu bolest, od kojih su najčešće sistemski eritemski lupus (SLE), Sjőgrenov sindrom (SS), polimiozitis (PM), miješana bolest vezivnog tkiva (MCTD), reumatoidni artritis (RA) i jatrogeni lupus, karakterizira određeni profil autoantitijela. U rutinskom radu imunološkog laboratorija koriste se brojne tehnike i metode za dokazivanje ponajprije autoantitijela, a rjeđe antigena (2). U detekciji antinuklearnih autoantitijela (ANA) metoda indirektne imunofluorescencije (IIF) je metoda izbora ako se koristi u pretraživanju seruma (3). Detekcija ANA IIF je mikroskopska tehnika u kojoj iskustvo analitičara ima važnu ulogu, nije standardizirana, omogućava približnu detekciju specifičnog antitijela temeljem specifične fluorescencije, dijagnostički je zahtjevna i vremenski dugo traje. Zbog potreba bržeg dijagnosticiranja bolesti i određivanja specifičnih antitijela razvile su se metode koje je moguće automatizirati i donekle standardizirati (4). Enzimimunokemijska metoda (ELISA) za otkrivanje specifičnog autoantitijela koristi antigene pripremljene iz humanih epiteloidnih stanica (HEp-2) stanica ili rekombinantnim tehnikama. Različiti načini dobivanja antigena često su uzrok različitih rezultata između testova (5). Specifičnost određenog ELISA-testa za određeno autoantitijelo ovisi o antigenu, te je važno da antigeni koji se koriste imaju istu sekvencu, konformaciju i post-translacijsku modifikaciju te da su što sličniji humanom antigenu (6). U novije vrijeme razvoj i primjena tehnika protočne citometrije u kombinaciji s homogenim fluoroimunokemijskim metodama omogućava istovremeno određivanje većeg broja autoantitijela iz jednog uzorka seruma. Polistirenske mikročestice označene su s dva fluorofora. Svaki fluorofor sadrži 10 precizno definiranih različitih koncentracija, odnosno intenziteta fluoroscencije, što omogućava kombinaciju od 100 različito obojenih mikročestica. To bi značilo da se, kada se svaka mikročestica obilježi specifičnim antigenom u homogenoj imunofluorokemijskoj reakciji, može dokazati i do 100 antitijela. Suspenzija čestica prolazi kroz protočnicu, obasjava se s dvije laserske zrake od kojih crvena grupira čestice po boji, tj. specifičnom antitijelu, a zelena ekscitira fluorescenciju specifičnog antitijela te ih kvantificira (7). Jedan od primjera primjene tih tehnika je i test *AtheNA Multi-Lyte ANA* kojim se kvalitativno određuje nazočnost antinuklearnih antitijela te polukvantitativno istovremeno devet pojedinačnih autoantitijela.
Rezultati se očitavaju na sustavu *Luminex 100*, protočnom citometru prilagođenom za tehnologiju xMAP.

Introduction

Detection of antinuclear antibodies (ANA) is extensively used for establishing a diagnosis in patients with clinical features suggestive of non-organ specific (systemic) or organ-specific autoimmune disorders. Among the systemic autoimmune diseases that are characterized by the presence of ANA there are systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), systemic sclerosis (SSc), inflammatory myositis (IM), mixed connective tissue disorder (MCTD), rheumatoid arthritis (RA) and drug induced SLE. The diagnosis of these diseases depends on the identification of disease-associated clinical symptoms and signs, and is associated with the detection of autoantibodies directed against nuclear or cytoplasmic antigens (1). Autoimmunity laboratories analyze and measure an increasing number of autoantibodies, employing a broad spectrum of techniques and methods. The main characteristic of the autoimmunity laboratory, and indeed the one that differentiates it from other laboratories that use immnunoassays as the basic technique, is that it determines antibodies (autoantibodies) and not antigens (2).
The most common method for the identification of positive patient's sera for ANA is indirect immunofluorescence (IIF), but this method gives high positive (false) responses among healthy individuals and variability in results between laboratories (3). Detection of ANAs by IIF is a microscopic technique, in which reader agreement and reliability are of great importance. IIF provides an approximation to the identification of possible autoantigens by means of their fluorescence patterns. Furthermore, the manual IIF procedure is relative labor-intensive. Considerable effort has been made in developing simpler automated assays for routine laboratory use. Several different forms of enzyme immunoassays have been developed which were simpler, high-throughput analyses that can be automated and standardized. (4) Of these, enzyme-linked immunosorbent assay (ELISA) based either on antigens prepared from human tumor cell line (HEp-2) nuclear extracts or from recombinant and highly purified nuclear antigens has been the most promising, but substantial differences in terms of positivity among various enzyme immunoassay methods have been described (5). The specificity of ELISAs for autoantibody measurements is strongly dependent on the quality of antigens used, and it is important that an antigen should have exactly the same sequence, conformation and post-translational modifications as the human antigen. Flow cytometry for the analysis of microsphere-based immunoassays has been developed for the simultaneous determination of different substances (6). Recently a commercially available microsphere-based fluorescent assay has been introduced for the detection of ANA. Among applications of this technology there is simultaneous measurement of several autoantibodies.

U detekciji antinuklearnih antitijela važno je znati kada je potrebno zamijeniti IIF ANA s novom tehnologijom mnogostrukog određivanja. Cilj našeg ispitivanja bio je utvrditi karakteristike nove metode mnogostrukog određivanja autoantitijela (engl. *AtheNA Multi-Lyte ANA test system*) u odnosu na dosad korištene metode indirektne imunofluorescencije (IIF ANA) i enzimimunološke metode (ELISA).

Materijali i metode

Ispitanici

Ispitivanje je obuhvatilo 897 ispitanika čije smo uzorke seruma obradili u našem laboratoriju u vremenskom intervalu od 7 mjeseci (od lipnja 2006. do prosinca 2006. godine). Ispitanici su odabrani na temelju dijagnoze ili sumnje na autoimuni poremećaj. Uzorci su podijeljeni u tri skupine:

Prva skupina obuhvaćala je 301 ispitanika s dijagnozama: reumatoidni artritis (N = 122), SLE (N = 101), Sjögrenov sindrom (N = 9), sistemska skleroza (N = 34) i različiti oblici vaskulitisa (N = 35).

