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Comparison of second- and third-generation anti-cyclic citrullinated peptide antibodies assays for detecting rheumatoid arthritis

Laurence Lutteri [a,*], Michel Malaise [b], Jean-Paul Chapelle [a]

[a] Laboratory of Clinical Chemistry, TSI 2, 5th floor, CHU, University of Liège, Sart-Tilman, 4000 Liège, Belgium
[b] Department of Rheumatology, CHU, University of Liège, Belgium

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Abstract

Background: The aims of the present study were to compare the diagnostic and clinical value of seven commercially available assays for second-generation anti-cyclic citrullinated peptide (CCP2) antibodies, anti-keratin antibodies (AKA) and rheumatoid factor (RF) determination in patients with rheumatoid arthritis (RA), and to check the potential advantages of a third-generation anti-CCP (CCP3) antibodies assay.
Methods: Serum samples from 120 RA patients and from 170 controls were used to determine the sensitivity, the specificity, the positive (PPV) and negative predictive values (NPV) of CCP2 and CCP3 antibodies, AKA and RF assays. The respective performances of these tests were compared using a receiver-operating characteristics (ROC) curves methodology.
Results: We found no significant differences in sensitivity and specificity between the tested anti-CCP assays. The CCP3 antibodies assay we evaluated was not significantly more sensitive than those of the second generation. Compared with RF technique, all anti-CCP assays showed better specificity, but lower sensitivity. In contrast, while of equivalent specificity, they proved to be more sensitive than AKA in the discrimination of RA from other rheumatic diseases.
Conclusions: Anti-CCP antibodies determination proved to be a powerful diagnostic tool, especially in RA patients with negative AKA or RF. The investigated CCP3 antibodies assay had no better diagnostic performances than the second-generation assays.
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Keywords: Anti-cyclic citrullinated peptide antibodies; Anti-keratin antibodies; Rheumatoid arthritis; Rheumatoid factor; Sensitivity; Specificity

1. Introduction

Rheumatoid arthritis (RA) is the most frequent systemic autoimmune disease. The prevalence is about 1% worldwide and RA is occurring more frequently in women than in men (2.5:1 ratio). RA is a chronic inflammatory disease characterized by joint inflammation, progressive erosions and cartilage destruction [1]. During the first stages of the disease, the 1987 revised criteria of the American College of Rheumatology (ACR) [2] are rarely met.

The presence of rheumatoid factor (RF) is included as the laboratory criterion of RA in the ACR criteria. RF consists of antibodies directed against the Fc portion of the IgG immunoglobulins and can be detected in 60–80% of patients affected by RA. However RF is not specific for this disease and can also be detected in other rheumatic disorders, especially connective tissue diseases, other chronic inflammatory diseases, infections and even in healthy individuals, particularly the elderly [3]. The most routinely used laboratory tests for RF determination are agglutination methods based on the ability of RF to agglutinate sheep red cells, latex or polystyrene particles coated with IgG. They detect the IgM isotype. The development of an enzyme linked immunosorbent assay (ELISA) for RF permits the detection and quantitative measurement of RF in various immunoglobulin classes.

Abbreviations: CCP, Cyclic citrullinated peptide; AKA, Anti-keratin antibodies; RF, Rheumatoid factor; RA, Rheumatoid arthritis; CCP2, Second-generation anti-cyclic citrullinated peptide; CCP3, Third-generation anti-cyclic citrullinated peptide; PPV, Positive predictive value; NPV, Negative predictive value; ACR, American College of Rheumatology; APF, Anti-perinuclear factor antibodies; MCV, Anti-mutated citrullinated vimentin; AUC, Area under the curve.

* Corresponding author. Tel.: +32 43668822; fax: +32 43668823.
E-mail address: laurence.lutteri@chu.ulg.ac.be (L. Lutteri).

