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Original Research Article

Comparative study of rheumatoid factor with anti - CCP antibodies by ELISA method in the diagnosis of rheumatoid arthritis

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ABSTRACT

Rheumatoid Arthritis is a chronic inflammatory disease of unknown aetiology marked by a symmetric peripheral polyarthritis. It is the most common form of chronic inflammatory arthritis and often results in joint damage and physical disability. Because it is a systemic disease, RA may result in a variety of extra-articular manifestations, including fatigue, subcutaneous nodules, lung involvement pericarditis, peripheral neuropathy, vasculitis, and hematologic abnormalities. AIM: To compare the Rheumatoid factor by latex agglutination method with anti-cyclic citrullinated peptide antibodies by Enzyme Linked Immunosorbent Assay method in Rheumatoid Arthritis. Statistical Analysis: The results of anti-CCP antibody test and RF were expressed as positive or negative. Chi-square test was used to determine the statistical difference between the sensitivity, specificity, positive and negative predictive values of Anti-CCP and RF tests.

KEY WORDS: Rheumatoid Factor, Anti - CCP Antibodies, Rheumatoid Arthritis

INTRODUCTION

The science of RA has taken a major leap forward with the identification of new disease related genes and further deciphering of the molecular pathways of disease pathogenesis. The relative importance of these different mechanisms has been highlighted by the observed benefits of the new class of highly targeted biologic therapies. Despite these gains incomplete understanding of the initiating pathogenic pathways of RA remains a sizable barrier to its cure and prevention. The incidence of RA increases between 25 and 55 years of age after which it plateaus until the age of 75 and then decreases [1]. The disease onset is usually gradually, with predominant symptoms being pain, morning stiffness, and swelling of many joints. Early tends to affect smaller joints of hand and feet on as the disease progresses symptoms often spread to the knees, ankles, elbows, hips and shoulders [2]. Early and aggressive intervention with new and effective biological treatment can alter the course of the disease, lengthen life, and improve function. Better molecular markers for diagnosis and prognosis are needed to identify RA patient [3].
Anti-CCP positivity is added to the new RA diagnosis criteria of the American College of Rheumatology (ACR) in 2010 [4]. Greater sensitivity and specificity than IgM RF and probable predictability of erosive disease in RA or the eventual development of undifferentiated arthritis into RA makes anti‑CCP antibodies potentially important surrogate markers for the diagnosis and prognosis in RA.

The aim of present study is to compare the Rheumatoid factor by latex agglutination method with anti-cyclic citrullinated peptide antibodies by Enzyme Linked Immunosorbent Assay method in Rheumatoid Arthritis.

MATERIALS AND METHODS

The study was conducted in the department of Microbiology, **S**. V. Medical College, Tirupati from the date of approval by institutional Ethical committee of S.V.M.C from November 2014 to October 2015. Samples were collected under aseptic precautions from the patients attending Orthopaedic department and General Medicine department S.V.R.R.G.G. Hospital, Tirupati. The total serum samples were 186 and were tested by Latex agglutination method (SPAN DIAGNOSTICS, SACHIN SURAT) for

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Rheumatoid factor and anti-CCP antibodies by IMUNOSCAN CCPLUS ELISA (EURODIAGNOSTICA, AB, SWEDEN).

Processing of Sample:

Collection of blood sample: The blood samples were obtained from the patients by **vein** puncture following strict aseptic precautions and allowed to clot at room temperature and then centrifuged. The serum was separated.

Storage of serum sample: Serum samples were refrigerated (2-8 ^{0}) or stored frozen in a deep freezer (-20 ^{0}C) if not tested within two days.

Inclusion Criteria:

- Clinically suspected rheumatoid arthritis patients.
- Age group of 20 to 60 years.

Exclusion Criteria:

- Age of less than 20 & more than 70 years
- Other established cases of Arthritis.

LATEX AGGLUTINATION METHOD FOR RA FACTOR

Principle: RA test antigen consists of polysterene latex particles coated with specially modified preparation of human gammaglobulin (IgG) in order to avoid non- specific agglutination. The suspension of coated latex particles agglutinate visible when mixed with serum containing RA factor. The sensitivity of the reagent has been adjusted to detect 10 IU/ml of RF calibrated against an International standard.

Procedure:

Qualitative method:

1. Samples were allowed to room temperature
2. By using disposable plastic dropper one drop of test serum (40-50 ml) placed within the circled area marked test on the slide.
3. After shaking the vial gently one drop of latex gamma-globulin reagent added to the above drop and mixed well with a disposable applicator stick.
4. Slide is rocked gently to and fro two minutes and examined for the agglutination within 2 minutes.
5. Clearly visible agglutination has been taken as positive.