U drugoj je skupini bilo 398 ispitanika s kliničkom sumnjom na određenu autoimunu bolest: reumatoidni artritis (N = 47), SLE (N = 22), Sjögrenov sindrom (N = 7), sistemska skleroza (N = 23), vaskulitis (N = 29) i sumnju na autoimuni poremećaj (N = 270).

U trećoj je skupini bilo 198 ispitanika bez dijagnoze.

U svim pozitivnim uzorcima određena su specifična autoantitijela protiv antigena SS-A/Ro (SSA), SS-B/La (SSB), Smith (Sm), U1-snRNP (RNP), antitijela protiv topoizomeraze 1 (Scl-70), anti-histidil-tRNA (Jo-1), antitijela protiv dvostruke uzvojnice DNA (dsDNA), antitijela protiv centromerskog proteina B (Centromere B), te antitijela protiv histona.

Nakon centrifugiranja odvojeni serum je pohranjen na temperaturi –20 °C do provođenja analize.

Ispitivanje je odobrilo Etičko povjerenstvo Kliničke bolnice Split (13).

Metode

Test AtheNA Multi-Lyte ANA

897 uzoraka seruma ispitano je metodom mnogostrukog određivanja autoantitijela (AtheNA Multi-Lyte ANA test system, Zeus Scientific, Inc., Raritan, NJ, 08869 USA). Test *AtheNA Multi-Lyte ANA* se primjenjuje za polukvantitativno određivanje autoantitijela usmjerenih na devet različitih antigena (SSA, SSB, Sm, RNP, Scl-70, Jo-1, dsDNA, centromera B i histona te kvalitativno dokazivanje ANA u serumu. Na polistirenskim mikročesticama vezani su pročišćeni antigeni SSA, Sm i histoni, dok su SSB, RNP, Jo-1 i centromera B rekombinantni antigeni. U kvalitativnoj detekciji ANA koriste se ekstrakti stanica HEp-2. Uzorak seruma i kontrole razrijedi se 1:21 s odgovarajućim razrjeđivačem. Sustav koristi polistirenske mikrosfere obojene internalno s različitim omjerima dva različita fluorokroma. Svaki fluorokrom može imati bilo koju od 10 mogućih razina intenziteta fluorescencije, čime nastaje obitelj od 100 spektralno adresiranih setova mikrosfera. Antigeni koji odgovaraju autoantitijelima vežu se na mikrosfere. Svaka od 100 mikrosfera koje se mogu razlikovati po fluorescenciji nosi specifični imobilizirani antigen za jedno autoantitijelo. U isto vrijeme zeleni laser pobuđuje vanjsku reporter fluorescenciju za kvantifikaciju specifične reakcije vezane za svako autoantitijelo (7). Test AtheNA Multi-Lyte ANA test system je multiplexirani homogeni, fluorescencijski mikročestični imunoanalizator, dizajniran za detekciju i razlikovanje autoantitijela prisutnih u humanom serumu. Luminex 100 System (Luminex Corporation, Austin, TX, USA) je protočni citometar analizator specifično dizajniran za xMAP tehnologiju.

U detekciji antinuklearnih antitijela važno je znati kada je potrebno zamijeniti IIF ANA s novom tehnologijom mnogostrukog određivanja. Cilj našeg ispitivanja bio je utvrditi karakteristike nove metode mnogostrukog određivanja autoantitijela (engl. *AtheNA Multi-Lyte ANA test system*) u odnosu na dosad korištene metode indirektne imunofluorescencije (IIF ANA) i enzimimunološke metode (ELISA).

The system uses polystyrene microspheres labelled internally with different ratios of two different fluorochromes. Each fluorochrome can have any of the 10 possible levels of fluorescence intensity, thereby creating a family of 100 spectrally addressed bead sets. The antigens corresponding to autoantibodies are bound to the microspheres. Each of the 100 microbeads that can be differentiated by their fluorescence carries a specific immobilized antigen for a single autoantibody. At the same time, a green laser excites the external reporter fluorescence to quantify the specific reaction related to each autoantibody (7). The AtheNA Multi-Lyte ANA test system is a multiplexed homogenous, fluorescence-based microparticle immunoassay, designed to detect and distinguish the autoantibodies present in human serum. The Luminex 100 System (Luminex Corporation, Austin, TX, USA) is a flow cytometer analyzer specifically designed for xMAP technology.

In detection of an antinuclear antibody, it is important to know when the initial IIF ANA screening on HEp-2 cells can be replaced with multiplexed bead-based immunoassay without loss of any important results. The objective of the study was to determine the performance of multiplexed bead based immunoassay (AtheNA Multi-Lyte ANA test system) relative to established, commercial IIF ANA HEp-2 and ELISA currently used in our laboratory.

Materials and methods

Patients

The study included 897 serum samples submitted to our laboratory for autoimmune testing over a period of 7 months (from June 2006 to December 2006). The sera were selected on the basis of established diagnosis or indication of autoimmune disease. All samples were divided into three groups.

One group consisted of 301 patients with established diagnosis: rheumatoid arthritis (N = 122), SLE (N = 101), Sjögren syndrom (N = 9), systemic sclerosis (N = 34) and different types of vasculitis (N = 35).

The second group consisted of 398 patients with clinical suspicion of certain autoimmune disease: rheumatoid arthritis (N = 47), SLE (N = 22), Sjögren syndrome (N = 7), systemic sclerosis (N = 23), vasculitis (N = 29) and with suspicion of autoimmune disorder (N = 270).

The third group consisted of 198 patients without diagnosis.