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These methods however lack sensitivity and specificity. The need for more efficient diagnostic tests for selecting the optimal treatment in early stages of the disease, encouraged the search towards more discriminant serological markers [4,5]. A recently discovered family of auto-antibodies, directed at citrullinated epitopes carried on proteins/peptides, may represent more sensitive and specific markers of RA. Anti-perinuclear factor antibodies (APF), anti-Sa and anti-mutated citrullinated vimentin (MCV), anti-keratin antibodies (AKA) and anti-cyclic citrullinated peptide (CCP) antibodies have an adequate specificity for supporting clinical and therapeutic decisions [6].

The aim of this study was to evaluate different anti-CCP assays of second and third generation by ELISA, immuno-enzymofluorimetry and line immunoassay in order to compare the technical and diagnostic performances of these tests. The results of the anti-CCP assays were also compared with the results obtained for AKA by immunofluorescence and for IgM-RF by nephelometry using coated particles.

2. Materials and methods

2.1. Study population

We addressed ourselves to 290 patients who attended the Department of Rheumatology of the University Hospital of Liège. All patients had a standardized interview, a general physical examination and a rheumatological examination. One hundred and twenty patients were diagnosed as RA according to the ACR criteria [2] and constituted the RA study population [35 men (29%) and 85 women (71%); median age: 56 years (range: 20–79 years)]. The control group consisted of 170 non-RA patients [66 men (39%) and 104 women (61%); median age: 51 years (range: 16–86 years)]. Among the non-RA patients were 37 patients with autoimmune disorders including systemic lupus erythematosus ($n=13$), Sjögren's syndrome ($n=5$), polymyositis or dermatomyositis ($n=3$), antiphospholipid primary syndrome ($n=1$), CREST syndrome ($n=1$), Crohn's disease ($n=5$), scleroderma ($n=3$), ulcerative colitis ($n=4$) and vasculitis ($n=2$); 41 patients with other inflammatory rheumatic disorders including arthritis ($n=6$), ankylosing spondylitis ($n=4$), psoriatic arthritis ($n=25$) and rhizomelic pseudopolyarthritis ($n=6$); 49 patients with other non-inflammatory rheumatic disorders included osteoarthritis ($n=35$), fibromyalgia ($n=4$), osteoporosis ($n=7$) and Paget disease ($n=3$); 9 patients with Lyme disease and 34 patients with other entities (gout, sarcoidosis, asthma...).

Blood was taken to a serum tube from an antecubital vein. All serum specimens were aliquoted within 5 h following sampling; they were stored at −80 °C and thawed only once before being assayed with all the kits investigated.

The study was approved by our hospital ethics committee. Patients' informed consent for the usage of sera was obtained prior to sample collection.

2.2. Methods for biochemical markers determination

2.2.1. Rheumatoid factor

Serum RF concentrations were quantified using the BN II nephelometer (Dade Behring). The reagent contains polystyrene particles coated with antigen–antibody complexes of human IgG/sheep anti-human IgG (Dade Behring Marburg GmbH, Marburg, Germany). The reaction between RF and the coated particles results in a light scatter in the nephelometer of which the intensity is proportional to the content of RF in the serum. The RF content of each serum was determined by comparison to standards of known concentrations. The results are given as IU/ml. Expected values in normal individuals were <9.9 IU/ml.

2.2.2. Anti-keratin antibodies

A semi-quantitative indirect immunofluorescence method [7] was used for the specific detection and titration of AKA. AKA were detected using sections obtained from the middle third of rat (*Rattus* sp.) oesophagus (The Binding Site, Birmingham, UK). Serum samples, diluted to 1/5 in phosphate buffered saline (PBS), pH 7.2, were applied to the tissue and incubated at room temperature for 30 min in a moist chamber. After two washings, fluorescein conjugated rabbit anti-human IgG (H+L) was added and incubated for 30 min, washed and mounted.

AKA positive serum gave distinct laminar or speckled fluorescent staining of the superficial layer (stratum corneum) of the rat oesophagus epithelium. Serum samples with positive fluorescence were subsequently titrated.

