

ORIGINAL ARTICLE

Analytical and diagnostic performance of an automated anti-CCP assay

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Abstract

Aim: Autoantibodies against cyclic citrullinated peptide (anti-CCP) are considered to be a sensitive and specific marker for rheumatoid arthritis (RA). This study evaluated the diagnostic and analytical performances of the automated anti-CCP assay. **Materials and Method:** Sera from 80 patients with established RA, 65 from other rheumatic diseases (non-RA) and 55 from healthy controls were studied using second generation anti-CCP. Rheumatoid factor (RF) was also assayed in each sample, and the results were compared to the anti-CCP findings. Serum pools were used to determine the precision and linearity. **Results:** At a cut-off of 7.4 U/ml for anti-CCP, the sensitivity and specificity for RA were 65% and 98% respectively. RF had a sensitivity of 58% and a lower specificity of 93 % than anti-CCP. **Conclusion:** The high specificity of the assay suggests that anti-CCP is useful in the diagnosis of rheumatoid arthritis and in our cohort of study population anti-CCP exhibits a better diagnostic value than RF. A considerable proportion (28%) of RF-negative RA patients were anti-CCP positive. Based on analytical performance of the assay, we conclude that full automation and high throughput features of AxSYM makes it an ideal platform for routine testing of anti-CCP.

Key words: rheumatoid arthritis, systemic lupus erythematosus, psoriatic arthritis, anti-CCP, rheumatoid factor

INTRODUCTION

Rheumatoid arthritis (RA) is the most common autoimmune disease, affecting approximately 1% of the world's population. RA is a systemic inflammatory disease characterised by chronic and erosive polyarthritis caused by abnormal growth of synovial tissue or pannus, and causes irreversible joint disability. The diagnosis of RA depends primarily on clinical manifestations, but laboratory results are helpful in the differential diagnosis and disease management. Historically, rheumatoid factor (RF) has long been the serological indicator for RA. Rheumatoid factor (RF) is an antibody directed against the Fc region of IgG that has been used as a diagnostic marker for RA. However, it is non-specific and may be present in healthy elderly persons or in patients with other autoimmune and infectious diseases.¹ Other RA-associated autoantibodies known to be specific for RA include anti-perinuclear

factor (APF) and anti-keratin antibodies (AKA); both recognize the antigenic protein filaggrin.^{2,3} Despite their high specificity, APF and AKA have not been widely used as markers for rheumatoid arthritis because of technical difficulties in substrate standardization and the subjective nature of the interpretation of the immunofluorescence tests.

Recently it was determined that AKA recognize an epitope that contains citrulline, the deiminated form of arginine, a post-translational modification of the amino acid arginine.⁴ In recent years, many studies on antibodies against cyclic citrullinated peptide (CCP) have demonstrated that these antibodies are highly specific and predictive for RA,⁵ that they can be detected years before onset,⁶ and also that they are associated with joint erosions.⁷ In this study we evaluated the diagnostic and analytical performances of the automated anti-CCP assay.

MATERIALS AND METHOD

We recruited 145 (21 male and 124 female) patients who attended the outpatient rheumatology clinic at University Malaya Medical Centre. 80 patients had definite RA diagnosed according to the criteria of the American College of Rheumatologists.⁸ To provide data on specificity, 65 patients with other rheumatic diseases (49 with systemic lupus erythematosus (SLE) and 16 patients with psoriatic arthritis (PsA)) were included for the study. 55 healthy hospital workers between the ages of 18 to 66 years, who did not suffer from any rheumatological diseases, were also included in this study. They were matched with the study group for age and sex.

Serum samples were collected from all these individuals and stored at -20°C until further analysis. Anti-CCP (second generation) was tested on the automated Abbott AxSYM analyser. Rheumatoid factor was measured in all the samples by Beckman Coulter Immage system. Imprecision was calculated that conform to CLSI documents EP05-A2 (Precision Performance of Clinical Chemistry Devices). Linearity study was performed using the sample with higher concentration of anti-CCP. Samples with anti-CCP concentrations more than 200 were serially diluted with AxSYM negative control and the concentration of the diluted samples were measured. The measured results were plotted on the y-axis vs the known values on the x-axis. The

reportable range was then assessed by drawing the best straight line through the linear portion of the data.

Statistical analysis was performed using SPSS for windows statistical package. Parametric method was used to calculate the reference range for anti-CCP. Receiver operator characteristic analysis was used to calculate the cut off values for optimal sensitivity and specificity. This study was approved by the medical ethics committee of our institution (Institutional Review Board).