All positive specimens were tested for specific autoantibodies directed against anti Sjőgren's syndrome SS-A/Ro (SSA), SS-B/La, Smith antigen (Sm), ribonucleoprotein (RNP), antitopoisomerase I (Scl-70), autoantibodies directed against hystidyl-tRNA syntetase (Jo-1), autoantibodies to native double stranded DNA (dsDNA), centromere B and histone.

vačem. Suspenzija mikročestica miješa se s razrijeđenim uzorkom i inkubira. Ukoliko su prisutna, autoantitijela iz uzorka vežu se za odgovarajući antigen. Nakon ispiranja nevezanoga materijala doda se konjugat (kozji anti-humani IgG obilježen fluoresceinom). Nakon druge inkubacije pristupa se čitanju rezultata testa *AtheNA Multi-Lyte ANA* prilagođenom protočnom citometru *Luminex 100* (Austin, SAD).
Rezultat je pozitivan ako je interna kontrola (HEp-2) ili bilo koji od devet parametara ≥ 120 AU/mL. Za granični rezultat smatra se vrijednost od 100 do 120 AU/mL, dok su negativni svi uzorci čiji intenzitet fluorescencije odgovara vrijednosti < 100 AU/mL.

IIF ANA

Autoantitijela su određena imunofluorescentnom tehnikom u 897 seruma. IIF ANA koristi stanice HEp-2 kao supstrat, a kozji anti-humani IgG obilježen fluorescein-izocijanatom kao konjugat (BioSystem S.A. Costa Brava 30, Barcelona, Španjolska). S uzorkom seruma razrijeđenim fosfatnim puferom u omjeru 1:80 prelije se supstrat te nakon inkubacije od 30 minuta i pažljivog ispiranja nevezanog materijala podloga se prekrije konjugatom. Po završetku inkubacije od 30 minuta i ispiranjem preparata s fosfatnim puferom prisutnost ANA se detektira pod fluorescentnim mikroskopom. Po tipu fluorescencije može se približno odrediti o kojem bi se antitijelu moglo raditi, a primjenom potvrdne metode odredi se točno specifično antitijelo. Uz svaku seriju određivane su pozitivna i negativna kontrola. Pozitivnim rezultatom smatra se razrjeđenje ≥ 1:80.

ELISA

Metodom ELISA smo u 200 seruma pozitivnih na antinuklearna antitijela odredili specifična antitijela za SSA, SSB, Sm, RNP, Scl-70, Jo-1, dsDNA (Hycor Biomedical Ltd Pentlands Science Park, Bush Loan, Penincula, V. Britanija), centromere B i histone (DiaSorin S.p.A. Via Corescentino 13040 Sallugia, Italija). Jažice mikrotitarskih pločica obilježene su antigenima različitoga podrijetla. SSA, SSB, Sm, RNP, Scl-70 i Jo-1 su goveđeg podrijetla, dsDNA iz telećeg timusa, centromeri B su rekombinantni antigen, a histoni su iz pilećih matičnih stanica. Sve su analize izvedene na automatskom analizatoru (miniBOS, Biomedica Gruppe, GMBH, A-1210, Divischgasse 4, Beč, Austrija).
Nakon automatskog razrjeđenja kontrole i uzorka (1:100) u jažice se pipetira standard, uzorak i kontrola te se za vrijeme inkubacije od 30 minuta specifično vežu autoantitijela za odgovarajući antigen. Po završetku inkubacije suvišak se ispere i dodaje konjugat (anti-humani IgG obilježen peroksidazom) te inkubira. Po završetku inkubacije, reakcija peroksidaze na dodani supstrat (tetra-metil benzidin) se prekida nakon 15 minuta s 0,25 M H_2SO_4. Apsorbancija se očita na 450 nm, a jačina obojenja propocionalna je

After blood clotting, serum was obtained by centrifugation and stored at –20 °C until further analysis.
Local Ethics Committee of the Split University Hospital approved the study (13).

Methods

AtheNA Multi-Lyte ANA

A total of 897 sera were tested using multiplex technology based tests (AtheNA Multi-Lyte ANA test system, Zeus Scientific, Inc., Raritan, NJ, 08869 USA). AtheNA Multi-Lyte ANA test system was utilized for the simultaneous semiquantitative determination of autoantibodies to nine different antigens: (SSA, SSB, Sm, RNP, Scl-70, Jo-1, dsDNA, Centromere B, Histones) and qualitative measurement of ANA. The assay is based on a microsphere-based technology**.** Antigen source for SSA, Sm, and histone antigens are purified from natural sources while SSB, RNP, Scl-70, Jo-1 and Cent B are human recombinant antigens. The qualitative ANA preparation is derived from the nuclei of HEp-2 cells. Sera and controls were diluted 1:21 with specimen diluent. Bead suspensions were thoroughly mixed with diluted sera and controls and incubated in a microtitre plate. If present in sera, specific autoantibodies bind to the antigen. The conjugate (goat anti-human IgG conjugate with phycoerythrin) is added after incubation and washing away of unbound material. After second incubation, the bead suspensions were thoroughly mixed and then placed on a microplate and into AtheNA reader (within 60 minutes) to read the requested results. The AtheNA Multi-Lyte instrument utilizes Luminex 100 technology (Austin, USA) which is based on flow cell fluorometry. Positivity of tests was calculated according to the manufacturer's instructions as follows: a serum was considered positive if at least one individual parameter was > 120 AU/mL, or an internal HEp-2 extract (internal control sample) was > 120 AU/mL. A serum was considered equivocal if any test value ranged from 100 to 120 AU/mL, and negative if the corresponding fluorescence was < 100 AU/mL.

IIF ANA

A total of 897 sera were tested using convetional immunoassay including ANA HEp-2 test system (BioSystems S.A., Costa Brava 30, Barcelona, Spain). The IIF ANA uses HEp-2 cells as a substrate and goat anti-human IgG conjugated with fluorescein isothiocyanate as a conjugate. Sera were diluted 1:80 with phosphate-buffered saline and overlaid on the substrate for 30 min at room temperature. Slides were washed twice for 5 min each with phosphate-buffered saline, overlaid with conjugate and incubated for additional 30 min. Subsequently, slides were washed twice and examined with a fluorescence microscope at x 40 magnification. Patient samples were considered positive when a positive signal was obtained at dilution ≥1:80. Negative and positive controls were used to check accuracy.

količini antitijela u serumu. Prema navodu proizvođača za nepreciznost unutar serije koeficijent varijacije (CV%) kreće se u rasponu za: SSA od 2,8% do 3,1%, SSB od 5,5% do 9,1%, Sm od 5,4% do 8,6%, RNP od 2,9% do 11,2%, Scl-70 od 3,0% do 5,1%, Jo-1 od 3,6% do 6,1%, dsDNA od 3,9% do 6,9%, centromere B od 4,8% do 8,6%, te za histone od 6,6% do 10,6%. CV% za nepreciznost između serija kreće se u rasponu za: SSA od 3,5% do 7,9%, SSB od 3,4% do 6,0%, Sm od 3,2% do 5,1%, RNP od 4,9% do 7,4%, Scl-70 od 6,7% do 9,4%, Jo-1 od 2,7% do 8,3%, dsDNA od 8,4% do 9,8%, centromere B od 6,2% do 11,0%, te histone od 8,3% do 12,9%.

