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Simultaneous comprehensive multiplex autoantibody analysis for rapidly progressive glomerulonephritis

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Abstract

Rapidly progressive glomerulonephritis (RPGN) is mainly caused by anti-glomerular basement membrane (GBM) antibody-mediated glomerulonephritis, immune-complex or anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides and leads to rapid loss of renal function. Detection of ANCA and autoantibodies (autoAbs) to GBM and dsDNA enables early diagnosis and appropriate treatment of RPGN aiding in preventing end-stage renal disease.

Determination of ANCA on neutrophils (ANCA) as well as autoAbs to myeloperoxidase (MPO-ANCA), proteinase 3 (PR3-ANCA), GBM, and dsDNA was performed by the novel multiplex CytoBead technology combining cell- and microbead-based autoAb analyses by automated indirect immunofluorescence (IIF). Forty patients with granulomatosis with polyangiitis (GPA), 48 with microscopic polyangiitis (MPA), 2 with eosinophilic GPA, 42 with systemic lupus erythematosus (SLE), 43 with Goodpasture syndrome (GPS), 57 with infectious diseases (INF), and 55 healthy subjects (HS) were analyzed and findings compared with classical single testing.

The CytoBead assay revealed for GPA, MPA, GPS, and SLE the following diagnostic sensitivities and for HS and INF the corresponding specificities: PR3-ANCA, 85.0% and 100.0%; MPO-ANCA, 77.1% and 99.1%; anti-GBM autoAb, 88.4% and 96.4%; anti-dsDNA autoAb, 83.3% and 97.3%; ANCA, 91.1% and 99.1%, respectively. Agreement with classical enzyme-linked immunosorbent assay and IIF was very good for anti-GBM autoAb, MPO-ANCA, PR3-ANCA, and ANCA, respectively. Anti-dsDNA autoAb comparative analysis demonstrated fair agreement only and a significant difference ($P = 0.0001$).

The CytoBead technology provides a unique multiplex reaction environment for simultaneous RPGN-specific autoAb testing. CytoBead RPGN assay is a promising alternative to time-consuming single parameter analysis and, thus, is well suited for emergency situations.

Abbreviations: AAV = ANCA-associated vasculitis, ANCA = anti-neutrophil cytoplasmic antibody, autoAb = autoantibody, CV = coefficient of variation, EGPA = eosinophilic granulomatosis with polyangiitis, ELISA = enzyme-linked immunosorbent assay, ethN = ethanol-fixed neutrophils, GPA = granulomatosis with polyangiitis, GPS = Goodpasture syndrome, HS = healthy subjects, IIF = indirect immunofluorescence, INF = infectious diseases, MFI = median fluorescence intensity, MPA = microscopic polyangiitis, ROC = receiver operating characteristic, RPGN = rapidly progressive glomerulonephritis, RT = room temperature, SLE = systemic lupus erythematosus, TIF = tagged image file.

Keywords: anti-neutrophil cytoplasmic antibody, digital fluorescence, immunoassay, microbead, rapidly progressive glomerulonephritis

1. Introduction

Rapidly progressive glomerulonephritis (RPGN) is a kidney syndrome clinically characterized by rapid decline of renal function, microscopic hematuria, mild (or non-nephrotic) proteinuria, and active urinary sediment. In patients with RPGN, the glomerular filtration rate decreases over a short period of time ranging in general from a few days to 3 months.[1] Light and electron microscopy analysis reveals glomerular crescent formation as the main histopathological finding in RPGN.[2,3] Specific autoantibody (autoAb) testing is an integral part of the serological diagnosis of RPGN and enables appropriate treatment to avoid progression to end-stage renal disease.[4,5]

From a pathological point of view taking into account the presence of autoAbs, RPGN can be stratified into 3 major groups: anti-glomerular basement membrane (GBM) autoAb disease (type I), immune complex disease (type II), and pauci-immune disease (type III).[6,7] Of note, a proper classification is difficult and many RPGN cases remain idiopathic.

Type I is caused by the deposition of autoAbs interacting with the noncollagenous region of the type IV collagen α_3 chain of GBM. When additional lung involvement occurs, this anti-GBM

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autoAb RPGN with pulmonary hemorrhage is named Goodpasture syndrome (GPS). RPGN patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) are classified as type III or pauci-immune because immune deposits are absent or scanty. Type III RPGN accounts for more than 50% of all RPGNs, especially in older ages. Of note, roughly 10% to 30% of patients with anti-GBM autoAb positivity demonstrate ANCA additionally, indicating a more progressive disease.[4,8–11]

Around 30% to 40% of patients suffering from RPGN have immune-complex disease due to the presence of systemic autoimmune rheumatic disease in particular systemic lupus erythematosus (SLE). Thus, patients suffering from SLE should be checked for renal involvement, because early detection and following appropriate treatment improves the renal outcome. Furthermore, 5% to 25% of patients with immune complex glomerulonephritis show ANCA positivity.[12–15]

