

## Anti-CCP: History and its usefulness

M. HEROLD, V. BOESER, E. RUSSE, & W. KLOTZ

*Rheumatology Unit, Clinical Department of General Internal Medicine, Innsbruck Medical University, A-6020 Innsbruck, Austria*

### Abstract

Antibodies directed to cyclic citrullinated peptides (anti-CCP) are highly specific for rheumatoid arthritis (RA) and can easily be detected in sera by using commercially available immunoassays. The second version of the anti-CCP test (anti-CCP2) demonstrated high specificity (89–98%) and good sensitivity (41–88%) for RA. Commercially available ELISA methods from three different companies are on the market. All three CCP2 assays show similar results as all CCP2 assays use the same antigen-coated plates. This study was an evaluation of a new automated method for the determination of anti-CCP2 in a routine laboratory setting. Five hundred and forty three serum samples were tested for anti-CCP2 within normal routine diagnostic using a commercially available ELISA and retested with a prelaunch version of a new and fully-automated method (*EliA™*). The results were comparable. The new automated assay is easy to use and demonstrated a diagnostic sensitivity of 80% and specificity of 97%.

**Keywords:** *Anti-CCP, automated determination, rheumatoid arthritis, enzyme-linked immunosorbent assays*

**Abbreviations:** *ACR, American College of Rheumatology; AFA, antifilaggrin antibodies; AKA, antikeratin antibodies; anti-CCP, anti cyclic citrullinated peptide; APF, antiperinuclear factor; ELISA, enzyme-linked immunosorbent assays; RA, rheumatoid arthritis; RF, rheumatoid factor*

### Introduction

Rheumatoid arthritis (RA) is the most common inflammatory joint disease with a prevalence between 0.5 and 1% worldwide (Lawrence et al. 1998). The diagnosis is mainly based on clinical signs and symptoms according to latest recommendations in 1987 (Arnett et al. 1988). Within these seven diagnostic criterias, detection of IgM rheumatoid factor (RF) in serum is the only recommended laboratory marker. Though IgM-RF is measured in most studies and is the most often ordered autoantibody test in laboratory diagnosis, its specificity for diagnosing RA is limited. At very low levels IgM-RF is present in sera of most people. High concentrations of IgM-RF are not only detected in RA but also in other conditions with polyclonal stimuli to B-cells like viral and bacterial infections or chronic inflammations other than RA. The need for a better laboratory marker with a higher disease-related specificity and sensitivity was always evident.

A very specific antibody for RA was first described by Nienhuis and Mandema (1964) which is called antiperinuclear factor (APF) as these antibodies bound to constituents of the keratohyaline granules which are located close to the nucleus of buccal mucosa cells of adult people. The APF showed an acceptable sensitivity and compared to RF a much higher specificity (Hoet et al. 1991). Nevertheless, the test was never used for routine testing in case of practical inconvenience. The test required carefully selected buccal mucosa cells which were differentiated enough to contain the perinuclear factor. Experienced laboratory technicians were needed to perform the test based on indirect immunofluorescence and to recognize the different immunofluorescent patterns.

In 1979, another group of RA specific antibodies, the so-called antikeratin antibodies (AKA) was described (Young et al. 1979). The AKA bind to keratin-like structures in the cornified layer of stratum

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Correspondence: M. Herold, Innsbruck Medical University, Clinical Department of Internal Medicine, Anichstrasse 35, A-6020 Innsbruck, Austria. Tel: 43 512 504 23321. Fax: 43 512 504 24213. E-mail: manfred.herold@uibk.ac.at

corneum. These autoantibodies could easily be detected by indirect immunofluorescence on rat oesophagus cryostat sections and some laboratories started to offer AKA measurement within normal routine diagnostics. The specificity for RA was comparable to APF (Hoet and van Venrooij 1992) and it could be demonstrated that AKA also precede the onset of RA (Kurki et al. 1992).

It was shown that APF and AKA target the same antigen (Sebbag et al. 1995) identified as the epithelial protein filaggrin (filament aggregating protein) which is involved in the organization of cytoskeletal structures. Several filaggrin subunits result from proteolytical cleavage of profilaggrin during differentiation of epithelial cells. Profilaggrin is first dephosphorylated and about 20% of the basic arginine residues are converted into neutral citrulline residues by the enzyme peptidylarginine deiminase. It has been documented that the modification to a citrulline containing protein is essential for the autoantigenicity of filaggrin (Schellekens et al. 1998) and citrullinated filaggrin is the antigen targeted by APF and AKA. Immunoblotting assays and enzyme-linked immunosorbent assays (ELISA) using filaggrin purified from human skin as antigen detected antifilaggrin antibodies (AFA) in 42% of patients with RA with a specificity of 99% (Vincent et al. 1998). Using *in vitro* deiminated recombinant filaggrin increased the number of positive RA sera to 52% (Aho et al. 1999). Sensitivity and specificity of AFA seemed to be dependent on the method of filaggrin purification and on the difficulty to obtain antigen preparations with reproducible citrullin content. This technical problem could be solved by using isolated citrullinated filaggrin peptides as antigen but it became apparent that sera from different RA patients show different patterns of reactivity indicating the heterogeneity of the autoimmune response. Using a combination of nine citrullinated peptide variants a sensitivity of 76% and a specificity of 96% were detected. The test was