Nalaz je pozitivan ako su rezultati za autoantitijelo na SSA, SSB, Sm, RNP, Scl-70 i Jo-1 > 15 U/mL, graničan ako je između 10 i 15 U/mL, a negativan ako je < 10 U/mL. Za dsDNA vrijednosti > 60 U/mL su pozitivne, od 40 do 60 U/mL granične, a < 40 U/mL su negativne, za centromere B vrijednosti > 11 U/mL su pozitivne, od 8 do 11 U/mL granične, a < 8 U/mL negativne i vrijednosti za histone > 21 U/mL pozitivne, od 15 do 21 U/mL granične, a < 15 U/mL negativne.

U ovom smo radu primijenili navedene referentne vrijednosti proizvođača testova.

Statistička analiza

Rezultati su obrađeni pomoću statističkog programa SPSS V 8.0 (SPSS Inc, Chicago, IL). Izračunani su koeficijenti korelacije (Spearman rho), uz prihvaćenu razinu značajnosti za P < 0,001, te Cohenov kapa-koeficijent kojim je izražena podudarnost između dvije različite metode za svako antitijelo pojedinačno uz razinu značajnosti P < 0,001.

Osjetljivost i specifičnost testa računane su u odnosu na metodu ELISA koju smo prihvatili kao referentnu metodu i gdje smo definirali: stvarno pozitivne rezultate testa (TP), stvarno negativne rezultate testa (TN), lažno negativne rezultate testa (FN), te lažno pozitivne rezultate testa (FP). Osjetljivost (TP/[TP+FN] x 100) je izražena kao postotak pozitivnih rezultata u odnosu na pozitivne rezultate testa ELISA, a specifičnost (TN/[FP+TN] x 100) kao postotak negativnih rezultata u odnosu na negativne rezultate testa ELISA.

Rezultati

Antinuklearna antitijela određivali smo kod 897 bolesnika sa sumnjom na autoimune bolesti. Rezultati dobiveni testom *AtheNA Multi-Lyte ANA* i testom indirektne imunofluorescencije prikazani su u Tablici 1. Kvalitativnom analizom uzoraka s ova dva testa podudarnost rezultata je nađena kod 92,3% uzoraka (70% negativnih i 22,3% pozitivnih). Neslaganje rezultata nađeno je kod 59 (6,6%) uzoraka od kojih je 6 (0,7%) uzoraka bilo lažno pozitivno, 35 (3,9%) lažno negativno, a 18 (2%) je dalo granične rezultate testa *AtheNA Multi-Lyte ANA*. Svih 35 negativnih uzoraka testa

ELISA

A total of 200 serum samples positive to ANA were analyzed by single-antigen ELISA for SSA, SSB, Sm, Sm/RNP, Scl-70, Jo-1, dsDNA (Hycor Biomedical Ltd, Pentlands Science Park, Bush Loan, Penincula, UK), Histone and Centromere B (DiaSorin S.p.A., Via Corescentino 13040 Sallugia, Italy). Microtitre wells were coated with antigens from the following sources: for SSA, SSB, Sm, RNP, Scl-70 and Jo-1 from bovine sources, for dsDNA the wells were coated with purified dsDNA from calf thymus, for Cent B recombinant CENT-P (bacillovirus) and for Histone from chicken bone tissue (blood stem cells). All single-antigen ELISA tests were performed at an automatic microplate analyzer (miniBOS, Biomedica Gruppe, GMBH, A-1210, Divischgasse 4, Vienna, Austria).

In principle, dilutions of controls and samples (1:100) are performed automatically by the instrument. On adding standards, diluted controls and serum to the wells, the antibodies present bind to the antigen. After incubating and washing away unbound material, a conjugate (peroxidase-labeled anti-human IgG mouse monoclonal antibody) is added. Following further incubation and washing, a substrate (tetra-methyl benzidine) is added to each well. The presence of the antigen-antibody-conjugate complex changes the substrate. The reaction is stopped by adding 0.25 M H_2SO_4. Absorbance is measured at 450 nm. Color intensity is proportional to the amount of the autoantibodies present in a serum sample. Test results were calculated according to manufacturer's instruction and range for the single-antigen ELISA. Intra assay coefficients of variation (CV%) ranged for SSA from 2.8% to 3.1%, SSB from 5.5% to 9.1%, Sm from 5.4% to 8.6%, RNP from 2.9% to 11.2%, Scl-70 from 3.0% to 5.1%, Jo-1 from 3.6% to 6.1%, dsDNA from 3.9% to 6.9%, Centromere B from 4.8% to 8.6%, and Histone from 6.6% to 10.6%, while inter assay CVs ranged for SSA from 3.5% to 7.9%, SSB from 3.4% to 6.0%, Sm from 3.2% to 5.1%, RNP from 4.9% to 7.4%, Scl-70 from 6.7% to 9.4%, Jo-1 from 2.7% to 8.3%, dsDNA from 8.4% to 9.8%, Centromere B from 6.2% to 11.0%, and Histone from 8.3% to 12.9%.

A serum was considered positive if autoantibodies to SSA, SSB, Sm, Sm/RNP, Scl-70 and Jo-1 were > 15 U/ml, equivocal if any test value was from 10 to 15 U/mL, and negative if < 10 U/mL. Results for autoantibody to dsDNA > 60 U/mL were considered positive, the value from 40 to 60 U/mL equivocal, and < 40 U/mL negative; for Centromere B > 11 U/mL was considered positive, the value from 8 to 11 U/mL equivocal, and < 8 negative, and for Histone > 21 U/mL value was considered positive, the value from 15 to 21 U/mL equivocal, and < 15 U/mL negative.

