

Combination of Serological Tests (Anti-CCP Antibody, Rheumatoid Factor IgM ELISA and Latex Test) are more Useful in Detection of Rheumatoid Arthritis

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Article history

Received: 15-11-2016

Revised: 27-01-2017

Accepted: 18-09-2017

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Abstract: Rheumatoid arthritis is a polyarticular and chronic inflammatory disease occurring throughout the world. To prevent significant joint damage, early diagnosis and proper treatment is of paramount importance. Though patients are diagnosed clinically supported by radiography and serological tests, early disease may present with non-specific arthritis and absence of specific radiographic findings. Though anti-CCP antibody is used for the diagnosis and may be found in early disease, recently some variability of results has been observed in some studies. In this context present study was carried out to combine anti-CCP antibody, rheumatoid factor IgM ELISA and Latex agglutination test to observe the combined specificity and sensitivity of the tests and the tests were compared with each other to examine the correlation between them.

Keywords: Anti-CCP Antibody, Rheumatoid Factor, Latex Agglutination Test, Combined Specificity, Resultant Sensitivity, ELISA

Introduction

Rheumatoid Arthritis (RA) may produce in many cases a significant level of morbidity (Arnett *et al.*, 1988). Though the diagnosis of rheumatoid arthritis is primarily clinical, based on signs and symptoms of chronic inflammatory arthritis with laboratory radiographic results providing important supplemental information, in many patients early disease presents with non-specific arthritis. Recently there is significant motivation among clinicians to diagnose rheumatoid arthritis patients, early in the course of the disease, because the recently developed disease modifying drugs and biological agents are notably helpful in long term outcomes of the patients (O'Dell, 2003). However, the overenthusiastic initiation of treatment with disease modifying agents, without diagnosing RA accurately can cause a lot of harm to the patient. It is also very important to identify the patients who will have progressive, erosive disease, as early aggressive treatment may help them most (Lee and Schur, 2003). Though radiography is the most common imaging modality in RA patients and allows easy serial comparison for assessment of disease progression, the main disadvantage is the absence of specific radiographic findings in early disease, since visualization of erosions may only be seen later (Guerhazi *et al.*, 2004).

The first autoantibody in RA, Rheumatoid Factor (RF), was described by Dr Waaler (1940). IgM, IgG and IgA isotypes of RF occur in sera from patients with RA, although the IgM isotype is the one, most frequently measured by commercial laboratories. The IgM or IgG RFs in the joints activate complement and recruit macrophages, neutrophils and lymphocytes and induce inflammation (Soltys and Axford, 1997). However, RF has been observed in many other autoimmune diseases, infectious diseases and even in healthy people. So, not all rheumatoid factors cause disease and many studies are recently going on to characterize the difference between "pathological" and "physiological" RFs (Brian *et al.*, 1998).

Nienhuis and Mandema (1964) described a specific antibody for rheumatoid arthritis called Antiperinuclear Factor (APF) as these antibodies combine with the components of the keratohyaline granules which are located close to the nucleus of buccal mucosa cells of adult people. Another group of antibodies found in RA patients, named Antikeratin Antibodies (AKA), which bind to keratin-like structures of stratum corneum of epidermis, was mentioned by Young (1979). Sebbag *et al.* (1995) showed that antiperinuclear factors and antikeratin antibodies combine with the same antigen identified as filaggrin (filament aggregating protein).

Schellekens *et al.* (1998) documented that, citrullination of filaggrin plays an important role in autoantigenic property. APF and AKA mainly target the citrullinated filaggrin antigen. Based on that finding, Schellekens GA first prepared an enzyme immunoassay, using human filaggrin as substrate (Schellekens *et al.*, 1998). Through several stages of development the assay was later improved as second generation anti-CCP test with a sensitivity of 70-80% and specificity of 95-98% in established rheumatoid arthritis (van Venrooij *et al.*, 2011).