Approximately 50% of RPGN patients suffer from pauci-immune disease whereas 80% to 90% of them have elevated ANCA levels. Of note, patients suffering from AAV, particularly those with progressive granulomatosis with polyangiitis (GPA) show renal involvement in most cases (70–77%).[4,16–18] Moreover, almost all patients suffering from other AAV like microscopic polyangiitis (MPA) show renal involvement.[4,19–21]

Patients with RPGN alone or those with additional pulmonary hemorrhage require immediate diagnosis and treatment due to the life-threatening prognosis.[22] Since clinical symptoms do not allow an appropriate differential diagnosis, fast analysis of above-mentioned autoAbs plays a pivotal role. For adequate ANCA testing, as a fact, the international consensus statement requires indirect immunofluorescence (IIF) on ethanol-fixed human neutrophils (ethN) confirmed by specific immunoassays for autoAbs to proteinase 3 (PR3-ANCA) and myeloperoxidase (MPO-ANCA).[18,23–26] All in all, up to 5 different tests with varying assay techniques should be performed to achieve a complete serological workup of patients with RPGN. Thus, a multiplex autoAb analysis combining these different techniques should be the method of choice.[27] To date, only the CytoBead technology enables such multiplex quantitative autoAb testing by digital IIF and automated IIF pattern interpretation.[28–30]

Consequently, a multiplex CytoBead assay was developed to determine ANCA on neutrophils, MPO-ANCA, PR3-ANCA, and autoAbs to GBM (anti-GBM) and dsDNA (anti-dsDNA) simultaneously in patients and controls. Findings were compared with classical testing by single assays.

2. Methods

2.1. Patients and controls

In total, 287 patients and controls, including 40 patients suffering from GPA, 48 from MPA, 2 from eosinophilic GPA (EGPA), 42 from SLE, 43 from GPS, 57 from infectious diseases (INF), and 55 healthy subjects (HS), were included into the study (Table 1). Specific laboratory tests for PR3- and MPO-ANCA as well as anti-GBM autoAb determination were performed in the Center of San Carlo Borromeo Hospital (Milan/Italy), where the patients were diagnosed and followed-up. Further, renal biopsies were performed on all GPS patients. Anti-dsDNA analysis was performed in Germany, Brandenburg-Technical University Cottbus-Senftenberg.

The diagnosis of clinical entities has been performed according to specific classification criteria.[25,26,31] The study was approved by the local ethics committee of Milano (CE Milano-Area B 8/7/2014, CS-GA-115565) and complies with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects and/or animals. Aliquots were stored at −20°C until used to detect antibody reactivity.

Table 1

Characteristics of patients and controls.

Diagnosis	N (F/M)	Median age (IQR)
ANCA associated vasculitis		
Granulomatosis with polyangiitis	40 (12/28)	58 (26)
Microscopic polyangiits	48 (30/18)	56 (22)
Eosinophilic granulomatosis with polyangiitis	2 (0/2)	73 (3)
Systemic autoimmune rheumatic disease		
Systemic lupus erythematosus	42 (35/7)	43 (18)
Infectious diseases		
HCV infection	25 (12/13)	74 (26)
HBV infection	3 (1/2)	33 (21)
EBV infection	3 (0/3)	26 (9)
Anti-mycoplasma positive	1 (0/1)	5 (0)
Undefined infectious disease	25 (16/9)	74 (22)
Rapidly progressive glomerulonephritis		
Goodpasture syndrome/anti-GBM nephritis	43 (20/23)	68 (22)
Healthy subjects	55 (5/50)	46 (14)

ANCA = anti-neutrophil cytoplasmic antibody, EBV = Epstein-Barr virus, F = female, GBM = glomerular basement membrane, HBV = hepatitis B virus, HCV = hepatitis C virus, IQR = interquartile range, M = male.

2.2. Determination of autoAb with antigen-specific ELISA

Specific autoAb to GBM (for GPS), PR3-ANCA (for GPA), and MPO-ANCA (for MPA) as well as dsDNA (for SLE) were detected using commercially available antigen-specific enzyme-linked immunosorbent assay (ELISA) (Phadia [Uppsala/Sweden], EuroDiagnostica [Lundavägen/Sweden] and GA Generic Assays GmbH [Dahlewitz/Berlin/Germany]). Assay performance was done according to the instructions of the manufacturers.

2.3. Detection of ANCA by IIF

The detection of ANCA (ethanol and formalin fixed) was performed by using a commercially available assay according to the instructions of the manufacturer.