The results obtained in this study were compared to the reference values declared by manufacturers.

Statistical analysis

Statistical analysis was performed with SPSS V 8.0 (SPSS Inc, Chicago, IL). Correlations were calculated using the

Tablica 1. Prikaz rezultata dobivenih metodom imunofluorescencije i testom *AtheNA Multi-Lyte ANA*

Table 1. Cross-tabulation of results obtained with immunofluorescence and the AtheNA Multi-Lyte ANA test System

		IIF ANA			Total
		Negative N (%)	Equivocal N (%)	Positive N (%)	N (%)
AtheNA Multi-Lyte ANA II Test System	Negative	631 (70)	2 (0.2)	35 (3.9)	668 (74.5)
	Equivocal	3 (0.3)	0 (0)	15 (1.7)	18 (2)
	Positive	6 (0.7)	5 (0.6)	200 (22.3)	211 (23.6)
Total N (%)		640 (71)	7 (0.8)	250 (27.9)	897 (100)

AtheNA Multi-Lyte ANA dalo je negativne rezultate kod testa ELISA, dok su kod testa IIF svi bili pozitivni i to 7 uzoraka je imalo točkastu, a 28 uzoraka homogenu imunofluorescenciju. Svih 6 uzoraka koji su nakon testa *AtheNA Multi-Lyte ANA* bili pozitivni, a imunofluorescencijom negativni, s ELISA testom su bili pozitivni kao SSA.
Između 18 graničnih uzoraka nakon testa *AtheNA Multi-Lyte*, 3 su uzorka bila negativna s IIF ANA, s ELISA-testom SSA pozitivna, a 15 uzoraka je bilo nakon IIF ANA pozitivno. Među 15 uzoraka koji su nakon IIF ANA bili pozitivni, nakon testa ELISA 6 je uzoraka bilo dsDNA pozitivno, 4 Scl-70 pozitivna, dok je 5 uzoraka bilo nakon testa ELISA negativno. Osjetljivost između testa *AtheNA Multi-Lyte ANA* i testa ELISA je bila od 80% za Scl-70 do 100% za SSA, specifičnost od 92,3% za dsDNA do 98,3% za Sm (Tablica 2).
Antinuklearna antitijela kod 200 pozitivnih uzoraka koji su usporedno ispitani testovima *AtheNA Multi-Lyte ANA* i IIF izražena su kao kvalitativni rezultati. Svi pozitivni serumi su ispitani s ELISA-pojedinačnim antigenom i testom *AtheNA Multi-Lyte ANA* na pojedinačne antigene: SSA, SSB, Sm, RNP, Scl-70, Jo-1, dsDNA, centromere B i histone i izra-

Spearman rho test and P value < 0.001 was considered statistically significant. Agreement among methods was measured by calculating the kappa coefficient, P < 0.001 was considered statistically significant.
Sensitivity and specificity of the AtheNA Multi-Lyte ANA test was calculated by using the single-antigen ELISA results as the reference standard and the following definitions: true positive test result (TP), true negative test result (TN), false negative test result (FN), false positive test result (FP). Sensitivity (TP/[TP+FN] x 100) was computed as the percentage of positive test responses in sample with positive ELISA. Specificity (TN/[FP+TN] x 100) was computed as the percentage of negative tests in a sample with negative ELISA.

Tablica 2. Osjetljivost i specifičnost metoda *AtheNA Multi-Lyte ANA* i ELISA

Table 2. Sensitivity and specificity of the methods: AtheNA Multi-Lyte ANA test system and single-antigen ELISA

	SSA N = 76	SSB N = 74	Sm N = 75	RNP N = 75	Scl-70 N = 54	Jo-1 N = 54	dsDNA N = 54	Cent B N = 54	Histone N = 54
Sensitivity %	100	92	84.6	85.7	80	83.3	86,7	83.3	90
Specificity %	97.4	98	98.3	97.1	97.0	97.9	92.3	97.9	95.5

Results

A total of 897 serum samples from patients with suspected autoimmune disorders were delivered to our laboratory for ANA testing. The data obtained by the AtheNA Multi-Lyte ANA test system (HEp 2 cell lysate only) and IIF ANA are presented in Table 1. On comparing the results obtained by the AtheNA Multi-Lyte ANA test system

ženi su kao polukvantitativni rezultati. Kada su uspoređeni rezultati za pojedinačna autoantitijela testova *AtheNA Multi-Lyte ANA* i ELISA, za svako autoantitijelo dobivena je značajna korelacija (P < 0,001) (Tablica 3). Za procjenu slaganja dviju metoda pomoću Cohenovog kapa-koeficijenta za svako je pojedinačno antitijelo dobivena također značajna korelacija (P < 0,001) (Tablica 3).

Kada su uspoređeni rezultati svih 200 uzoraka pozitivnih prema metodama A*theNA Multi-Lyte ANA* i ELISA, bez obzi-

(expressed as qualitative results) and IIF ANA, a complete concordance was observed in 92.3% samples (70% of total samples were negative and 22.3% were positive). Fifty-nine (6.6%) samples were disconcordant, 6 (0.7%) false positive, 35 (3.9%) false negative, and 18 (2%) were equivocal. All 35 negative samples obtained with AtheNA Multi-Lyte ANA test system and IIF ANA were negative with single antigen ELISA. Fluorescence pattern observed in this samples was noted as unusually speckled (N = 7) or ho-

Tablica 3. Korelacija specifičnih autoantitijela između *AtheNA Multi-Lyte ANA* i ELISA i procjena slaganja dviju metoda

Table 3. Correlation of specific autoantibodies assayed by AtheNA Multi-Lyte ANA test system and single-antigen ELISA, and agreement between the methods

Test	Positive N	Negative N	Spearman R	P	Kappa value ± SD	Kappa significance
SSA	138	62	0.924	< 0.001	0.834 ± 0.035	< 0.001
SSB	77	123	0.911	< 0.001	0.742 ± 0.041	< 0.001
Sm	22	178	0.762	< 0.001	0.723 ± 0.071	< 0.001
RNP	21	179	0.716	< 0.001	0.726 ± 0.053	< 0.001
Scl-70	39	161	0.612	< 0.001	0.586 ± 0.061	< 0.001
Jo-1	11	189	0.659	< 0.001	0.637 ± 0.101	< 0.001
dsDNA	51	149	0.714	< 0.001	0.531 ± 0.059	< 0.001
Centromere B	15	185	0.718	< 0.001	0.507 ± 0.094	< 0.001
Histone	48	152	0.776	< 0.001	0.673 ± 0.053	< 0.001