Table 1
Main technical characteristics of the nine anti-CCP tests evaluated in this study

Manufacturer	Antigen	Technique	Incubation time (serum, conjugate, substrate, stop), min	No. of calibrators	Conjugate	Substrate	Proposed cut-off	Measuring range
INOVA	CCP 2nd generation	ELISA	30; 30; 30	5	HRP anti-human IgG (goat)	TMB	20 U/ml	0–200 U/ml
Euroimmun	CCP 2nd generation	ELISA	60; 30; 30	5	AP anti-human IgG (mouse)	pNPP	5 U/ml	0–100 U/ml
Axis-Shield Diagnostics	CCP 2nd generation	ELISA	60; 30; 30	5	AP anti-human IgG (murine)	Phenolphtalein monophosphate	5 U/ml	0–100 U/ml
Euro-Diagnostica	CCP 2nd generation	ELISA	60; 60; 30	5	HRP anti-human IgG (goat)	TMB	25 U/ml	25–1600 U/ml
Edia Euro-Diagnostica	CCP 2nd generation	ELISA	60; 60; 30	5	AP anti-human IgG (goat)	PMP	5 U/ml	0–100 U/ml
Pharmacia Diagnostics	CCP 2nd generation	Immuno-enzymo-fluorimetry	180 max.	6	β-galactosidase anti-IgG (mouse)	4-Methylumbelliferyl-β-D-galactoside	10 EliA U/ml	0.4–340 EliA U/ml
INOVA	CCP 3rd generation	ELISA	30; 30; 30	5	HRP anti-human IgG (goat)	TMB	20 U/ml	0–250 U/ml
Triturus	Synthetic citrullinated peptides	ELISA	30;15;15	6	HRP anti-human IgG (goat)	TMB	15 U/ml	0–300 U/ml
Innogenetics	Filaggrin-derived citrullinated peptides	Line immunoassay	60; 30; 30; 10–30	/	AP anti-human IgG (goat)	5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium in dimethyl formamide	/	/

HRP = Horseradish peroxidase; AP = alkaline phosphatase; TMB = 3,3′,5,5′ tetra-methylbenzidine; pNPP = *p*-Nitrophenylphosphate; PMP = phenolphtalein monophosphate.

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Table 2
Comparison of RF and anti-CCP titres in the RA and non-RA patients investigated

	RA group (n=120)	Non-RA group (n=170)
	Median (standard deviation)	Median (standard deviation)
Euro-Diagnostica CCP (U/ml)	89 (3243)	25 (327)
Edia CCP (U/ml)	25.2 (140)	0.65 (1.3)
Euroimmun CCP (U/ml)	44 (190)	0 (36)
Axis-Shield CCP (U/ml)	28.2 (274)	0.4 (34)
INOVA CCP (U/ml)	89 (591)	20 (136)
INOVA 3 CCP (U/ml)	322.84 (1254)	15.62 (162)
Pharmacia CCP (EU/ml)	111 (1824)	1.6 (104)
Triturus CCP (U/ml)	30 (471)	2.1 (229)
RF by nephelometry (IU/ml)	100 (393)	10.9 (4046)

Median and standard deviation in the two groups are given for each technique.

2.2.3. Anti-cyclic citrullinated peptides antibodies

Anti-CCP antibodies were determined by five commercially second-generation ELISA's following the manufacturers' instructions: Quanta Lite CCP (INOVA, San Diego, USA); anti-CCP ELISA (Euroimmun, Lübeck, Germany); Immunoscan RA Mark 2 (Euro-Diagnostica, Arnhem, The Netherlands); Diastat anti-CCP (Axis-Shield Diagnostics, Dundee, UK) and Edia anti-CCP (Euro-Diagnostica, Arnhem, The Netherlands). All samples were tested in the EliA CCP automated test system (Pharmacia Diagnostics, Freiburg, Germany). While all kits make use of the same second-generation synthetic citrullinated peptide, the other components are different (e.g.: each kit includes its own standard reagents). The third-generation anti-CCP tested in this study was Quanta Lite CCP3 (INOVA, San Diego, USA).

We also used a new ELISA which detects antibodies to specific synthetic citrullinated peptides (RA/CP Detect first generation, Triturus, AutoImmune Diagnostic Assays, Bad Kreuznach, Germany) and a non-commercial line immunoassay for the detection of auto-antibodies against filaggrin-derived citrullinated peptides (Inno-Lia RA, Innogenetics, Ghent, Belgium) [8].