Citrullination occurs normally inside the damaged and dead cells of the body. High Ca^{2+} concentration ($\geq 10^{-5}$ mol/l) activates the enzyme Peptidyl Arginine Deiminase (PADI) and stimulates the conversion of arginine into citrulline. Normal level of Ca^{2+} inside the body is about 100 times lower. In damaged and dead cells, the cell membrane loses its selective permeability and allows excess entry of extracellular Ca^{2+} ions. Intracellular proteins like vimentin, nuclear histone become rapidly citrullinated and citrullination enhances cell-death process (van Venrooij *et al.*, 2011).

But mere presence of citrullinated proteins does not stimulate the immune system to produce anti-CCP antibodies, and several genetic factors of the patient are thought to influence it strongly. Many studies have indicated the association between HLA-DRB1 Shared Epitope (SE) alleles and RA (Klareskog *et al.*, 2009). Hill *et al.* (2009) demonstrated that the conversion of arginine to citrulline at the peptide side-chain position that interacts with the SE, significantly increases peptide-MHC affinity and leads to the activation of CD4+ T cells in DR4 IE transgenic mice.

Conversion of arginine into citrulline generates 'altered self' peptides that can be bound and presented by DRB1*1001, one of several SE alleles that is also strongly associated with RA and anti-CCP antibodies. Gyetvai *et al.* (2010) showed that, in particular, the S2 and S3P alleles (both associated with increased risk of RA) predisposed individuals to the production of anti-CCP and anti-MCV (Mutated Citrullinated Vimentin) antibodies.

In June, 2010 the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) revised the 1987 ACR classification criteria for RA and introduced anti-CCP antibody estimation in an effort to improve early diagnosis of RA (Kasper *et al.*, 2015). However in the early stages of the disease, it becomes difficult to make the diagnosis (Vallbracht and Helmke, 2005). Furthermore, in several studies conducted by Kroot *et al.* (2000), Quinn *et al.* (2006) and Kashyap *et al.* (2015) some variabilities in the results of anti-CCP positivity in rheumatoid arthritis patients have been observed.

Considering all these variability, present study was carried out to combine anti-CCP ELISA, RF IgM ELISA and RF latex agglutination tests to observe the combined

sensitivity and specificity of the tests to diagnose rheumatoid arthritis more accurately.

Materials and Methods

Study Design

The study was carried out over a period of one year from August 2014 to July 2015. It was conducted on a total of 88 patients presenting with history of polyarthritis and for selection of cases and controls, they were subjected to detailed history, clinical examinations and necessary laboratory investigations. All the patients under study were divided into two groups of which the study group was composed of 57 patients with rheumatoid arthritis. The diagnosis was based on clinical features and "The New 2010 American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) criteria" for the diagnosis of rheumatoid arthritis and on the expert opinion of attending physician of Rheumatology OPD. The control group was composed of 31 patients having non-RA rheumatic diseases with joint pain. The demographic profile of the patient such as age, sex, religion, residence, education and occupation was taken. The study group consisted of 42 (73.68%) females and 15 (26.32%) males with a ratio of 2.8:1, while the control group consisted of 12 (38.71%) females and 19 (61.29%) males with a ratio of 1:1.6. In the control group males were more than the females.

Ethical Clearance

The study proposal with relevant documents was submitted to the Institutional Ethics Committee (IEC) for review. The study commenced after receiving ethical approval and clearance certificate. Participation in the study was voluntary and a signed consent form was obtained from all the patients.

Sample Collection

About 5 ml of venous blood was collected from each patient aseptically in a sterile vial and the blood was allowed to clot for separation of serum. Then in the Microbiology laboratory the serum was completely separated by centrifuging the sample vials in a centrifuge machine at 3000 revolutions per minute (rpm) for 5 min. The separated serums were then transferred to sterile vials, labeled properly with serial numbers and stored at -80°C till the assay was done.

Laboratory methods: All the sera of the patients were examined by anti-CCP ELISA Kit, (Omega Genesis, BioMerieux) for detection of anti-CCP IgG antibody. The results were expressed in Optical Density (OD) along y-axis and corresponding antibody titer of the six standards, plotted along x-axis of the curve. Subsequently the sera were examined for IgM Rheumatoid Factor by

AutostatTMII Rheumatoid Factor IgM ELISA Kit (Hycor Biomedical, Garden Grove, California, USA). Rheumatoid Factor (RF) assay was also done by Latex Agglutination Slide Test with RHELAX-RF reagent Kit, (Tulip Diagnostics (P) Ltd. India).