Tablica 4. Podudarnost rezultata među pozitivnim uzorcima seruma za *AtheNA Multi-Lyte ANA* i metodu ELISA

Table 4. Correspondence for positive antinuclear antibody in serum between AtheNA Multi-Lyte ANA test system and single-antigen ELISA

Test	Total N	Positive AtheNA	Positive ELISA	Concordance %
SSA	139	138	137	99.3
SSB	79	77	77	100
Sm	22	20	22	90.9
RNP	21	19	21	90.5
Scl-70	39	36	39	92.3
Jo-1	12	11	10	90.9
dsDNA	52	47	51	92.2
Centromere B	15	15	14	93.3
Histone	48	48	45	93.8

ra na različitost u metodologiji dobivena je podudarnost rezultata od 90,5% do 100% (Tablica 4).

Rasprava

Detekcija antinuklearnih antitijela provodi se kod različitih autoimunih sistemskih i organospecifičnih bolesti, uz primjenu brojnih tehnika i metoda kao što su imunofluorescencija, imunodifuzija, enzimska imunometoda, imunoblot i u zadnje vrijeme analiza mnogostrukog određivanja utemeljena na načelu protočne citometrije. Na laboratoriju je zadatak da zajedno s kliničarima odredi metodu koju će primijeniti u detekciji brojnih autoantitijela kako bi se ustanovila ona najznačajnija. Istovremeno primijenjena metoda mora biti brza, učinkovita, jednostavne izvedbe i isplativa. U našem smo radu ispitivali kombinaciju testova kako bismo utvrdili koja od metoda je najučinkovitija za pretraživanje, a potom i utvrđivanje specifičnih autoantitijela. Ipak, potrebno je napomenuti da je jedino zajedno s kliničkom validacijom dobivenih rezultata moguće zaključiti o kliničkoj specifičnosti i osjetljivosti testa.
Indirektna imunofluoroscencija (IIF) je osjetljiva metoda s poznatim ograničenjima kao što su vrsta supstrata, način izvođenja testa, subjektivna interpretacija, niska reproducibilnost i nedostatak standardizacije.
Metoda mnogostrukog određivanja autoantitijela kao test *AtheNA Multi-Lyte ANA* priređena je za kvalitativnu detekciju ANA i istovremeno za polukvantitativno određivanje IgG-razreda specifičnih antitijela na SSA, SSB, Sm, RNP, Scl-70, Jo-1, dsDNS, centromere B i histone antigene u serumu. Sistemske autoimune bolesti karakterizira odnos pojedinih autoantitijela te je nakon pretraživanja uzorka bitno odrediti koja su specifična autoantitijela i u kojoj količini zastupljena.
Usporedbom kvalitativnih rezultata IIF ANA i *AtheNA Multi-Lyte ANA* podudarnost je iznosila 92,3%. Od 897 testiranih seruma 35 (3,9%) je bilo pozitivno s IIF ANA, a negativno s *AtheNA Multi-Lyte ANA* (Tablica 1). Oblik fluorescencije ovih uzoraka bio je nespecifičan i izražen kao točkasta i homogena fluorescencija. Pozitivni uzorci s IIF ANA nađeni su u skupini bolesnika sa sumnjom na autoimuni poremećaj i među uzorcima bolesnika bez dijagnoze, što je moguće s obzirom na nespecifičnost i kliničku nedefiniranost ovih uzoraka. IIF ANA detektira brojna autoantitijela na jezgrine centriole, signalne molekule te stanične sastojke koji također mogu biti od koristi u kliničkoj dijagnostici (8,9). Imajući u vidu da veliki broj uzoraka nema utvrđenu dijagnozu te da se među njima nalaze i bolesnici kod kojih postoji sumnja na organospecifičnu bolest, koristili smo razrjeđenja uzorka 1:80, iako je preporuka da se za detekciju kod sistemskih bolesti koristi razrjeđenje 1:160 (10,11).
Nalaz 6 (SSA) pozitivnih i 3 (SSA) granična rezultata primjenom *AtheNA Multi-Lyte ANA*, a negativnih s IIF ANA potmogenous (N = 28). All six samples positive according to AtheNA Multi-Lyte ANA test system and IIF ANA negative were positive in single-antigen ELISA as SSA. Among 18 equivocal samples obtained with AtheNA Multi-Lyte ANA test system, three samples were IIF ANA negative (single antigen ELISA SSA were positive) and 15 samples IIF ANA positive. Of these 15 IIF ANA positive samples tested with single antigen ELISA, 6 were ds DNA positive, 4 Scl-70 positive, while 5 samples were negative. For a specific autoantibody tested using AtheNA Multi-Lyte ANA test system and single antigen ELISA, sensitivity was from 80.0% (Scl-70) to 100% (SSA) and specificity from 92.3% (dsDNA) to 98.3% (Sm), as presented in Table 2.
Antinuclear antibodies were positive in 200 serum samples tested with AtheNA Multi-Lyte ANA test system and IIF ANA, and expressed qualitatively. All positive sera were tested with single-antigen ELISA for the same autoantibodies obtained with AtheNA Multi-Lyte test system reacting with SSA, SSB, Sm, RNP, Scl-70, Jo-1, dsDNA, Centromere B and Histones expressed semi-quantitatively. When multiplexed-assay results were compared to single antigen ELISA for all tested parameters, a significant correlation was obtained ($P < 0.001$). Two methods correlated significantly for all autoantibodies (kappa value from 0.834 for SSA to 0.507 for centromere B; Table 3).
Differences in methodology between multiplexed and single ELISA assays were taken into consideration and comparison of 200 positive serum samples included combined results for single autoantibodies. When multiplexed-assay results were compared to the single antigen ELISA, there was an agreement from 90.5% to 100% (Table 4).