The technical details of the investigated anti-CCP assays are summarized in Table 1.

2.3. Statistical analysis

The various statistical tests used in this study (Mann–Whitney test, Chi-square test, Spearman's correlation, inter-rater agreement Kappa, ROC curves) were performed using MedCalc® for Windows (MedCalc Software, Mariakerke, Belgium).

3. Results

We evaluated for each kit the intraassay imprecision using a sample with high concentrations of antibodies from an RA patient and a negative specimen from a healthy subject. Interassay imprecision was determined using the negative and positive internal controls of the respective kits. The CVs ranged from 8.8 to 17.9%.

Serum levels of anti-CCP and RF antibodies were compared in RA (n=120) and non-RA (n=170) patients for all techniques (Table 2). Antibody concentrations were significantly higher in the RA subjects than in the control group ($p<0.0001$).

The cut-off values recommended by the respective manufacturers were used to determine the sensitivity and specificity of the different assays for diagnosing RA. We found positive AKA results in 45.5% of the RA patients, positive anti-CCP antibodies in 57.7 to 68.5% according to the different kits and positive RF values by nephelometry in 77.9% (Table 3). 23 to 44% of AKA negative and 13 to 19% of RF negative RA patients yielded positive results with the various CCP assays.

Among these, the new CCP3 assay did not prove to be significantly better in sensitivity than CCP2 techniques. The RF test was the most efficient for pointing out RA patients.

Anti-CCP antibodies (86.2 to 97.7%) and AKA (97.7%) demonstrated the best specificity for RA, with no significant differences being recorded between the different kits. The specificity of the RF assay was lower. The PPV of the anti-CCP assays (80.6 to 96.2%) and AKA (94.6%) were markedly higher than that of the RF method (77.9%). In contrast, no significant differences were observed for NPV between the various assays.

Despite the high specificity of anti-CCP, false positive results for at least one of the kits were recorded in 14 patients with: Crest syndrome (n=1, very high anti-CCP antibodies concentration), Sjögren's syndrome (n=1), systemic lupus erythematosus (n=2), rhizomelic pseudopolyarthritis (n=2), oligoarthritis (n=3), osteoporosis (n=1), Paget (n=1), Sapho (n=1) and ulcerative colitis diseases (n=2).

Table 3
Comparison of sensitivity, specificity, positive and negative predictive values of anti-CCP, AKA and RF tests in differentiating RA and non-RA patients, using the manufacturer's and ROC curves derived cut-off values

	Euro-Diagnostica	Edia	Euroimmun	Axis-Shield	INOVA	INOVA 3	Pharmacia	Triturus	Innogenetics	RF Nephelometry	Anti-keratin
Manufacturer's cut-off	25 [a]	5 [a]	5 [a]	5 [a]	20 [a]	20 [a]	10 [b]	15 [a]	/	10.9 [a]	/
Sensitivity (%) with manufacturer's cut-off	66.7	66.7	66.7	66.7	66.7	68.5	68.5	57.7	68.5	77.9	45.5
Specificity (%) with manufacturer's cut-off	92.2	97	97.7	97.7	94.4	95.3	96.5	88.8	86.2	78.6	97.7
PPV	87.7	94.3	96.2	96.2	90.9	92.6	94.3	84.8	80.6	77.9	94.6
NPV	76.9	76.6	77.1	77.1	77.3	78.1	78.3	65.8	76.5	79.5	69.4
Cut-off based on ROC curves	25 [a]	3.2 [a]	2.32 [a]	4.4 [a]	20 [a]	15.62 [a]	3 [b]	4.5 [a]	/	55 [a]	/
Sensitivity (%) with cut-off based on ROC curves	66.7	81.8	73.3	71	66.7	74.6	85.5	68	/	78.3	/
Specificity (%) with cut-off based on ROC curves	92.2	94.7	95.7	97.8	94.4	93.3	86.6	82	/	88.9	/

[a] U/ml.
[b] EU/ml.