RESULTS

Healthy control: The reference interval for anti-CCP was calculated in healthy individuals using the parametric method. The lower limit was observed to be 0.1 U/ml and the upper limit was 1.86 U/ml. Manufacturers' recommendation for the reference interval was 1 to 2.9 U/ml. The reference interval noted in our population was in agreement with the manufacturers' recommendation. None of the controls were noted to have elevated levels of anti-CCP but RF was elevated in 3 of them.

Sensitivity and specificity of anti-CCP

ROC curve analysis was performed to calculate the sensitivity, specificity, positive predictive value and negative predictive value for anti-CCP and RF (Figures 1 and 2). The area under the curve for anti-CCP and RF were 0.82 and

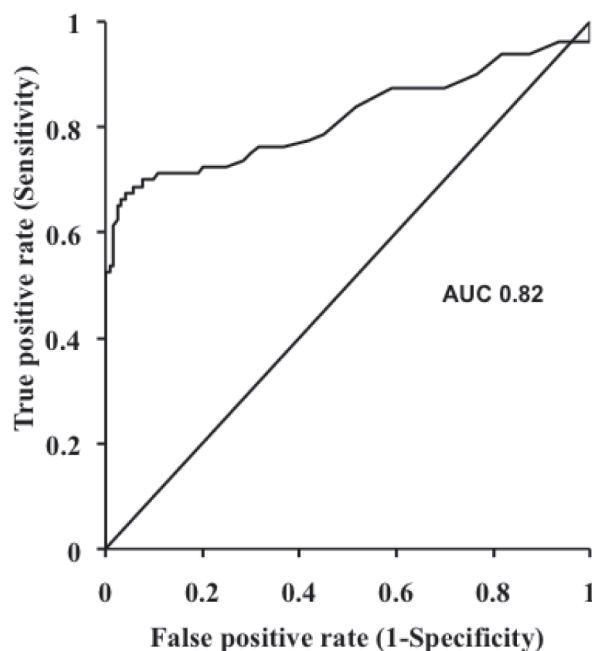


FIG. 1: ROC curve for anti-CCP

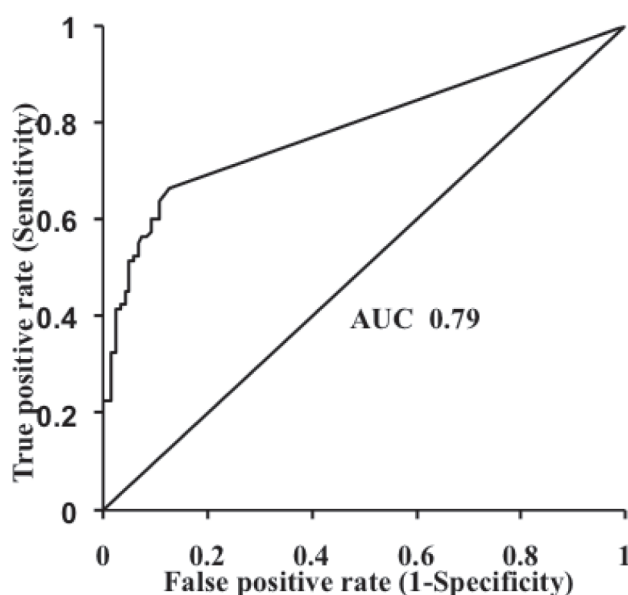


FIG. 2: ROC curve for rheumatoid factor

0.79 respectively. At a cut off value of 7.4 U/ml, anti-CCP had a sensitivity and specificity of 65% and 98% respectively. The PPV and NPV for anti-CCP were 95% and 81% respectively. The sensitivity and specificity for RF at a cut off value of 41 IU/ml were noted to be lower than anti-CCP (58% and 93 %). The PPV and NPV were 84% and 75% respectively (Table 1).

Anti CCP in RA and other rheumatic diseases
Fifty-two RA patients had anti-CCP level >7.4 U/ml (the cut off value) whereas only 43 patients had RF >41 IU/ml. Ten RF-negative patients were noted to be anti-CCP positive. Only three patients with other rheumatic diseases were noted to be positive for anti-CCP (2 SLE patients and 1 PsA patient), whereas seven patients (6 SLE and 1 PsA) had RF values more than the cut-off value (Figure 3).

Anti-CCP concentration

Anti-CCP levels were above the cut-off value of 7.4 U/ml in 52 RA patients. 28 patients had anti -CCP levels less than the cut -off value.