Discussion

Screening of antinuclear antibody is performed for a variety of systemic and organ specific autoimmune diseases. Different techniques have been used to develop specific tests for ANA detection including immunofluorescence, immunodiffusion, enzyme immunoassays, immunoblotting techniques and recently multiplexed bead base assays. The laboratory may have dilemmas about techniques that are relevant and reliable to detect all clinically significant autoantibodies. At the same time, the applied method must be a tool for high throughput, efficient, easy to use and inexpensive. In our study, we examined test combinations to find out the the best choice for screening antinuclear autoantibody and determination of a specific autoantibody. It is, however, only with clinical validation possible to conclude about clinical specificity and sensitivity of tests.
Indirect immunofluorescence (IIF) microscopy is a sensitive method, yet it has some limitations like substrate variations, manual performance, subjective result interpretation, low reproducibility and lack of standardization. The

vrđuje da je ANA HEp-2 supstrat nedovoljno osjetljiv za SSA (12). Razlika u negativnim rezultatima ostavlja mogućnost pogrešnog tumačenja rezultata (14-16). Usprkos nedovoljnoj osjetljivosti za SSA i Jo-1 antigen, IIF ANA rijetko daje lažno negativne rezultate. Martins i sur. uspoređivali su metodu ELISA za SSA, SSB, Sm, RNP i Scl-70 s metodom mnogostrukog određivanja testa *ENA 5 Luminex*. Podudarnost rezultata bila je od 93,6% ili naviše, osjetljivost 50% za Scl-70 do 100% za SSA i specifičnost od 97,9% za Sm do 99,5% za SSB (17). Naši su rezultati pokazali podudarnost od 90,5% za RNP do 100% za SSB, osjetljivost od 80,0% za Scl-70 do 100,0% za SSA i specifičnost 92,3% za dsDNA do 98,3% za Sm između testova *AtheNA Multi-Lyte ANA* i ELISA (Tablica 2, Tablica 4). Usporedbom testa *AtheNA Multi-Lyte ANA* i pojedinačnih testova ELISA dobili smo značajnu korelaciju (Spearman R od 0,612 do 0,924, $P < 0,001$), što je u suglasnosti i s brojnim studijama koje su usporedbom istih metoda pokazale dobru podudarnost (17-21). Nifli i sur. uspoređivali su testove ELISA i *AtheNA Multi-Lyte ANA* za SSA, SSB, Sm, RNP i dobili značajnu korelaciju (kapa od 0,347 do 0,764, $P < 0,001$) izuzev za citoplazmatski antigen Jo-1. Zaključili su da test *AtheNA Multi-Lyte ANA* može zamijeniti pojedinačni test ELISA u mjerenju specifičnih autoantitijela izuzev za antigen Jo-1 kod kojega se vjerojatno koriste različiti epitopi na molekuli antigena u navedenim metodama (20). Usporedba dviju metoda u našem određivanju za sva autoantitijela daje značajnu korelaciju (kapa-koeficijent od 0,834 za SSA do 0,507 za centromere B). Shovman i suradnici uspoređivali su rezultate dobivene metodom ELISA za pretraživanje ANA i test *AtheNA Multi-Lyte ANA* kod zdravih osoba i dobili su podudarnost u 99% slučajeva, dok je podudarnost iz uzoraka dobivenih iz drugih laboratorija bila 97,7%. Zaključili su da je test *AtheNA Multi-Lyte ANA* osjetljiv za detekciju ANA (18). Međutim, metoda ELISA za pretraživanje ANA može propustiti neke antigene jer je test pripremljen iz ekstrakta stanica HEp-2 s ograničenim brojem antigena. Usporedbom pojedinačnih rezultata za specifična autoantitijela dobili smo neke razlike (Tablica 4). Svi rezultati koji se nisu podudarali bili su oko graničnih vrijednosti pojedinačnih testova deklariranih prema proizvođačima, izuzev 3 uzorka za Scl-70 i 4 za dsDNA. Razlika u rezultatima za 3 Scl-70 i 4 dsDNA dobivenih kao pozitivni testom ELISA i negativni testom *AtheNA Multi-Lyte ANA* može biti zbog različite pripreme antigena, prezentacije antigena autoantitijelu, različitih uvjeta u odvijanju reakcije među metodama, razlici u metodologiji, razlici u pripremi antigena (pročišćeni ili rekombinantni), kapacitetu vezanja i koncentraciji drugog antitijela te konjugatu (20,22). Uvjeti testa kao ionska jakost i granična vrijednost (engl. *cut off*) također utječu na razlike među testovima. Antigeni Scl-70 i dsDNA vezani su kovalentnom vezom za karboksilnu skupinu istaknutu na površini mikročestice te, ukoliko nisu uravnoteženi postupak vezanja, aktivacije, koncentracije mikročesti-

multiplexed bead base assay, AtheNA Multi-Lyte ANA test system, is designed for the qualitative measurement of ANA while simultaneously permitting the semi-quantitative detection of IgG class antibodies against SSA, SSB, Sm, U1-RNP, Scl-70, Jo-1, Centromere B, dsDNA and histones in human serum. These nine parameters permit the detection and quantification of the main autoantibody of systemic autoimmune disseases. This test system has been intended to become a potential replacement for conventional IIF and ELISA methods. In the present study, we compared 897 serum samples using IIF ANA and AtheNA Multi-Lyte ANA test for the qualitative measurement of ANA; the agreement between these methods was 92.3%. Among 897 tested serum samples, 35 (3.9%) samples were positive with IIF ANA test but negative with AtheNA Multi-Lyte test system (Table 1). The fluorescence pattern in these samples was observed as unusually speckled or homogeneous. IIF ANA positive samples were from a group with suspicion of autoimmune disorder and among the sera that were without diagnosis. IIF ANA testing on HEp-2 cells lacks specificity because of a considerable overlap in the clinical features of systemic autoimmune diseases, and because of the presence of various extractable nuclear antigen (ENA) antibodies. Also, IIF ANA method offers a broad screening capability and detects rarely-seen antibodies against centrioles, signal recognition particles and many other cellular targets which may be useful in clinical diagnostics (8,9). We used IIF ANA positive samples with 1:80 titer despite the recommendation by an international serology committee (10,11) because we had a great number of specimens without diagnosis or samples with possible diagnosis of an organ specific disorder. Thus we obtained high sensitivity and minor possibility to miss positive results, but also low throughput and the need for further testing with single autoantibodies. The finding of 6 (SSA) positive and 3 (SSA) equivocal results according to AtheNA Multi-Lyte test system and negative according to IIF ANA method suggested that IIF ANA HEp-2 substrate lacked sensitivity for SSA (12). Also, the samples with bordeline ANA fluorescence might contain anti SSA. Discrepant negative results present a problem because a potentially relevant antibody may be missed (14,15,16). Although screening with conventional IIF ANA HEp-2 cells misses some antigens such as SSA and Jo-1, false negative results are infrequent. Martins et al. compared single-antigen ELISAs for SSA, SSB, Sm, RNP and Scl-70 with multiplexed ENA 5 Luminex based assay. They obtained 93.6% agreement or higher for all antibodies, the sensitivity ranging from 50% for Scl-70 to100% for SSA and the specificity from 97.9% for Sm to 99.5% for SSB (17). Our results showed agreement from 90.5% for RNP to 100% for SSB, sensitivity from 80.0% for Scl-70 to 100% for SSA, and specificity from 92.3% for dsDNA to 98.3% for Sm for AtheNA Multi-Lyte ANA test system and ELISA (Table 2, Table 4).