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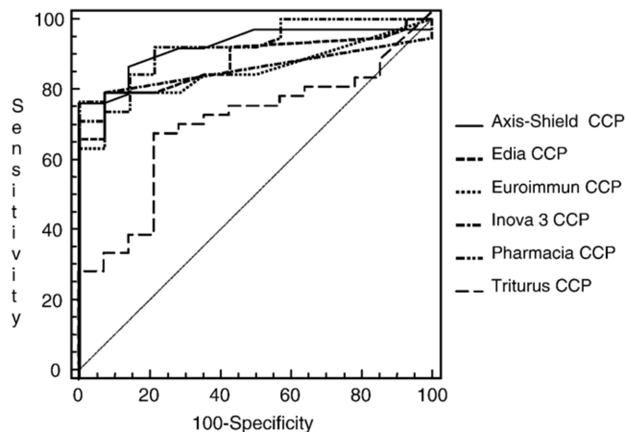

Fig. 1. Receiver-operating characteristic curves of Axis-Shield CCP, Edia CCP, Euroimmun CCP, INOVA 3 CCP, Pharmacia CCP and Triturus CCP used to discriminate rheumatoid arthritis and non-rheumatoid arthritis.

The performance characteristics of the tests for discriminating between RA and non-RA subjects were evaluated using ROC curves analysis (Fig. 1). The areas under the curve (AUC) ranged between 0.73 and 0.92. We found no significant differences between the different assays, except for anti-CCP Triturus which showed a lower AUC value (0.73). The ROC curves were also used to determine the optimum cut-off points (with the best sensitivity and specificity). As can be seen in Table 3, adapting the cut-off value proposed in the kit insert may be desirable for most assays. New cut-offs improve sensitivity without important loss in specificity.

In spite of differences between antigens (2nd-generation versus 3rd-generation versus specific synthetic citrullinated peptides) or techniques (ELISA, immuno-enzymo-fluorimetry), most anti-CCP assays were well correlated ($p<0.001$), with correlation coefficient ranging from 0.59 to 0.96 (Table 4). The worst correlation coefficients were observed between anti-CCP assays and RF technique. Semi-quantitative tests (AKA and Innogenetics CCP) were compared by means of an inter-rater agreement Kappa test; correlation coefficients ranged from 0.47 to 0.82.

4. Discussion

Anti-citrullinated peptides antibodies were first described by Schellekens et al. [9]. These components were determined by ELISA based on highly purified synthetic linear peptides containing modified arginine residues (citrulline) serving as antigen. This test proved to be very specific in the diagnosis of RA [10–12] but could detect only 48% of RA patients. To increase the sensitivity (up to 69%), peptides were modified to a structure in which citrulline is optimally exposed for antibody binding [10]. These cyclic variants of citrullinated peptides were used as antigen in the first-generation CCP test, an assay whose sensitivity was higher than that of APF and AKA assays, but lower than that of RF [6]. The currently available second-generation tests use highly reactive peptides, identified from dedicated libraries of citrullinated peptides, screened with RA sera [13]. Recent studies indicate that the CCP2 test is very specific (98%) and has a sensitivity of 80% for RA [9,10,12,14–21]. Recently, a third-generation peptide was developed by INOVA and claimed to be a new more sensitive and specific ELISA.

The study at hand is the first to compare eleven techniques: six commercially available second-generation anti-CCP antibody tests, a line immunoassay for the detection of autoantibodies against filaggrin-derived citrullinated peptides, an ELISA which detects antibodies to specific synthetic citrullinated peptides, a new third-generation anti-CCP, an immunofluorimetric assay for anti-keratin antibodies and an immunonephelometric assay for RF.

We showed in this study that anti-CCP antibodies are highly specific markers for diagnosing RA; their specificities ranged from 89 to 98%, confirming results obtained by others [4,10,15,22,23].