Statistical Analysis

GraphPad inStat software and online Med Calc. statistical calculator were used to do statistical analyses of the test results. The distribution of laboratory test results were compared between study group and control group using the Fisher's exact test where necessary. Diagnostic utility of the tests were described by determining the sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) obtained with the cut-off value mentioned by the respective commercial kits. Assuming the prevalence of RA in the community as 0.5% corrected PPV and NPV was calculated using Bayes' theorem. Correlation between variables was assessed by Pearson correlation.

Results

Anti-CCP antibody was positive in 52(91.23%) out of 57 cases in the study group and was negative in only 5(8.77%) out of 57cases. The picture was different in case of control group, as anti-CCP antibody was positive in 9(29.03%) out of 31cases in this group. Anti-CCP antibody was negative in 22 out of 31(70.97%) cases in the control (Table 1). When the serological profile in the study group and control group was compared it was found to be significant.

So according to the formula sensitivity, specificity, positive predictive value and negative predictive value of anti-CCP test is 91.23%, 70.97%, 75.77% and 89% respectively. Here, specificity of anti-CCP antibody test is showing lower value though the sensitivity of the test is high.

Rheumatoid Factor profiles as done by IgM ELISA was positive in 43(75.44%) and negative in 14(24.56%) out of a total of 57cases in the study group (Table 2). Rheumatoid Factor (IgM ELISA) in cases of control group was positive in 10(32.26%) and negative in 21(67.74%) cases respectively. When the serological profile of Rheumatoid Factor IgM ELISA in the study group and control group was compared, it was found to be significant. (p = 0.0002).

After application of formula the sensitivity of Rheumatoid Factor IgM ELISA is 75.44% and specificity is 67.74%. Positive Predictive Value (PPV) is 70.07% and Negative Predictive Value (NPV) of the test is 73.31%.

Similarly the sensitivity of Rheumatoid Factor Latex agglutination test is 40.35% and specificity is 90.32%. Positive Predictive Value (PPV) of the test is 80.65% and Negative Predictive Value (NPV) is 60.22%.

Table 1. Serological profile in the study and control group

Test	Study Group	Control Group
anti-CCP positive	52 (91.23%) (True Positive)	9 (29.03%) (False Positive)
anti-CCP negative	5 (8.77%) (False negative)	22 (70.97%) (True Negative)
Total (n = 88)	57	31

p<0.0001

Table 2. Serological profiles of RF IgM ELISA in the study and control group

Test	Study Group	Control Group
RF IgM positive	43 (75.44%) (True Positive)	10 (32.26%) (False Positive)
RF IgM negative	14 (24.56%) (False Negative)	21 (67.74%) (True Negative)
Total (n = 88)	57	31

p=0.0002

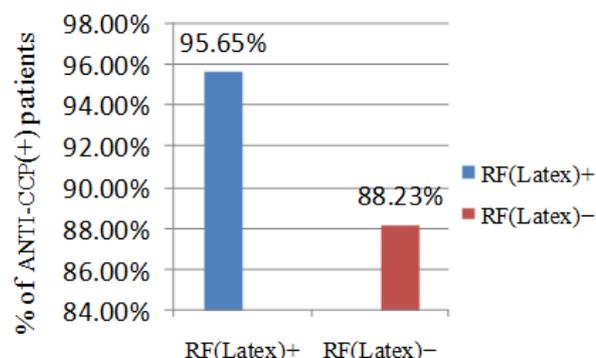


Fig. 1. Percentage of anti-CCP positive(+) patients in Latex test + and - patient

Here sensitivity of RF IgM ELISA (75.44%) is higher than the sensitivity of RF Latex agglutination test (40.35%) which is statistically extremely significant with p value of 0.0003 using Fisher's exact test. However specificity of RF IgM ELISA (67.74%) is lower than the specificity of RF Latex agglutination test (90.32%) which is statistically not quite significant (p = 0.0586).