ELISA METHOD FOR anti- CCP ANTIBODIES:

Principle: The test utilizes microtitre plate wells coated with citrullinated synthetic peptides (antigen). Diluted Patient serum is applied to the wells and incubated. If specific antibodies are present, they will bind to the antigen in the wells. Unbound material is washed away and any bound antibody is detected by adding horse raddish peroxidise (HRP) labelled anti-human Ig G, followed by a second washing step and an incubation with substrate. The presence of reacting antibodies will result in the development of colour, which is proportional to the quantity of bound antibody, and this is determined photometrically.

Materials and methods: One sealed (96 wells) CCP peptide coated microtitre plate ready to use. Unused microwells should be resealed immediately and stored in the presence of a desiccant. It is stable at 2-8 C until expiry.

i. 5 vials containing calibrators (positive human serum pool) ready to use.
ii. 1 vial containing reference control human serum.
iii. 1 vial containing positive control human serum.
iv. 1 vial containing negative control.
v. 1 vial containing conjugate solution (peroxidise conjugated to antihuman IgG Antibodies).
vi. 1 vial containing TMB (3, 3',5,5'-tetramethyl benzidine) substrate.
vii. 2 vials dilution buffer.
viii. 1 vial containing stop solution contains 0.5 M sulphuric acid.
ix. wash buffer:

Procedure:

Qualitative Assay for the detection of anti-CCP antibodies: Ensured all the reagents were equilibrated to room temperature before commencing assay. The test protocol was prepared.

Sample dilution: The test is performed on serum samples. Dilute patient sample 1:50 (10 µL sample in a tube with 490 µL dilution buffer).100 µL diluted sample has been used for the test.

Wash buffer preparation: 35 ml wash buffer with 665 ml distilled water.

Before performing the procedure all the reagents should bring to the room temperature.

1. A 1 kept as blank.
2. 100 µl of negative control, positive control and reference controls were added into their respective microwells (i.e. from B1 to D 1) of the assay plate, as per the protocol. From E 1 samples were added.)
3. Plate was covered and incubated at room temperature (18 to 25 degrees) for 1 hour
4. Plate was washed 3 times with diluted wash buffer.
5. 100 µl conjugate which contains peroxidise conjugated to anti human Ig G antibodies.
6. The plate was covered and incubated at room temperature for 30 minutes.
7. The plate was washed 3 times

8. 100µL TMB substrate solution was added to each well and Incubated at room temperature for 30 minutes
9. 100 µL stop solution added to each well after 10 minutes of incubation.
10. The absorbance value of each well was read within 30 minutes at a wavelength of 450 nm filter.
11. Absorbance values has been read at 450 nm

Washing procedure

1. All wells were aspirated completely.
2. All the wells are filled during wash cycle.
3. After completion 3 times the plate was inverted and taped firmly on absorbent paper towel to ensure all wash buffer is removed.
4. Automated plate washers were all maintained to ensure efficient washing. Manufacturers cleaning instructions were followed of all times

QUALITY CONTROL

Each kit contains calibrator, positive and negative control, reference control sera. For the qualitative procedure ratio of the positive control versus the reference control should be within the range 4.0-6.2. The ratio of the negative control versus the reference control should be <0.95.

TABLE 1: Results Interpretation table

ABSORBANCE RATIO	RESULT INTERPRETATION
<0.95	Negative
0.95 - >1.0	Borderline
>1.0	Positive

RESULTS AND DISCUSSION

This study was conducted in the department of Microbiology, S. V. Medical College, Tirupati. This is a case control study. Total 186 samples, among these 80 patients were taken as Cases of suspected Rheumatoid Arthritis(i.e. Morning stiffness of >1 h most mornings for at least 6 weeks, Arthritis of hand joints, present for at least 6 weeks. swelling of >3 of 14 joints present for at least 6 weeks. Symmetric arthritis for at least 6 weeks). In 80 clinically suspected RA cases 58 (72.5%) females and 22 (22.5%) are males. The mean age was 42.21 ± 10.33 years. 106 patients who presented with nonspecific joint paints were included in this study as controls, among these 82(77.35%) are females and 24 (22.64%) are males. The mean age was 41.8 ± 10.62 years.