2.4. Multiparametric autoAb detection with CytoBead technology

ANCA on ethN, MPO-ANCA, PR3-ANCA, and autoAbs to GMB and dsDNA were determined simultaneously by the CytoBead RPGN assay employing ethN from freshly donated human blood along with PR3 (human native), GBM antigen (human recombinant; type IV collagen α_3 chain,[32–34] MPO (human native), and dsDNA (salmon native) covalently linked to fluorescent microbeads of 9 and 15 μm (PolyAn, Berlin, Germany; excitation 610 nm/emission 690 nm) as autoantigenic targets on glass slides with compartmented wells (Fig. 1).[35] Fixation of neutrophils and immobilization of autoantigen-coated fluorescent beads was performed as described elsewhere.[30]

A serum dilution of 1/20 was incubated 30 min at room temperature (RT). After washing, secondary antihuman IgG conjugated to AlexaFluor488 in combination with 4′,6-diami-

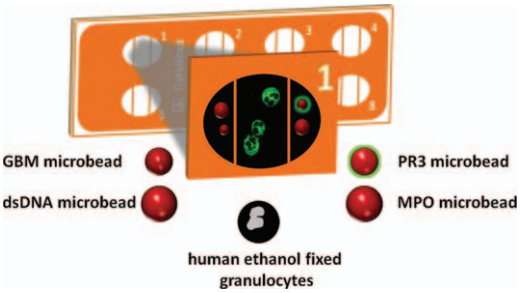

Figure 1. CytoBead RPGN glass slide for multiplex autoantibody (autoAb) analysis. Neutrophils isolated from donated human blood are fixed by ethanol in the middle compartment of each well for the detection of classical anti-neutrophil cytoplasmic antibodies (ANCA). Proteinase (PR3) and myeloperoxidase (MPO) are coated covalently on fluorescent microbeads of 9 and 15 μm, respectively, and immobilized on the right well compartment. Likewise, glomerular basement membrane (GBM) antigen and dsDNA are covalently linked to aforementioned microbead populations, respectively, and coated onto the left well compartment. The figure shows the reactivity pattern of a PR3-ANCA positive sample with a cytoplasmic fluorescence ANCA pattern on the neutrophils and a positive rim-like fluorescence signal on PR3-coated microbeads.

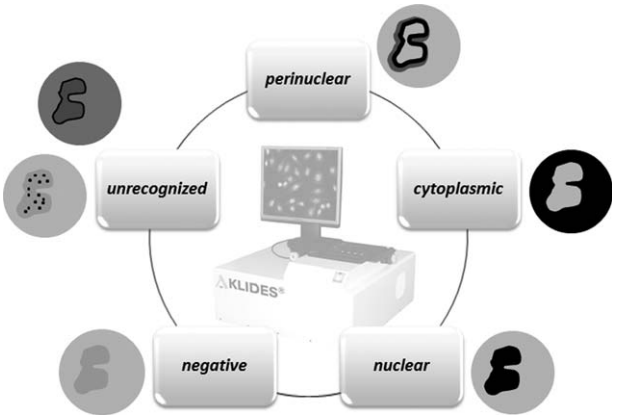

Figure 2. Anti-neutrophil cytoplasmic antibody (ANCA)-pattern recognition of indirect immunofluorescence (IIF) images on neutrophils by AKLIDES. The automated IIF interpretation system AKLIDES classifies cytoplasmic, perinuclear, nuclear, unrecognized, and negative fluorescence ANCA on ethanol-fixed neutrophils according to international guidelines[22,36,37,45].

dino-2-phenylindole was added and incubated for 30 min at RT, followed by a second washing step. Subsequently, slides were mounted either for automated evaluation with the IIF interpretation system AKLIDES (Medipan, Berlin, Germany) or manual analysis using a standard fluorescence microscope with green fluorescence channel (Carl Zeiss, Jena, Germany) as described elsewhere.[36–40] Fluorescence patterns of ethN were evaluated according to the international guidelines by AR.[25,26]

The final automated read-out was expressed in international units per mL (IU/mL) for PR3-ANCA, MPO-ANCA, and anti-dsDNA antibodies calibrated against the international reference sera of the Centers for Disease Control and Prevention (Serum 16 and 15, Atlanta, GA) and Wo/80, respectively. Furthermore, anti-GBM levels were determined in units per mL (U/mL) in accordance with internal standard material. All digital IIF images were captured and stored in lossless compressed tagged image file (TIF) format as reported earlier. Automated pattern recognition of ANCA IIF images was conducted as described elsewhere (Fig. 2).[30,37,38]

2.5. Analysis of coefficient of variation

Coefficient of variation (CV) was analyzed by using in-house reference sera. Each reference serum was diluted 3 times in order to get high, moderate, and low antibody concentrations.

Intra-assay CV was determined by 8 measurements for each serum while inter-assay CV was assessed by analyzing 8 determinations for each serum on 5 different days in accordance with the clinical and laboratory standards institute protocol EP15-A2. Microbead and ethN fluorescence analysis for the determination of median fluorescence intensity (MFI) was performed using AKLIDES.