The correlation coefficient of anti-CCP ELISA and rheumatoid factor Latex test is 0.3551 (95% CI, 0.1573 to 0.5255, $r^2 = 0.1261$). The two tailed p value is 0.0007 which is highly significant. The same for anti-CCP ELISA and rheumatoid factor IgM ELISA is 0.6318 (95% CI, 0.4867 to 0.7430, $r^2 = 0.3992$). The two tailed p value is <0.0001 which is extremely significant. The correlation coefficient for RF IgM ELISA and RF Latex test is 0.7679 (95% CI 0.6655 to 0.8420, $r^2 = 0.5897$). The two tailed p value is <0.0001 which is extremely significant.

In this study, citrullinated peptide antibody test is positive in 95.65% RF Latex positive patients versus 88.23% RF Latex negative patients (Fig.1).

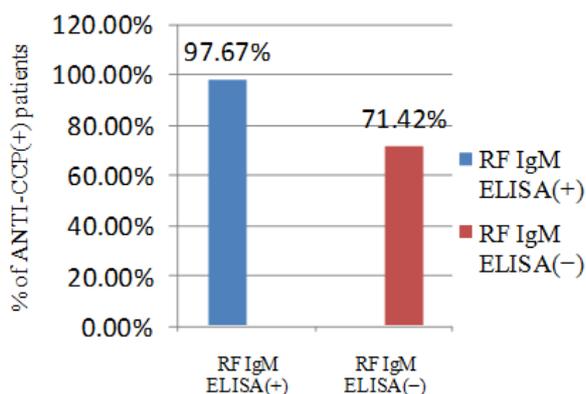


Fig. 2. Percentage of anti-CCP positive(+) patients in RF IgM ELISA + and - patients

Table 3. Sensitivity and specificity of different tests in the present study

Test	Sensitivity	Specificity
Anti-CCP IgG ELISA	91.23%	70.97%
RF IgM ELISA	75.44%	67.74%
RF Latex test	40.35%	90.32%
Combined anti-CCP ELISA + RF IgM ELISA	68.82%	90.64%
Combined anti-CCP ELISA + RF Latex test	36.81%	97.19%

Similarly anti-CCP antibody is positive in 97.67% RF IgM ELISA positive patients versus 71.42% RF IgM ELISA negative patients (Fig. 2). So we can say that anti-CCP antibody can be found more often in RF Latex test and RF IgM ELISA positive patients.

In normal clinical practice, to make a diagnosis more confidently, the results of two independent tests can be combined. To increase the specificity of a test, two tests can be combined by using the following formula:

$$\text{Specificity of combined test} = 1 - (1 - \text{specificity of test 1}) \times (1 - \text{specificity of test 2})$$

Here combining anti-CCP assay and Rheumatoid Factor Latex test, the combined specificity is increasing to 97.19% (Table 3) which indicates that, in a polyarthritis patient if both anti-CCP antibody and RF (Latex test) is negative then there is 97.19% chance that the patient is negative for rheumatoid arthritis.

But one drawback in combining these two tests in this way is that here the resultant sensitivity of the two tests is 36.81% which is less than the individual sensitivity of anti-CCP and RF (Latex test) assay.

Still if the specificity of the combined test is 97.19%, we can safely say that in a polyarthritis patient if both anti-CCP and RF (Latex test) is negative then there is 97.19% chance that the patient is negative for rheumatoid arthritis.

Similarly if anti-CCP antibody test is combined with RF IgM ELISA then combined specificity of the two tests is 90.64% and the resultant sensitivity is 68.82% (Table 3).

After comparing all the results it is seen that combining these two results can more efficiently diagnose rheumatoid arthritis because there is 90- 97% chance of the patient not having rheumatoid arthritis though the resultant sensitivity is reduced (Table 3).

Similarly by combining the anti-CCP ELISA, RF IgM ELISA and RF (Latex test), the combined specificity of the three tests can be calculated using the following formula:

$$\text{Specificity of three combined tests} = 1 - (1 - \text{specificity of test 1}) \times (1 - \text{specificity of test 2}) \times (1 - \text{specificity of test 3})$$

Combined specificity of the three tests is 99.09% which means that if a patient is negative for all the three tests of anti-CCP ELISA, RF IgM ELISA and RF Latex test, then we may be 99.09 % sure that the patient is not having rheumatoid arthritis.