improved and simplified using a cyclic variant of a citrullinated peptide called anti-cyclic citrullinated peptide (CCP) test. This first anti-CCP test revealed a high diagnostic specificity of about 98% and a sensitivity around 70% (Boekel et al. 2002). To improve, the anti-CCP test peptides from dedicated libraries of citrullinated peptides were tested with RA sera to select the most reactive species. The selected peptides were made cyclic to ensure the exposure of the antigenic citrulline structure. This investigation resulted in the second generation anti-CCP assay which is sold worldwide as CCP2 assay (Vossenaar and Venrooij 2004).

Till the end of 2003, there were three distributors (Euro-Diagnostica, Arnhem, The Netherlands; Axis-Shield Diagnostics Ltd., Dundee, UK; Inova Diagnostics Inc., San Diego, CA) selling the CCP2 test. CCP2 is a brand name and all commercial available immunoassays use the same antigen and the same antigen-coated plates but differ in working procedures. The results of all three assays are similar (Dubucquoi et al. 2004, Garcia-Berrocal et al. 2005). The anti-CCP test has a comparable sensitivity but a much higher specificity than the IgM-RF test in diagnosing RA (Table I) and it is not surprising that anti-CCP testing has increasing acceptance in laboratory medicine. In November 2004, Sweden Diagnostics affiliate of Pharmacia Diagnostics AB Freiburg, Germany, launched the first fully-automated testing system for CCP antibodies called *ELLA*<sup>TM</sup> CCP. This assay was tested in a prelaunch version on samples collected consecutively from patients coming to the Rheumatology Unit of our hospital.

## Materials and methods

Five hundred and forty three serum samples (395 female, 148 male) from outpatients of our hospital were tested for anti-CCP routinely by a commercially available ELISA (Inova Diagnostics Inc.) and retested

Table I. Sensitivity and specificity of anti-CCP and IgM-RF antibody measurements in sera of patients with RA.

Source	Anti-CCP		IgM-Rf	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Schellekens et al. (2000)	68	98	54	91
Goldbach-M. et al. (2000)	50	90	66	87
Bizzaro et al. (2001)	41	98	62	84
Bas et al. (2002)	68	96	75	74
Jansen et al. (2002)	43	98	50	93
Lee and Schur (2003)	66	90	72	80
Suzuki et al. (2003)	88	89	70	82
Zeng et al. (2003)	47	97	59	
Saraux et al. (2003)	47	93	41	
Araki et al. (2004)	81	92		
Dubucquoi et al. (2004)	65	96	60	70
Girelli et al. (2004)	71	95	91	31
Vallbracht et al. (2004)	64	97	66	82

bulkwise by *EliA*<sup>TM</sup>-CCP (Sweden Diagnostics affiliate of Pharmacia Diagnostics AB Freiburg), an automated test system for the determination of anti-CCP. The tests of both companies correspond to the so-called anti-CCP2 test. Results were classified according to the recommendations of the manufacturers. The ELISA from Inova categorizes negative (<20 U/ml), weak positive (20–39 U/ml), moderate positive (40–59 U/ml) and strong positive (>60 U/ml). *EliA*<sup>TM</sup> from Pharmacia gave a preliminary recommendation with negative (<7 U/ml), borderline positive (7–10 U/ml) and positive (>10 U/ml).

Serum samples were drawn from patients consecutively coming to our hospital with joint problems. CCP was routinely tested in all patients with clinical suspect for RA or other inflammatory joint diseases and from patients with prediagnosed RA but not yet tested on anti-CCP. After detection of anti-CCP with both methods, patients' files were screened to evaluate the diagnosis. RA was diagnosed according to the revised ACR criteria (Arnett et al. 1998).

## Results

Overall, there was a good agreement in the results of both assays (Table II). Among the positive samples detected by ELISA, 12 samples were negative by *EliA*<sup>TM</sup>. These samples were from patients who were diagnosed in 1 case as possible RA, in 4 cases as non-RA and in 7 cases a diagnose was not known.

Thirteen out of 88 positive samples tested by *EliA*<sup>TM</sup> were negative in the Inova assay. Twelve of these samples were only weak positive with concentrations below 29 U/ml and only one sample from a patient diagnosed as non-RA was strong positive. The 12 weak positive samples were in 8 cases from patients with RA (Table III). Three samples were moderate to strong positive in ELISA but negative in *EliA*<sup>TM</sup>. None of these three samples was from a patient with definite or possible RA.

