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Review Article

The Origin of Life on Earth-Viruses and Microbes

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Abstract

Origin of life on the earth is a mystery. Nobody exactly knows how the first life appeared on the earth. However, it can be assumed that the basic constituents of life like Carbon, Hydrogen, Oxygen, Nitrogen, Phosphorous and other inorganic substances combined in a proportion to create first life on earth. Probably the origin of life started from the production of purine, pyrimidine rings, amino acids, sugar alcohols, nucleic acid chains and the first life on earth is prokaryotic microbes which probably evolved from the virus like particles. Structure of viruses which are lacking the cytoplasm and definite cellular structure but containing nucleic acid core and protein covering are the proof that nucleic acid strands were first produced and later on, in presence of water they evolved to produce the desired products to form cytoplasm and biological membrane to create primitive cell. To explain the systematic assembling of all the basic structural elements leading to a structural unit with functional autonomy with bio-chemical synthesis, degradation (metabolism) , energy production, self-replication which can be called "nano-scale organization", a hypothesis has been proposed which is called Random Collision, the Association, Rejection with the Acquisition of Added characteristic Hypothesis which can explain the creation of first functional unit of life as a virus like particle and later on prokaryotic microbes and with the help of Darwin's theory of evolution it can be explained that this first functional unit of life later evolved into more complex multi-cellular living forms.

Keywords: Origins of Life; Random Collision; Acquisition of Added Characteristic; Functional Autonomy; Purine and Pyrimidine Ring; Systematic Assembling

Introduction

Origin of life on earth is the most mysterious & debatable thing in the field of scientific research. From several evidences of the fossilized microbes (cyanobacteria) in Stromatolytes it can be presumed that first life in earth probably originated in between 3.5 to 4.4 billion years ago [1,2]. But it is sure that the basic constituents of life like Carbon, Hydrogen, Nitrogen, Phosphorous, Oxygen and other inorganic substances combined in a proportionate way to create first life in earth, though there is a controversy between "Genetic-first" approach or "Metabolic-first" (Energy acquisition) approach or the "Compartmentalization first" approach for the emergence of life on earth. From several evidences it can be assumed that oxygen was not present in early earth's atmosphere and probably the primitive organism in earth was the heterotrophic bacteria. It is known that for any chemical reaction to occur an optimum temperature, pressure, catalyst, reagents are required. When present in suitable proportion and the required activation energy is reached, the reaction can occur and the desired product is formed. In the pathway of gradual transformation of Abiotic to Biotic earth, probably the origin of life started from the first production of amino acids, purine, pyrimidine rings, sugar alcohols, nucleic acid chains and other basic components of life. The first life on earth was prokaryotic microbes which probably evolved from the virus like particles. When a process progresses gradually step by step through a long period of time, the intermediary steps may persist as a proof that the process progressed through these steps. Following this logic, it can be said that the structure of viruses which are lacking the cytoplasm, definite cellular structure but containing nucleic acid core, protein covering are the proof that nucleic acid strands were first produced. Later on, in presence of water they created the desired products to form cytoplasm only after being enclosed by a biological membrane to create primitive cell or protocell. But before that chemical evolution took place leading to the embedment of structural information in chemical entities with

information storage, retrieval, transfer and processing of information through charge transfer in donor-accepter sequences which is a well-organized, highly efficient, directional and specific electron transfer process [3-5].

Moving backwards to the history of research in the field of origin of life, early philosophers and naturalists believed in spontaneous generation to explain the origin of life, but later on Lewis Pasture disproved the idea by showing that bacteria can grow only from bacteria or bacterial spores. After that, scientists adopted the idea that living organisms were the historical outcome of gradual transformation of lifeless matter. The heterotrophic origin of life was proposed by Russian biochemist Alexander Ivanovic Oparin [6] and British biologist B.S. Haldane [7]. They proposed the idea of a primordial protoplasm and hypothesized that life had been preceded by a lengthy period of abiotic syntheses and accumulation of organic compounds from the "hot primitive soup". At the initial stage earth's environment was anaerobic and the primitive organisms must have been heterotrophic bacteria. Later on, Miller-Urey experiment [8] strengthened the hypothesis that "generation of the first living organisms might have taken place if large quantities of organic compounds had been present in the oceans of the primitive earth." Electric discharge of thunder storm and UV radiation from our solar system might have played a significant role in the primitive atmosphere containing ammonia, methane, nitrogen, hydrogen, water etc. to produce amino acids and other complex components of the life in soupy sea [8,9].