Mean and 2SD anti-CCP level (median) in RA patients were 197.8 ± 351 U/ml (45.7U/ml). In SLE patients and PsA patients the mean \pm 2SD (median) anti-CCP levels were 3.9 ± 4.7 U/ml (2.2 U/ml) and 1.12 ± 0.49 U/ml (1.1 U/ml) respectively.

Precision study

The precision was assessed by analysing samples of two different levels in replicates at two different times of the day for 20 days (n=80). The levels chosen were 7 U/ml and 100 U/ml and the total imprecision were 7.57% and 8.3% respectively. The AxSYM anti-CCP was designed to have a precision <15%.

Linearity study

The analytical range of the test stated by the manufacturer was 1-200 U/ml. Data from our study showed a linear response for concentrations from 6 to 180 U/ml ($Y=0.962x + 2.9727$; $r^2 = 0.9948$) and the linearity hypothesis was accepted (Figure 4).

TABLE 1: Comparison of analytical parameters of anti-CCP and RF

	AUC	Sensitivity	Specificity	PPV	NPV
Anti-CCP	0.82	65%	98%	95%	81 %
RF	0.79	58 %	93 %	84 %	75 %

AUC- area under curve, PPV-positive predictive value, NPV-negative predictive value

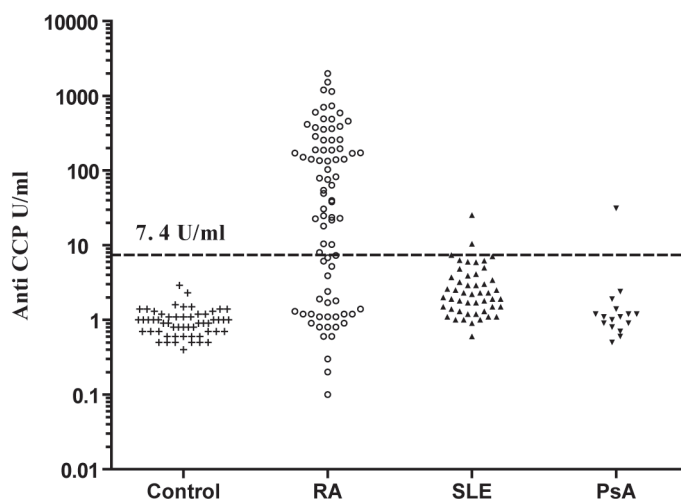


FIG. 3: Anti-CCP levels in control and study populations.
 RA-Rheumatoid arthritis, SLE-Systemic lupus erythematosus, PsA-psoriatic arthritis

DISCUSSION

The ideal diagnostic marker for RA should fulfil at least four requirements, (1) good sensitivity to detect higher percentage of patients (2) good specificity: to limit false positive results; (3) early response: to facilitate early diagnosis and (4) prognostic abilities: to identify those patients who will develop more severe disease.

Rheumatoid factor has long been the serological marker most commonly used to diagnose RA. In fact, although high titers of IgM-RF are relatively specific for diagnosing RA in a picture of chronic polyarthritis, RF is also present in some healthy individuals, in

immune-mediated diseases and some infections;¹ hence these antibodies are not specific for RA. Other RA-associated antibodies include anti-RA 33, anti-calpastin, anti-neutrophil cytoplasmic antibodies, antibodies to nuclear antigen, anti-collagen type II, anti-glucose 6-phosphate isomerase, anti-heavy binding protein and anti-phospholipid antibodies.^{9,10} Some of these RA-associated antibodies can be of additional use in the identification of subsets of patients. However, given their lack of specificity for RA, most of them are of little use as a reliable marker for RA.¹¹

Biochemical characterisation of APF and

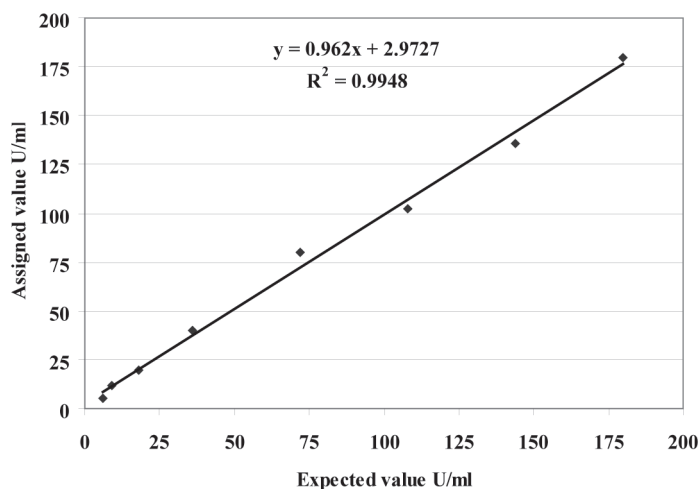


FIG. 4: The linearity test showed linear response for concentrations from 6 to 180 U/ml

AKA demonstrated that the main activity of these antibodies resides in citrulline.¹² The development of synthetic peptide containing citrulline⁴ has enabled the development of the ELISA test.