ca, kvaliteta vezanja antigena u stabilizirajućoj otopini i postupak ispiranja filtracijom, može doći do lažno negativnog rezultata za specifično antitijelo. Lažno negativni rezultat može prouzročiti pogreške u postavljanju kliničke dijagnoze ili, ukoliko se radi o terapiji, dovesti do pogrešnoga liječenja. Granični se rezultati moraju ili potvrditi kao pozitivni ili odbaciti kao negativni. Važno je znati da se testom *AtheNA Multi-Lyte ANA* može odrediti 9 specifičnih autoantitijela, dok IIF ANA na stanicama HEp-2 ima veću mogućnost detektiranja brojnih antigena. Razumijevanje razlika u metodama važno je za tumačenje rezultata u kliničkom kontekstu.

Zaključak

Testovi *AtheNA Multi-Lyte ANA* i IIF ANA pokazuju dobru podudarnost kao i usporedba rezultata s pojedinačnim ELISA u sistemskim autoimunim bolestima. Test *AtheNA Multi-Lyte ANA* omogućava brzu identifikaciju klinički relevantnih autoantitijela u sistemskim autoimunim bolestima bez potrebe daljnjeg testiranja. Za laboratorij je učinkovita, jednostavna za rad i omogućava izradu velikoga broja autoantitijela istovremeno iz jednog uzorka. Važno je znati može li se reći za testove da se dobro podudaraju ili ne, ali bez kliničke evaluacije koja uključuje bolesnike s potvrđenom dijagnozom za autoimunu bolest i one iz zdrave populacije, vrijednosti testa su nepotpune.

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We compared AtheNA Multi-Lyte ANA test with single-antigen ELISA and found good correlation between all tested parameters (Spearman R from 0.612 to 0.924 and $P < 0.001$) (Table 3). A high concordance was obtained with the study that used the same technology, multiplex bead base and ELISA (17-21). Nifli et al. compared results of ELISA and AtheNA methods for SSA, SSB, Sm, RNP, Scl-70 and the correlation was significant (kappa values from 0.347 to 0.764, $P < 0.001$), but no correlation was established for cytoplasmic antigen Jo-1. Despite the latter, results indicate that AtheNA Multi-Lyte ANA could replace single-antigen ELISA for the measurement of specific autoantibodies (20). In the present study, two methods correlated significantly for all autoantibodies (kappa value from 0.834 for SSA to 0.507 for centromere B). Shovman et al. compared the results obtained for ANA in healthy donors, using ELISA and AtheNA Multi-Lyte ANA and demonstrated high concordance rate of 99%, with 97.7% rate found for ANA determined on requested samples provided by other laboratories. They concluded that AtheNA Multi-Lyte ANA test system is a sensitive screening method for ANA (18). The ELISA method for ANA detection could, however, miss some antigens because antigens were prepared from HEp-2 cells and were defined with a limited number of specific antigens.

Comparing single sample results for specific antigens, we found some discrepant results (Table 4). All discrepant results except three serum samples for autoantibody to Scl-70 antigen and four for dsDNA antigen were near bordeline positive or negative results. The assays possess a separate cut off value which allow comparison of obtained results. The discrepant results for three ELISA positive results for Scl-70 and four AtheNA Multi-Lyte ANA negative results for dsDNA can be explained by the source of antigens, presentation of antigens, reaction conditions of these two methods, diferences in test methodology, diferences in recombinant or purified antigens, their binding capacity and concentration in secondary antibodies and fluorescent conjugates (20, 22). The test condition including ionic strengths and cut off determination may account for differences in test results between assays. Scl-70 and dsDNA antigens were conjugated by a covalent bound to an individual set of microspheres through its surface-functional carboxyl groups. If the coupling procedure, activation, concentration of microspheres or quality of coupling antigen stabilizing solution and washing by filtration are not balanced, we can lose antigens and results will be a negative. Discrepant negative results present a problem because potentially relevant antibodies may be missed. Bordeline results must be confirmed or refuted by use of an independent method and retesting on a new serum sample during a month period. It is important to note that the AtheNA Multi-Lyte ANA test is composed of nine individual analytes while IIF ANA test is composed

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of numerous components present in HEp-2 cells. The difference in assays must be understood, and results from multiplex assay should always be interpreted within the clinical context.

Conclusion

The AtheNA Multi-Lyte ANA test system showed good agreement with IIF ANA and excellent results in comparison with the conventional single-antigen ELISA methods for sera with defined systemic autoimmune disease. The AtheNA Multi-Lyte ANA test supports the immediate identification of autoantibodies of clinical significance in serum samples without additional testing. For laboratory, it is more efficient, easy-to-use, and a high-throughput technique. It is important to note that the assays can be expressed in terms of agreement rates and discrepancies but, without proper clinical evaluation including population with confirmed autoimmune disease and healthy population, the value of the data obtained is rather limited.

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