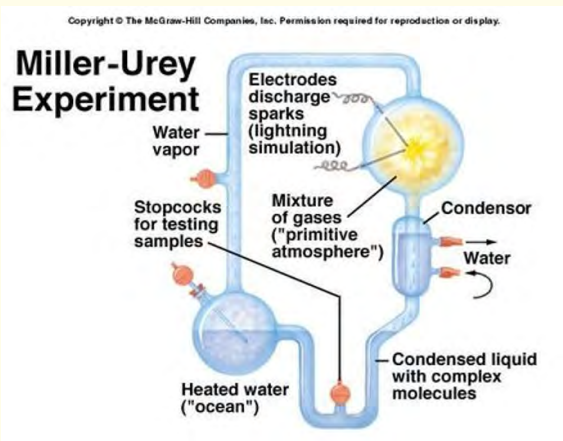

Figure 1: Miller- Urey Experiment (copyright @ McGraw-Hill Companies, Inc).

The Miller's paper (1953) was published only a few weeks after Watson and Crick's [10] classic article revealed their double helix model for the structure of DNA [10]. So, when Oparin & B.S. Haldane proposed the idea of "hot primitive soup" and Miller-Urey performed their experiment, that time knowledge of Genetics was limited. So, later on with the development of Biochemistry and Molecular Biology, scientific trends towards the understanding biological phenomena at the molecular level led authors like Troland [11], Muller [12] and others to propose that single molecules or viruses represented primordial living systems [11,12]. Muller proposed the "naked gene" theory for the origin of life. Later on "RNA hypothesis" of origin of life first proposed in 1968 by Leslie Orgel, supported by Crick and later on the term coined by Walter Gilbert in 1986 favors the assumption that RNA can simultaneously act as an enzyme (Ribozymes) and also as a storehouse of genetic informations [13]. However, from the 1960s onwards, scientists studying the origin of life split into camps. According to Sutherland the basic polarization was between the proponents of "metabolism-first" versus "genetics-first" theory. Meanwhile, a third group proposed the idea that "Compartmentalization must have come first, because there is no point doing metabolism unless it is compartmentalized." "Compartmentalization-first" – has its supporter in Pier Luigi Luisi of Roma Tre University in Rome, Italy. Luisi's reasoning was simple and hard to be argued as the evidence supporting the presence of lipid molecules in the prebiotic environment and their natural ability to self-organize into vesicular compartments cannot be denied [14]. Though till now the researches in these three directions have not shown any promising results towards unraveling the mysteries of first origin of life on earth, in this current paper a new theory has been proposed following the rules of nature, which may help in making the total process of creation logically more comprehensible and the theory is- Random Collision, the Association, Rejection with the Acquisition of Added characteristic Hypothesis.

Metabolism-first theory vs Genetic-first description of life

Metabolic-first approach for the origin of life requires the confirmation that metabolic (or protometabolic) routes can replicate and evolve. But so far there are no indications that this is the case and on the contrary the available experimental evidences strongly suggest that the prebiotic environment was already endowed with a wide range of monomers of biochemical significance, many organic and inorganic catalysts, purines and pyrimidines, i.e., the potential for template-directed polymerization reaction, and membrane-forming compounds, so the genetic first description of life

is more pragmatic. Indeed, the evidence supporting the presence of lipid molecules in the prebiotic environment and their natural ability to self-organize into vesicular compartments underlines the significance of theoretical models of simple cells involving an evolving ribozymic RNA polymerase [15].

Genetic-first description of life in the context of present hypothesis

In this paper to make the total process of creation logically more comprehensible, the origin of life has been divided into three steps- Step I – Formation of basic structural elements or building blocks of life like purine and pyrimidine rings, amino acids, glucose, phosphate energy bonds etc. Step II – Formation of more complex structural forms by chain elongation of basic structural molecules. Step III –Systematic assembling of all these structural elements leading to a structural unit with functional autonomy where all the biochemical reactions can occur automatically, repeatedly in an organized way, making it an autonomic functional unit (nano-scale organization) capable of recognition, sensing, signaling, bio-chemical synthesis, degradation (metabolism), energy production, self-duplication (reproduction), homeostasis and information dissipation. To explain this nano-scale organization more easily, a new hypothesis has been created which is called Random Collision, the Association, Rejection with the Acquisition of Added characteristic Hypothesis.