Several studies have examined the performance characteristics of anti-CCP antibodies in RA, using both the anti-CCP1 and anti-CCP2 assays. When a filaggrin-based cyclic peptide (cyclisation increased the sensitivity) was applied in the first generation anti-CCP (CCP1) test, a sensitivity of 68% was obtained with very high specificity for RA (98%).¹³ Second- and third-generation tests (CCP2 and CCP3) using mixtures of synthetic cyclic peptides are now available, which have produced a further significant increase in sensitivity (around 80%), while maintaining very high specificity (98%–99%).^{14,15,16}

Our study was conducted to evaluate the diagnostic and analytic performance of fully automated anti CCP2 assay in our population. We found the analytic imprecision was less than 15% in two different serum pool concentrations, thus confirming the manufacturers claim. Our findings of sensitivity and specificity of 65 % and 98 % for anti-CCP antibodies was in agreement with the studies reported earlier.⁴ The high specificity of anti-CCP is particularly useful in RF-negative RA patients. In our study the frequency of anti-CCP antibodies in RF-negative RA patients was 28%, but this has been reported up to 40%.¹⁷

Increased concentration of anti-CCP has also been reported in other autoimmune disorders.¹⁸ Fifty-one SLE and 16 psoriatic arthritis patients were included in our study to study the specificity of anti-CCP. Anti-CCP was above the cut-off value in two SLE and one PsA patients. RA and SLE, especially in early stages, may present with similar symptoms. Most patients with RA develop erosions within the first three years of onset of the disease,¹⁹ whereas only 5% patients with SLE develop erosions.²⁰ In practice, musculoskeletal lupus may appear similar to RA. Given that the outcomes of these diseases are different, it would be helpful to have serological means to distinguish between them at onset. In our study we found only two patients with SLE had increased concentrations of anti-CCP. Mediawake *et al*²¹ observed that 20% erosive SLE patients were anti-CCP positive but only 0.5% non-erosive SLE patients were anti-CCP positive. The raised levels of anti-CCP in these two patients probably indicate erosive SLE.

Psoriatic arthritis (PsA) is an inflammatory joint disease that shares features of rheumatoid arthritis (RA). This makes the differential diagnosis between PsA and RA in a patient with psoriasis difficult.²² Moreover, a laboratory test with specificity for PsA is still unavailable. Anti-CCP has been also identified in PsA with controversies as regards its clinical and radiological associations.^{23,24} However, Abdel Fattah *et. al.* noted in their study, anti-CCP seropositivity in PsA contributes to disease severity.²⁵ In our study, we noted anti-CCP was increased in one PsA patient.

In conclusion, the sensitivity and specificity of anti-CCP noted in our study are in agreement with other studies. A considerable proportion (28%) of RF-negative RA patients, were anti-CCP positive. The high specificity of the assay suggests that anti-CCP is useful in the diagnosis of rheumatoid arthritis and in our cohort of study population anti-CCP exhibits a better diagnostic value than RF. The anti-CCP is a reliable tool to make the diagnosis of RA. Based on analytical performance of the assay, we conclude that full automation and high throughput features of AxSYM makes it an ideal platform for routine testing of anti-CCP.

REFERENCES

1. Dörner T, Egerer K, Feist E, Burmester GR. Rheumatoid factor revisited. *Curr Opin Rheumatol.* 2004; 16:246-53.
2. Nienhuis RL, Mandema E. A new serum factor in patients with rheumatoid arthritis; the antiperinuclear factor. *Ann Rheum Dis.* 1964; 23:302-5.
3. Young BJ, Mallya RK, Leslie RD, Clark CJ, Hamblin TJ. Anti-keratin antibodies in rheumatoid arthritis. *Br Med J.* 1979; 2:97-9.
4. Schellekens GA, de Jong BA, van den Hoogen FH, van de Putte LB, van Venrooij WJ. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. *J Clin Invest.* 1998; 101:273-81.
5. van Gaalen FA, Toes RE, Ditzel HJ, *et al.* Association of autoantibodies to glucose-6-phosphate isomerase with extraarticular complications in rheumatoid arthritis. *Arthritis Rheum.* 2004; 50:395-9.
6. Rantapää-Dahlqvist S, de Jong BA, Berglin E, *et al.* Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheum.* 2003; 48:2741-9.
7. Meyer O, Labarre C, Dougados M, *et al.* Anticitrullinated protein/peptide antibody assays in early rheumatoid arthritis for predicting five year radiographic damage. *Ann Rheum Dis.* 2003; 2:120-6.
8. Arnett FC, Edworthy SM, Bloch Da, *et al.* The