2.6. Statistical analysis

The statistical analysis was performed by using MedCalc software (Version 12.4.0; MedCalc, Mariakerke, Belgium). Kruskal–Wallis test was used to compare unpaired cohorts. P values < 0.05 were considered statistically significant. Specific cut-off data were determined using receiver operating characteristic (ROC) curve analysis. Furthermore, inter-rater agreement (Cohen's kappa [κ]) and McNemar test were used for testing concordance values of CytoBead RPGN and routine test as well as clinical findings.

3. Results

3.1. Analysis of assay parameters

The cut-off determination of each parameter analyzed by the CytoBead RPGN was performed by ROC curve analysis employing patients with GPA, MPA, GPS, and SLE as disease groups for PR3-ANCA, MPO-ANCA, anti-GBM, and anti-dsDNA, respectively, and disease controls as well as HS as negative groups. Cut-offs were determined to match at least 95.0% specificity and revealed for PR3-ANCA, MPO-ANCA, anti-GBM, and anti-dsDNA values of 5 IU/mL, 5 IU/mL, 7 U/mL, and 10 IU/mL, respectively (Fig. 3). For ANCA testing by IIF pattern analysis on ethN, 70 MFI was used as cut-off as determined in an earlier study.[30]

Coefficients of variation (CVs) were determined using intra- and inter-assay datasets as described in "Methods" section. Intra- and inter-assay CVs of specific autoAb testing to PR3-ANCA, MPO-ANCA, GBM, and dsDNA showed values <15.0% which is in line with food and drug administration criteria Q2B (Table 2). Furthermore, intra-assay CVs of neutrophil cytoplasmic, perinuclear, and nuclear fluorescence staining patterns were also below 15.0% whereas corresponding inter-assay CVs exceeded 20.0% for 2/9 serum samples but were <23.0% altogether (Table 2).

3.2. ANCA and specific autoAb analysis by CytoBead RPGN

In total, 287 serum samples (Table 1) were analyzed for the presence of ANCA on ethN, PR3-ANCA, MPO-ANCA, anti-GBM, and anti-dsDNA by CytoBead technology. All 5 parameter levels demonstrated significant differences in the patient and control cohorts tested (Kruskal–Wallis test, $P < 0.005$, respectively; Fig. 4).

IIF testing on ethN by CytoBead RPGN revealed prevalences between 77.1% and 100.0% in patients with SLE, GPA, MPA, and EGPA (Table 3). In contrast, HS and INF demonstrated prevalences of 0.0% and 1.7% only, respectively. Interestingly,

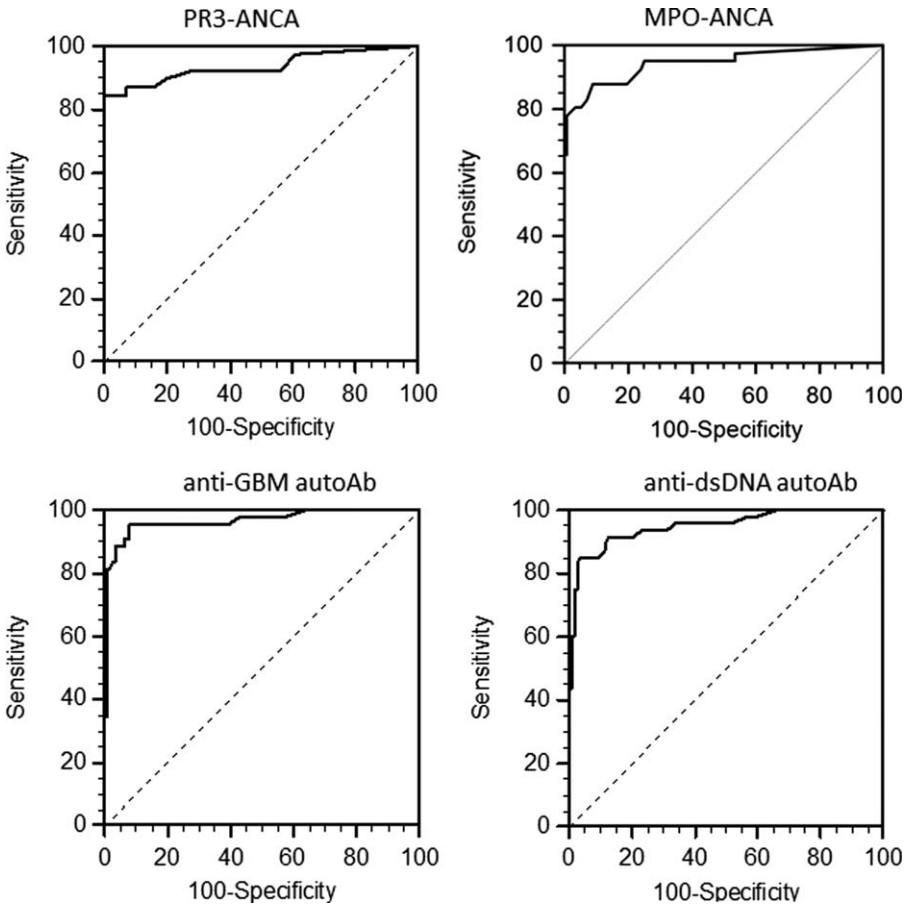

Figure 3. Receiver operating characteristic curve analysis for the determination of cut-off values of proteinase 3 (PR3)-anti-neutrophil cytoplasmic antibody (ANCA), myeloperoxidase (MPO)-ANCA, anti-glomerular basement membrane (GBM), and anti-dsDNA autoantibodies (autoAb).