TABLE 2: Tests properties of anti –CCP antibodies and RF

Parameter	Anti-CCP	RF	P-value
Prevalence	43.01 (35.85-50.46)	43.01 (35.85-50.46)	---
Sensitivity	60.00 (48.42-70.60)	63.75 (52.18-73.99)	0.625
Specificity	93.40** (86.40-97.08)	79.25 (70.05-86.27)	0.003
Positive Predictive Value	87.27* (74.91-94.31)	69.86 (57.85-79.76)	0.020
Negative Predicative Value	75.57 (67.15-82.47)	74.34 (65.11-81.88)	0.824

Anti CCP antibodies: Based on the cut off value >1 (absorbance ratio by manufacturer) among 80 clinically suspected cases of RA 48 sera were positive for anti- CCP antibodies. In 106 participants who are controls, 7 (6.6%) sera were positive. The sensitivity was 60%. The specificity was 93.4%.

TABLE 3: Test properties of anti-CCP antibodies

Prevalence	43%
Sensitivity	60%
Specificity	93.4%
Positive predictive value	87.2%
Negative predictive value	75.57%

TABLE 4: Anti- CCP assay results in Cases and Controls

Anti ccp	Suspected RA Cases	Controls	Total
Positive	48	07	55
Negative	32	99	131
Total	80	106	186

Among total 80 cases 48 (60%), and in 106 controls7 (6.6%) showed positive for anti CCP testing by ELISA

Rheumatoid Factor: Based on the cut off value >10 IU/ml among 80 clinically suspected cases of RA, 50 sera were positive for RF by latex agglutination In 106 participants who are controls, only 23 (21.7%) sera were positive. The prevalence is 43%. The sensitivity was 62.50%. The specificity was 78.3 %.

TABLE 5: Tests properties of Rheumatoid Factor

Prevalence	43%
Sensitivity	62.50%
Specificity	78.3%

TABLE 6: RF and Anti CCP positivity in Clinically suspected RA and non RA

	Positives in Cases	Positive in controls
ANTI CCP	48	7
RF	50	23

The results indicate that Anti-CCP had significantly ($P<0.05$) higher specificity (93.40%) and positive predictive value (87.27%) compared to RF test (Specificity: 79.25%; PPV: 69.86%). There was no significant difference between the sensitivity and negative predictive values of both tests. Some studies suggested that the anti-CCP antibody assay plays an important role in diagnosing RA patients who are RF-negative and have atypical clinical symptoms [5]. The high specificity of anti-CCP might appear to provide a definitive RA diagnosis. This study shows females were affected more than males. This is correlating with Rajiva Gupta, Molly M Thabah, et al. [6]. The present study shows 60% sensitivity closely resemble with 70% of Fariba Binesh et al., [10] and Sneka P et al [7], (2015). The low sensitivity of the anti CCP test may be due to sero negative rheumatoid Arthritis.

TABLE 7: Comparison of percentage of anti CCP Parameters in the present study with previous studies

STUDY	SENSITIVITY (in %)	SPECIFICITY (in %)
Present study (2015)	60	93.33
Rajiva Gupta et al., [6] (2009)	85	90.19
Sneka P, Sujith et al. [7] (2015)	76	97
S.Oommen, B.Appalaraju et al. [8] (2011)	81	98
Yadollah Shakiba et al. [9] (2014)	53.1	95.3
Fariba Binesh et al. [10].	70.76	85.07

TABLE 8: Comparison of percentage of Parameters of Rheumatoid Factor in the present study with previous studies

STUDY	**SENSITIVITY (in %)**	**SPECIFICITY (in %)**
Present study (2015)	62.5	78.3
Bizzaro et al., [11] (2001)	62	84
S Oommen, B. Appalaraju et al. [8] (2011)	77	76
I.G. Silveira et al.[12] (2007)	64	90
Shakineh-Khatoun SHARIF et al.,(2007) [13]	85.3%	64.7%
Yadollah Shakiba et al.[9]	61.8	82.3

TABLE 9: Comparison of percentage of Parameters of anti CCP& RF in the present study with previous studies

	Anti- CCP		RF	
Study	PPV (%)	NPV (%)	PPV (%)	NPV (%)
Present study(2015)	87.2	75.57	68.5	73.45
I.G. Silveira et al.[12] (2007)	79	92	56	92
Fariba Binesh et al. [10](2014)	90	59	90	45

The sensitivity of the present study correlates with Bizzaro et al., [11] I. G. Siveira et al., [12] and the specificity of RF was 78.3% is correlates with S Oommen et al [8] of 76%. The combined specificity of the test was 81% which is better than RF specificity. The positive predictive value and negative predictive value of the study for anti CCP antibodies 87.2% and 75.57% respectively. The PPV & NPV for the RF of this study is 68.5% and 73.45% respectively.