The sensitivity of the first-generation anti-CCP test was low (49%, data not shown); with the second-generation anti-CCP assay, sensitivity increases up to 70%. No significant differences in terms of sensitivity and specificity could be observed between the different anti-CCP tests probably because they use the same second-generation synthetic citrullinated peptide. The other components were nevertheless different and each kit included its own standard reagents; this probably explains the discrepancies. In particular, the RA/CP Detect ELISA had lower performances than the other kits. However it must be noted that after completion of our study, the Triturus company decided to introduce on the market an improved ELISA using new synthetic citrullinated peptides. The sensitivity of the third-generation anti-CCP (68.5%) was somewhat higher than INOVA second-generation anti-CCP but similar to some other CCP2 assays; its specificity (95.3%) was comparable to that of the second-

Table 4
Results of Spearman correlation (rho) between the different quantitative anti-CCP assays

	INOVA CCP	INOVA 3 CCP	Euroimmun CCP	Euro-Diagnostica CCP	Axis-Shield CCP	Edia CCP	Pharmacia CCP	Triturus CCP
INOVA 3 CCP	0.86							
Euroimmun CCP	0.84	0.76						
Euro-Diagnostica CCP	0.96	0.83	0.8					
Axis-Shield CCP	0.83	0.71	0.81	0.8				
Edia CCP	0.86	0.82	0.93	0.62	0.93			
Pharmacia CCP	0.82	0.66	0.71	0.79	0.8	0.87		
Triturus CCP	0.75	0.74	0.77	0.71	0.67	0.7	0.59	
IgM-RF nephelometry	0.67	0.64	0.63	0.65	0.59	0.73	0.58	0.48

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generation anti-CCP assays. Anti-CCP assays were in general well correlated but correlations were not close enough to allow direct comparison of laboratory results obtained with different kits in the same patient.

The high specificity of anti-CCP assays did not exclude some false positive results; some patients with various non-RA diseases (Crest syndrome, Sjögren's syndrome, systemic lupus erythematosus, oligoarthritis,...) demonstrated high anti-CCP titres. Similar results were previously reported in patients with psoriatic arthritis, systemic lupus erythematosus and inflammatory arthritis [24].

Confirming previous works [16,19], our study indicates that anti-CCP antibodies demonstrate the highest predictive values (PPV between 84.3 and 96.2% and NPV between 65.8 and 78.3%).

The diagnostic value of AKA has been assessed by several researchers [7,25,26]. AKA had a diagnostic specificity comparable to anti-CCP but its low sensitivity (46%) restricts its usefulness in diagnosing RA. Our data indicate that reactivity to CCP did not encompass the complete spectrum of AKA reactivity (2 RA patients were AKA positive but anti-CCP negative). This might be related to the fact that citrulline has been demonstrated as an essential constituent of the antigenic determinants recognized by AKA, but that individual RA patients respond to different antigenic determinants [27]. A potential advantage of quantitative values of anti-CCP antibodies in comparison with semi-quantitative results of AKA could be to monitor the treatment of RA patients. For the moment, however, the relation disease activity/antibody concentration is still questioned and few data have been published on this topic.

RF is the most commonly used serological criterion to diagnose RA. However, although high titres of IgM-RF are relatively specific for diagnosing RA, RF is also present in other diseases and even in a healthy population. In this study, we found that IgM-RF is the most sensitive marker and can be found in 77.9% of patients with RA. It must be noted however that the sensitivity of RF for RA diagnosed by ACR criteria cannot really be directly compared to that of other tests which are not included in the diagnostic criteria. IgM-RF was found less disease specific. Like others [21,24], we observed an additional value of anti-CCP as compared to IgM-RF: in 13–19% of RF negative RA patients, anti-CCP were positive.

In conclusion, the anti-CCP antibody test is useful for the diagnosis of RA. This assay has a high specificity, similar to AKA, and a better sensitivity than this test. IgM-RF, included as laboratory criteria of RA in the ACR, has, with the restriction cited above, the best sensitivity; it can however be found in cases other than RA.

This study also shows that CCP ELISA provided the best combination of sensitivity and specificity for detecting RA with no significant difference between the different anti-CCP assays from second generation whatever the technique used (ELISA, immuno-enzymo-fluorimetry and line immunoassay). The third-generation anti-CCP assay from INOVA didn't demonstrate a significant advantage in terms of sensitivity and specificity over the various kits of second generation.

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