Table 2

Intra- and inter-assay variation of (A) ANCA and (B) PR3-ANCA, MPO-ANCA, anti-GBM, and anti-dsDNA autoantibodies (autoAb) by CytoBead RPGN.

(A) ANCA	Cytoplasmic ANCA			Perinuclear ANCA		
Serum titer	High	Moderate	Low	High	Moderate	Low
Intra-assay CV, %	10.8	4.0	12.1	3.3	1.8	3.1
Inter-assay CV, %	19.9	19.9	22.3	15.1	12.1	7.8

	Nuclear autoAb		
Serum titer	High	Moderate	Low
Intra-assay CV, %	6.8	10.9	13.2
Inter-assay CV, %	12.6	19.2	22.7

(B) Specific autoAb	PR3-ANCA			MPO-ANCA		
Serum titer	High	Moderate	Low	High	Moderate	Low
Intra-assay CV, %	0.8	6.6	13.4	0.4	14.9	14.4
Inter-assay CV, %	2.2	1.4	8.5	9.5	10.1	13.1

	Anti-dsDNA autoAb			Anti-GBM autoAb		
Serum titer	High	Moderate	Low	High	Moderate	Low
Intra-assay CV, %	6.1	14.5	9.8	4.1	2.7	14.2
Inter-assay CV, %	10.8	10.1	10.0	13.4	14.5	14.4

ANCA = anti-neutrophil cytoplasmic antibody, CV = coefficient of variation, GBM = glomerular basement membrane, MPO = myeloperoxidase, PR3 = proteinase 3, RPGN = rapidly progressive glomerulonephritis.

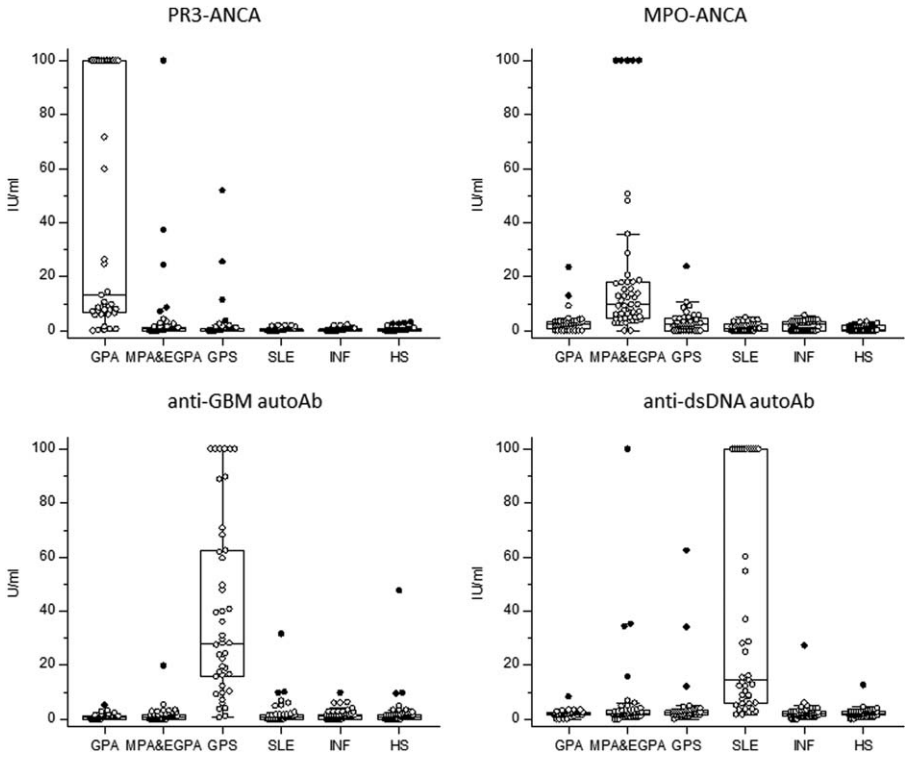

Figure 4. Detection of autoantibodies (autoAb) to dsDNA, glomerular basement membrane (GBM), myeloperoxidase (MPO), and proteinase 3 (PR3)-anti-neutrophil cytoplasmic antibody (ANCA) by CytoBead RPGN. Indirect immunofluorescence findings were interpreted on the automated interpretation system AKLIDES. EGPA = eosinophilic granulomatosis with polyangiitis, GPA = granulomatosis with polyangiitis, GPS = Goodpasture syndrome, HS = healthy subjects, INF = infectious diseases, MPA = microscopic polyangiitis, RPGN = rapidly progressive glomerulonephritis, SLE = systemic lupus erythematosus.