This study more concerned with specificity of the anti CCP assay. The result showed that anti CCP has better specificity than RF. Because of the low specificity of RF the diagnostic implication is lower. So positive RF must be interpreted with caution and other parameters like clinical features and inflammatory markers such as CRP, TNF-α to be taken in to consideration while diagnose a case of RA. An important fact should be mentioned regarding false positive (those not showing typical clinical RA) cases allocated by the anti CCP may have value since the more recent studies showed anti CCP could be detected 1.5 -9 years before onset of arthritis and progression from undifferentiated polyarthritis to RA in 93% of anti CCP positive patients [7].

In 48 anti CCP positive cases, 16 were RF negatives. 8 anti CCP negative patients have shown RF positives. 32 were positive for both RF and anti CCP antibodies. In controls 2 were positive for both tests. In total 80 suspected RA cases 48 were anti CCP poisitives and 50 were RF positives.

CONCLUSION

The anti- CCP antibody assay is a valuable tool for the classification criteria of RA as they may predict the eventual development into RA when found in undifferentiated arthritis patients. Because of its low sensitivity it does not allow its use as a screening test, but because of its high specificity when compared to Rheumatoid factor, it is one of the most useful serological test for the diagnosis of RA. Because of increasing burden of RA patients and its morbidity, anti-CCP antibody assay will help in predicting RA onset in the patients. Combined use of RF and anti- CCP is a better tool for the diagnosis of RA.

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REFERENCES

1. Fauci, Kasper, Hauser, Longo, et al. Harrison's Principles of internal medicine. 18th ed. Volume; 2:2738-2739.
2. Ali Aghuveri , Ahmed Marafi, Mansour Alhiary et al. Use of serological markers for evaluation patients with rheumatoid arthritis. Int J Biol Med Res.2012;3(1):1397-1398.
3. T.B. Niewold, M.J. Harrison and S.A. Paget. Anti-CCP antibody testing as a diagnostic and prognostic tool in rheumatoid arthritis.Q J Med 2007; 100:193–201.
4. Bridges SL (2001) Rheumatoid factor. In : Koopman WJ, ed. Arthritis and Allied Conditions,pp. 1223-44. Lippincott, Williams &Wilkins, Philadelphia.
5. Li JS. ELISA method was used to detect the significance of Anti- CCP antibody in rheumatoid arthritis. Modern Preventive Medicine 2010;37:3329.
6. Gupta R, Thabah MM, Aneja R, Kumar A, Varghese T, Chandrasenan PJ. Usefulness of anti-CCP antibodies in rheumatic diseases in Indian patients. Indian J Med Sci 2009;63:92-100)
7. A. Barakat, H.H asony, A.AL Khafaji,T. Mezal. Diagnostic value of antibodies to itrullin containing peptides in patients with rheumatoid arthritis in southern iraq. Bas J Surg, March15 2009.
8. Oommen S, Appalaraju B, Sivadarshini S, Jayashree. A combined diagnostic approach to rheumatoid arthritis using anti-cyclic citrullinated peptide and rheumatoid factor. Indian journal of medical microbiology2012; 29:195-196.
9. Yadollah Shakiba, Susan Koopah ,Ahmad Jamshidi, et al. Anti- Cyclic Citrullinated Peptide Antibody and Rheumatoid Factor Isotypes in Iranian Patients with RA: Evaluation of Clinical Value and Association with Disease Activity.Iran J Allergy Asthma Immunol,2014; 13(3): 147-156.
10. Fariba Binesh ,Hossein Soleimani Salehabadi, Nasim Behniafard., et al. A comparative Assessment of the Diagnostic Value of Anti-Cyclic Citrullinated Peptide Antibodies and Rheumatoid Factor in Rheumatoid Arthritis. J Clin Exp Pathol 2014, 4:2.
11. Nicola Bizzaro , Glovanni Mazzanti, Elio Tonutti, Danilo Villalta and Renato Tozzoli. Diagnostic Accuracy of the Antibody Assay for Rheumatoid Arthritis. Clin Chem 47, 1089-93.
12. I.G.Silveira, R.W. Burlingame, C. A. Von Muhlen. Anti –CCP antibodies have more diagnostic impact So anti CCP positivity is added to the new RA diagnosis criteria of the than rheumatoid factor(RF) in a population tested for RF.Clin Rheumatol,DOI 10.1007/s 10067-007-0601-6.
13. Sakineh-Khatoun Sharif, Shahin Eghbal, Farhad Gharibdoost, Mahmood A. Kbarian, Farhad Shahram,et al. Comparative study of anti-CCP and RF for the diagnosis of rheumatoid arthritis. APLAR Journal of Rheumatology 2007; 10: 121–124.