Discussion

The present study was done to compare citrullinated peptide antibody and rheumatoid factor assay, individually and in combination to detect rheumatoid arthritis patients. Several studies have shown that citrullinated peptide antibody test is highly specific and could be detected early in the disease course and also predict the trends of progressive erosive disease. However, some variability of the results has also been found.

In the present study, sensitivity of Rheumatoid Factor IgM ELISA is 75.44% and specificity is 67.74%. In a separate study conducted by Bas *et al.* sensitivity of Rheumatoid Factor IgM ELISA was 73% and specificity was 82% respectively (Bas *et al.*, 2003). In the present study, sensitivity of Rheumatoid Factor (Latex agglutination test) is 40.3% and specificity is 90.32% respectively. In another study conducted by Saraux *et al.* (2003) sensitivity of RF Latex test was 45%. In a different study Aflaky *et al.* (2010) reported similar values of RF Latex test. Nishimura *et al.* (2007) reported that sensitivity of RF is 69% and specificity is 85%.

In the present study, sensitivity of RF IgM ELISA (75.44%) is better than the sensitivity of RF Latex test (40.3%) which is statistically extremely significant ($p = 0.0003$). Swedler *et al.* (1997) has also mentioned that sensitivity of RF IgM ELISA is better than the sensitivity of RF Latex test. Niewold *et al.* (2007) has mentioned that in several studies it has been observed that rheumatoid factor showed a variable sensitivity of 31% to 54% and specificity of 91% to 93% for the eventual diagnosis of RA.

In the current study sensitivity of anti-CCP antibody is 91.23% and specificity is 70.97% respectively. In another study conducted by Gupta *et al.* (2009) sensitivity and specificity of anti-CCP antibody was 85 and 90.19% respectively. In a study conducted by Schellekens *et al.* (2000), sensitivity and specificity of anti-CCP antibody was 68 and 98% respectively. Goldbach-Mansk *et al.* (2000) reported that sensitivity and specificity of anti-CCP antibody was 50% and 90% respectively. According to Bizzaro *et al.* (2001) sensitivity and specificity of anti-CCP antibody was 41% and 98% respectively. In a study Bas *et al.* (2002) showed that sensitivity and specificity of anti-CCP antibody was 68% and 96% respectively. Suzuki *et al.* (2003) reported that sensitivity and specificity of anti-CCP antibody was 88% and 89% respectively. So it is quite obvious that in the present study, citrullinated peptide antibody test is showing reduced specificity in comparison to the other studies.

There may be several explanations for the difference between reported sensitivity and specificity of anti-CCP antibody test in different studies. One explanation for the discrepancy is that the differences in the patient populations (mainly disease duration) among these studies might have some influence on the results. Another study showed that the specificity and sensitivity of anti-CCP antibodies may depend on the patient's race (Binesh *et al.*, 2014). It is also probably inevitable that sensitivity and specificity of laboratory tests will vary between studies as there will be local variations in the application of clinical diagnostic criteria.

To overcome discrepancies of individual test results, if anti-CCP ELISA, RF IgM ELISA and RF Latex test are combined, then the combined specificity of the three tests is 99.09% which is quite helpful in establishing diagnosis. Parikh *et al.* has also shown that to make diagnosis of a disease more accurately three diagnostic tests can be combined (Parikh *et al.*, 2008).

Conclusion

Though citrullinated peptide antibody assay can help significantly in diagnosis of RA, all the present study can realistically do is provide a suggestion to clinicians that combining multiple blood test results like anti-CCP antibody, RF IgM assay and RF Latex agglutination tests may provide an output that more strongly predicts clinical diagnosis.

Acknowledgement

All the staffs of the Department of Microbiology and the Department of Medicine of Gauhati Medical College and Hospital, Guwahati, where this study was carried out.

Author's Contributions

Anindya Das: Performed the experiments, prepared data analyses report and wrote the manuscript.

Chimanjita Phukan: Assisted in revising, data analyses and improving the paper.

Chitrlekha Baruah: Assisted in collecting patients' history and data from Rheumatology Out Patient Department.

Ethics

The article is original in all respect. All the authors have participated and approved the manuscript and no ethical issues are related to it.

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