14/43 (32.6%) patients with GPS showed ANCA reactivity, of which 10 were confirmed by specific autoAb testing.

Specific autoAb testing by CytoBead RPGN demonstrated prevalences of 85.0%, 77.1%, 88.4%, and 83.3% for PR3-ANCA, MPO-ANCA, anti-GBM, and anti-dsDNA in patients with GPA, MPA, GPS, and SLE, respectively (Table 3). In contrast, HS showed prevalences between 0.0% and 5.4% and INF between 0.0% and 3.5% regarding these specific autoAbs.

3.3. Comparison of classical ANCA analysis with CytoBead RPGN testing

The performance of the multiplex CytoBead RPGN was further evaluated by comparison with classical ANCA testing by IIF and specific autoAb determination by solid-phase immunoassays. Consequently, 287 serum samples were analyzed with CytoBead RPGN and with classical tests employing IIF with ethanol and formalin-fixed neutrophils as well as specific ELISA (Table 4). Inter-rater agreement showed very good agreement for anti-GBM autoAb, PR3-ANCA, and MPO-ANCA and fair agreement for anti-dsDNA autoAbs (Table 4).

As a fact, comparison of anti-dsDNA autoAb testing revealed 52/287 (18.1%) discrepant results. Thus, whereas anti-GBM autoAb, PR3-ANCA, and MPO-ANCA comparative analysis of both methods did not reveal significant differences (McNemar test, $P > 0.05$, respectively), testing of anti-dsDNA autoAb did (difference 12.54%, 95% confidence interval: 7.94–15.62, $P <$

Table 3

Prevalence of ANCA by IIF, PR3-ANCA, MPO-ANCA, anti-GBM, and anti-dsDNA autoantibodies by CytoBead RPGN, classical testing employing ELISA and IIF on ethanol and formalin-fixed neutrophils, in 287 patients and controls.

	CytoBead RPGN, %						Classical tests, %					
Cohorts	**PR3-ANCA**	**MPO-ANCA**	**Anti-GBM**	**Anti-dsDNA**	**ANCA**	**Bead + IIF**	**PR3 ELISA**	**MPO ELISA**	**GBM ELISA**	**dsDNA ELISA**	**ANCA**	**ELISA + IIF**
GPA (n = 40)	**34 (85.0)**	3 (7.5)	0 (0.0)	1 (2.5)	35 (87.5)	33 (82.5)	**32 (80.0)**	3 (7.5)	0 (0.0)	7 (17.5)	39 (97.5)	35 (87.5)
MPA (n = 48)	4 (8.3)	**37 (77.1)**	0 (0.0)	6 (12.5)	45 (93.8)	38 (79.2)	4 (8.3)	**42 (87.5)**	0 (0.0)	17 (35.4)	47 (97.9)	44 (91.7)
EGPA (n = 2)	0 (0.0)	2 (100.0)	1 (50.0)	0 (0.0)	2 (100.0)	2 (100.0)	0 (0.0)	2 (100.0)	0 (0.0)	1 (50.0)	2 (100.0)	2 (100.0)
GPS (n = 43)	3 (6.9)	7 (16.3)	**38 (88.4)**	3 (6.9)	14 (32.6)	13 (30.2)	1 (2.3)	7 (16.3)	**41 (95.3)**	6 (14.0)	9 (56.25)	10 (23.3)
SLE (n = 42)	0 (0.0)	0 (0.0)	3 (6.25)	**35 (83.3)**	34 (81.0)	31 (73.8)	0 (0.0)	0 (0.0)	0 (0.0)	**36 (85.7)**	34 (81.0)	27 (64.3)
INF (n = 57)	0 (0.0)	1 (1.7)	1 (1.7)	2 (3.5)	1 (1.7)	1 (1.7)	0 (0.0)	0 (0.0)	0 (0.0)	14 (24.6)	1 (1.7)	2 (3.5)
HS (n = 55)	0 (0.0)	0 (0.0)	3 (5.4)	1 (1.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (5.4)	0 (0.0)	0 (0.0)

ANCA = anti-neutrophil cytoplasmic antibody, EGPA = eosinophilic granulomatosis with polyangiitis, ELISA = enzyme-linked immunosorbent assays, GBM = glomerular basement membrane, GPA = granulomatosis with polyangiitis, GPS = Goodpasture syndrome, HS = healthy subjects, IIF = indirect immunofluorescence, INF = infectious diseases, MPA = microscopic polyangiitis, MPO = myeloperoxidase, PR3 = proteinase 3, RPGN = rapidly progressive glomerulonephritis, SLE = systemic lupus erythematosus.