- American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;31:315-24
9. van Boekel MA, Vossenaar ER, van den Hoogen FH, van Venrooij WJ Autoantibody systems in rheumatoid arthritis: specificity, sensitivity and diagnostic value. *Arthritis Res*. 2002; 4:87-93.
 10. Steiner G, Smolen J. Autoantibodies in rheumatoid arthritis and their clinical significance. *Arthritis Res*. 2002; 4:S1-5.
 11. Vossenaar ER, Zendman AJ, van Venrooij WJ, Pruijn GJ. PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. *Bioessays*. 2003; 25:1106-18.
 12. Vossenaar ER, Nijenhuis S, Helsen MM, *et al*. Citrullination of synovial proteins in murine models of rheumatoid arthritis. *Arthritis Rheum*. 2003; 48:2489-500.
 13. Schellekens GA, Visser H, de Jong BA, *et al*. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum*. 2000; 43:155-63.
 14. Suzuki K, Sawada T, Murakami A, *et al*. High diagnostic performance of ELISA detection of antibodies to citrullinated antigens in rheumatoid arthritis. *Scand J Rheumatol*. 2003; 32:197-204.
 15. van Gaalen FA, Visser H, Huizinga TW. A comparison of the diagnostic accuracy and prognostic value of the first and second anti-cyclic citrullinated peptides (CCP1 and CCP2) autoantibody tests for rheumatoid arthritis. *Ann Rheum Dis*. 2005; 64:1510-2.
 16. Fernández-Suárez A, Reneses S, Wichmann I, Criado R, Núñez A. Efficacy of three ELISA measurements of anti-cyclic citrullinated peptide antibodies in the early diagnosis of rheumatoid arthritis. *Clin Chem Lab Med*. 2005; 43:1234-9.
 17. Kastbom A, Strandberg G, Lindroos A, Skogh T. Anti-CCP antibody test predicts the disease course during 3 years in early rheumatoid arthritis (the Swedish TIRA project). *Ann Rheum Dis*. 2004; 63:1085-9.
 18. Avouac J, Gossec L, Dougados M. Diagnostic and predictive value of anti-cyclic citrullinated protein antibodies in rheumatoid arthritis: a systematic literature review. *Ann Rheum Dis*. 2006; 65:845-51
 19. Bukhari M, Harrison B, Lunt M, Scott DG, Symmons DP, Silman AJ. Time to first occurrence of erosions in inflammatory polyarthritis: results from a prospective community-based study. *Arthritis Rheum*. 2001; 44:1248-53.
 20. Labowitz R, Schumacher HR. Articular manifestations of systemic lupus erythematosus. *Ann Intern Med* 1971; 74: 911-21.
 21. Mediwake R, Isenberg DA, Schellekens GA, van Venrooij WJ. Use of anti-citrullinated peptide and anti-RA33 antibodies in distinguishing erosive arthritis in patients with systemic lupus erythematosus and rheumatoid arthritis. *Ann Rheum Dis*. 2001; 60:67-8.
 22. Palazzi C, Olivieri I, Petricca A, Salvarani C. Rheumatoid arthritis or psoriatic symmetric polyarthritis? A difficult differential diagnosis. *Clin Exp Rheumatol*. 2002;20:3-4
 23. Bogliolo L, Alpini C, Caporali R, Scirè CA, Moratti R, Montecucco C. Antibodies to cyclic citrullinated peptides in psoriatic arthritis. *J Rheumatol*. 2005;32:511-5
 24. Vander Cruyssen B, Hoffman IE, Zmierzczak H, *et al*. Anti-citrullinated peptide antibodies may occur in patients with psoriatic arthritis. *Ann Rheum Dis*. 2005;64:1145-9
 25. Abdel Fattah NS, Hassan HE, Galal ZA, El Okda el SE. Assessment of anti-cyclic citrullinated peptide in psoriatic arthritis. *BMC Res Notes*. 2009; 2:44