In sera of 66 patients with definite RA (Table IV), the ELISA and *EliA*<sup>TM</sup> revealed 45 (68%) and 53 (80%) positive results, respectively. In the group of patients

Table II. Number of serum samples with negative, borderline or weak positive and positive results comparing ELISA and *EliA*<sup>TM</sup>.

ELISA Inova	Automated ( <i>EliA</i> <sup>TM</sup> Pharmacia)		
	Negative	Borderline	Positive
Negative	442	1	13
Weak positive	9	0	7
Moderate positive	2	0	5
Strong positive	1	0	63

The ELISA from Inova categorizes negative <20 U/ml, weak positive 20–39 U, moderate positive 40–59 U and strong positive (>60 U). The automated *EliA*<sup>TM</sup> from Sweden Diagnostics categorizes negative <7 U, borderline 7–10 U and positive >10 U.

Table III. Incidence of definite RA in those patients whose sera revealed different results in both anti-CCP2 assays.

ELISA	<i>EliA</i> <sup>TM</sup>	n	Rheumatoid arthritis			
			Yes	Possible	No	Unknown
Negative	Positive	13	8	1	2	2
Positive	Negative	3	–	–	1	2

with possible RA, anti-CCP results were identical. In the non-RA group results were almost identical with 11 positive and 317 negative samples in ELISA versus 10 positive and 318 negative samples in *EliA*<sup>TM</sup>. The diagnostic sensitivity and specificity were 68.2 and 96.9% in the ELISA and 80.3 and 97.0% in the *EliA*<sup>TM</sup>, respectively.

Sera were collected from 16 patients at two different times and analysed blinded. All results were highly reproducible in both assay systems though all samples were analyzed in different assay runs.

## Discussion

Antibodies directed to citrullinated proteins and their association to RA are well known since several years. These antibodies occur in patients with RA and can even be detected in sera years before onset of disease symptoms (Kurki et al. 1992, Rantapaa-Dahlqvist et al. 2003, Nielen et al. 2004). The development of synthetic citrullinated proteins provided the use as antigens in immunoassays. Since test improvement by making cyclic variants of citrullinated peptides as antigens in commercially available ELISAs, there is an increasing interest in testing these antibodies as diagnostic and prognostic markers in patients with RA (Kroot et al. 2000, Schellekens et al. 2000, Vasishta 2002, Bas et al. 2003, Meyer et al. 2003, Vencovsky et al. 2003, Berglin et al. 2004, Forslind et al. 2004, van Gaalen et al. 2004a,b, Kastbom et al. 2004, Lopez-Hoyos 2004, Maddali Bonghi et al. 2004, van Venrooij et al. 2004, Lindqvist et al. 2005, Nielen et al. 2005, Raza et al. 2005).

In 2004, there were three commercial second-generation ELISAs for the measurement of anti-CCP antibodies marketed as anti-CCP2 tests. All CCP-2

Table IV. Incidence of positive or negative results of anti-CCP antibodies in serum samples of different patient groups.

Diagnosis	n	ELISA anti-CCP		<i>EliA</i> <sup>TM</sup> anti-CCP	
		Positive	Negative	Positive	Negative
RA	66	45	21	53	13
Possible RA	42	12	30	12	30
Non RA	328	11	317	10	318
Unknown	107	19	88	14	93
Total	543	87	456	89	454

tests use the same second-generation synthetic citrullinated peptide antigen bound to the same surface of a microwell plate. The tests differ in handling procedures and other components of the assay system but reveal similar results. Comparing to one of these three ELISA's, we tested a new and fully automated CCP-2 assay in a prelaunch version. The results were comparable and both assays showed the same specificity. We found some difference between the two assays in sensitivity. This difference is mainly based on 8 samples of RA patients, which were negative in the ELISA and weak positive in the *EliA*<sup>TM</sup>. Comparing to data from literature, the sensitivity of 80% for the automated assay fits to results from anti-CCP2 ELISAs with optimum cut-off values (Garcia-Berrocal et al. 2005).

IgM rheumatoid factor has been commonly used as serological marker of RA. In all publications, anti-CCP demonstrated a higher specificity and a comparable or slightly higher sensitivity than IgM-RF. There is no doubt that anti-CCP is a valuable serum marker for the diagnosis of RA. Different analytical procedures may be one of the reasons that measurement of IgM-RF is still offered in most laboratories but not anti-CCP. IgM-RF is usually measured automatically on common analyzers, which are used in routine laboratory diagnostics. *EliA*<sup>TM</sup> anti-CCP is the first fully automated method for the determination of anti-CCP with all advantages of an automated analytical method like standardized conditions and short hands-on-time.

The new automated immunoassay *EliA*<sup>TM</sup> for measuring anti-CCP is easy to use and shows reliable results with sensitivity and specificity for diagnosing RA comparable to given anti-CCP2 ELISAs on the market.

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