Table 4

Comparison of ANCA by IIF, PR3-ANCA, MPO-ANCA, anti-GBM, and anti-dsDNA autoantibody (autoAb) analysis by CytoBead RPGN and classical testing employing ELISA in 287 patients and controls.

		CytoBead RPGN						
		PR3-ANCA				MPO-ANCA		
		Negative	Positive			Negative	Positive	
PR3-ANCA ELISA	Negative	243	7		MPO-ANCA ELISA	230	9	
	Positive	3	34			7	41	
				∑ 287				∑ 287
Weighted kappa	0.852				Weighted kappa	0.803		
Standard error	0.046				Standard error	0.047		
95% CI	0.762–0.941				95% CI	0.710–0.896		

		CytoBead RPGN						
		Anti-GBM autoAb				Anti-dsDNA autoAb		
		Negative	Positive			Negative	Positive	
GBM ELISA	Negative	237	9		dsDNA ELISA	195	8	
	Positive	4	37			44	40	
				∑ 287				∑ 287
Weighted kappa	0.824				Weighted kappa	0.500		
Standard error	0.047				Standard error	0.057		
95% CI	0.731–0.917				95% CI	0.387–0.612		

ANCA = anti-neutrophil cytoplasmic antibody, CI = confidence interval, ELISA = enzyme-linked immunosorbent assays, GBM = glomerular basement membrane, IIF = indirect immunofluorescence, MPO = myeloperoxidase, PR3 = proteinase 3, RPGN = rapidly progressive glomerulonephritis.

0.0001). The 8 positive anti-dsDNA autoAb findings by CytoBead RPGN and negative with solid-phase immunoassays belong to patients with SLE (3, 37.5%), GPS (2, 25%), INF (1, 12.5%), and HS (1, 12.5%). Only 3/44 (6.8%) sera with negative anti-dsDNA autoAbs by CytoBead RPGN and positive test results by solid-phase assays are from patients with SLE. The further discrepant 41 disease and healthy controls of this particular group contain 13 patients with INF and 11 with MPA. Of note, CytoBead RPGN revealed only 1 false positive each regarding the respective discrepant control patient groups.

Findings of the AKLIDES software for automated pattern recognition showed very good agreement ($\kappa = 0.885$) with manual reading by an expert in ANCA diagnostics (Table 5).

4. Discussion

A patient with RPGN suffering from selective or combined kidney and lung disease is classified as clinical emergency case and has to be treated very fast to avoid fatal progression of disease. In particular, patients with GPS are identified to have the worst prognosis of all RPGN patients without the correct medical treatment.[2] As a matter of fact, in such critical settings, autoAb analysis is crucial for diagnosing patients adequately. Thus, determination of anti-GBM autoAbs for GPS, ANCA for ANCA-associated RPGN, and autoAb to dsDNA are recommended for an appropriate serological diagnosis of RPGN.[34,41–45] However, the analysis of all these parameters requires different techniques and is time consuming. Hence, there is a need for 1 step multiplex analysis addressing the urgent need for express RPGN serology.

In this context, the present study evaluated the multiparametric assay CytoBead RPGN for the simultaneous analysis of ANCA on ethN, MPO-ANCA, PR3-ANCA, and autoAbs to GBM and dsDNA.

With regard to ANCA pattern interpretation, the majority of patterns interpreted by AKLIDES were in line with the findings of a human expert. The AKLIDES system gives the result "unrecognized," when the pattern is not a classical cytoplasmic, perinuclear, or nuclear one, thus further interpretation by an expert is possible using the saved TIF images afterwards.[30,46] In that case, the "unrecognized" pattern could be declared as atypical or classified as perinuclear, cytoplasmic, or nuclear. The very good concordance of automated and manually obtained fluorescence patterns in this study might provide the basis for a

Table 5

Comparison of automated and manual ANCA pattern evaluation by the AKLIDES system and an expert in ANCA diagnostics.

	AKLIDES system				
Manual	**Perinuclear**	**Cytoplasmic**	**Nuclear**	**Unrecognized**	**Negative**
Perinuclear	**36**	1	3	5	0
Cytoplasmic	3	**47**	2	3	2
Nuclear	0	0	**29**	0	0
Unrecognized	5	1	1	**10**	0
Negative	0	0	0	0	**145**

Fluorescence patterns were categorized according to international guidelines (25, 26, 30, and 46). The inter-rater agreement (Cohen's kappa) of the different evaluation strategies was very good 0.885. Weighted kappa: 0.885; standard error: 0.023; 95% CI: 0.841–0.93.
CI = confidence interval.

Table 6

Comparison of diagnostic performance parameters of autoantibodies detected by CytoBead RPGN in patients with GPA, MPA, anti-GBM nephritis/GPS, and SLE.

	Disease	Prevalence, %	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	+LR	−LR
PR3-ANCA	GPA	13.9	85.0 (70.2–94.3)	97.2 (94.3–98.9)	82.9 (67.9–92.9)	97.6 (94.8–99.1)	30.0 (14.28–63.0)	0.2 (0.1–0.3)
MPO-ANCA	EGPA and MPA	17.4	78.0 (64.0–88.5)	95.4 (91.9–97.7)	78.0 (64.0–88.5)	95.4 (91.9–97.7)	16.8 (9.3–30.5)	0.2 (0.1–0.4)
Anti-GBM	GPS	15.0	88.4 (74.9–96.1)	96.7 (93.6–98.6)	82.6 (68.6–92.2)	97.9 (95.2–99.3)	26.95 (13.5–53.7)	0.1 (0.1–0.3)
Anti-dsDNA	SLE	15.2	83.3 (68.6–93.0)	94.5 (90.7–97.0)	72.9 (58.2–84.7)	96.9 (93.8–98.8)	15.06 (8.7–26.0)	0.2 (0.1–0.4)

PR3-ANCA, MPO-ANCA, autoAb to anti-GBM, and anti-dsDNA were determined by CytoBead RPGN. Disease prevalence reflects the prevalence of the given disease in the whole study cohort.
−LR = negative likelihood ratio, +LR = positive likelihood ratio, ANCA = anti-neutrophil cytoplasmic antibody, CI = confidence interval, GBM = glomerular basement membrane, GPA = granulomatosis with polyangiitis, GPS = Goodpasture syndrome, MPA = microscopic polyangiitis, MPO = myeloperoxidase, NPV = negative predictive value, PPV = positive predictive value, PR3 = proteinase 3, RPGN = rapidly progressive glomerulonephritis, SLE = systemic lupus erythematosus.

successful introduction of automated ANCA reading into routine diagnostics of RPGN and AAV. The present data are corroborated by recent reports demonstrating the usefulness of the novel pattern recognition algorithms used by the automated interpretation system AKLIDES for ANCA reading.[30,46]

Digital fluorescence enables standardization and quantitative end-point titer reading for autoAb testing for the first time in autoimmune diagnostics and, thus, offers new exciting perspectives with regard to automation and multiplexing.[36–39]

For rapid simultaneous multiparametric quantitative determination of several specific RPGN-specific autoAbs, antigen-coated fluorescent microbeads, and lot-specific calibration curves fitted by asymmetric 5-parameter equations were employed.[28–30] Of note, obtained diagnostic parameters for MPO-ANCA, PR3-ANCA, anti-GBM, and anti-dsDNA autoAbs in MPA, GPA, GPS, and SLE matched literature data adequately (Table 6).[41,42,45,47–50] In fact, anti-dsDNA antibody detection by CytoBead RPGN showed a diagnostic sensitivity of 83.3% with a diagnostic specificity of 97.3% in patients with SLE compared to a routine ELISA used by default in the nephrology department demonstrating 85.7% diagnostic sensitivity along with a poorer diagnostic specificity of only 84.8%.[47] Indeed, comparative anti-dsDNA autoAb analysis revealed a significant difference for both techniques and a fair agreement only. As a fact, CytoBead RPGN determined significantly less false-positive anti-dsDNA autoAb findings compared to ELISA.

The better specificity of the CytoBead RPGN anti-dsDNA autoAb detection might be a result of the specific covalent coupling strategy of the complete and nonfragmented dsDNA molecules to the activated microbead surface.[6] In addition, the agreement of anti-GBM autoAb, PR3-ANCA, and MPO-ANCA testing by CytoBead RPGN with classical corresponding ELISA was very good.

These findings support the assumption that the CytoBead RPGN is an attractive alternative to classical single testing regarding the analysis of all diagnostic relevant antibody specificities for the correct serological diagnosis of RPGN variants.

Furthermore, CytoBead RPGN is characterized by a very low incubation time of 1 h in contrast to current single routine tests. Hence, treatment of RPGN patients could start much earlier by addressing the most critical limiting factor for patients well-being or even survival.

Another characteristic of the CytoBead RPGN assay is its flexibility with regard to the autoimmune laboratory. Indeed, the assay can be run manually and interpreted by a conventional fluorescent microscope for qualitative autoAb assessment. Thus, emergency diagnostics for RPGN can be run without the need of expensive equipment by retaining all the benefits of multiplex autoAb analysis.

Our study has certain limitations. HS are not age and gender matched with the study cohorts. Further, the relevant prevalences of the disease cohorts do probably not reflect the actual prevalences in most nephrology departments. In order to obtain quantitative data for further evaluation, an automated interpretation system would have been necessary.

5. Conclusions

The multiparametric CytoBead technology is a unique combination of screening and confirmatory autoAb testing for RPGN serology and might be a very promising alternative to classical time-consuming single parameter testing. In the present study, CytoBead RPGN demonstrated satisfactory assay performance of the multiplex reaction environment for the detection of ANCA, PR3-ANCA, MPO-ANCA, autoAb to dsDNA and GBM addressing the need for emergency testing in routine autoimmune laboratories.

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