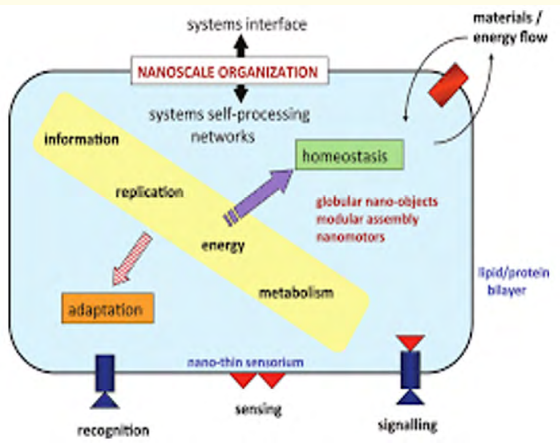

Figure 2: Cellular Organization (taken from- Stephen Mann, Systems of Creation: The Emergence of Life from Nonliving Matter, Accounts of Chemical Research, Nov 4, 2011).

In this context it is worth mentioning that the above mentioned Step I can be explained by the Miller-Urey Experiment where Stanley Miller in conjunction with fellow scientist Harold Urey, in 1953 first demonstrated the spontaneous synthesis of amino acids and organic compounds that are required as the basis of life on Earth, from the inorganic precursors and the simplest of organic molecules like ammonia, nitrogen, methane, hydrogen, water etc.

It can also be explained by the heterogeneous catalysis of the Fischer–Tropsch CO/H_2 reaction on silicon/metal oxides to form amino acids and nucleobases [16].

But the most difficult part is to explain the Step II and Step III. How the formation of more complex structural form by chain elongation & how the systematic assembly of these parts occurred to achieve functional autonomy, forming the first living form? To explain the formation of more complex structural form by chain elongation, it can be said that the purine, pyrimidine ring has some unusual property of organizing and assembling carbon, hydrogen, oxygen and nitrogen molecules in a systematic way. "RNA hypothesis" of origin of life favors the assumption that RNA can simultaneously act as an enzyme (Ribozymes) and also as a storehouse of genetic information's. It is also known that uridine ring, the phosphate bond (UMP, UDP and UTP) and adenine ring in Co-enzyme-A help in the polymerization of carbohydrate and fatty acid chains respectively. It is an important aspect that purine and pyrimidine ring creating the mRNA and tRNA helps in the polymerization of amino acids creating protein molecules which are the building blocks of life. The form in which the energy is stored in biological world (ATP) is made up of nucleotide and phosphate. So, it strengthens the idea that purine & pyrimidine rings, phosphate bonds are the key structures towards the creation of life. Though the chain elongation towards the formation of more complex form can be partly explained, the most difficult part is to explain how the systematic assembly of these parts occurred in such a complex, organized way to achieve the functional autonomy, forming the most incredible and mysterious form of thing in the universe which is life. It can be easily challenged that life as a single cell is several times more complex than a computer CPU!!

According to this newly created hypothesis- Random Collision, the Association, Rejection with the Acquisition of Added characteristic Hypothesis, it can be said that at the beginning of the creation, just like inorganic molecules randomly come in contact with

each other, in the similar way bio-molecules also came in contact with each other at random. As it is known that the bio-molecules have some affinity to each other if suitable condition prevails (just like a positively charged compound can be attached to a negatively charged compound), the combination occurred through charge transfer or electron sharing in donor-accepter sequences at random following the rules of permutation & combination in the primitive soup of Alexander Oparin [6] and B.S. Haldane [7]. For example---

"A" combining with "B" forming "AB", next "B" combining with "C" forming "BC" & so on

A + B = AB

B + C = BC

C + D = CD

D + A = AD

Now when "A" combines with "B" forming "AB" & if "AB" can acquire some extra property by which it can combine with another molecule "XY" to form "ABXY" with some added advantages then automatically it was selected by nature.

On the contrary, if "B" & "C" after combining to form "BC" do not acquire some extra property or added character to combine with "XY", then it was rejected. So this chain-wise selection & rejection processes can explain how it proceeds further to form more complex molecules. Whenever a new structural form was produced, if it acquired some extra functional capability or added advantages over other ones, it was automatically selected by nature. Whereas the combinations that did not acquire some extra functional capabilities, did not proceed further.

Example 1

AB+ XY ----------> ABXY +αβ ----------> ABXYαβ + γδ ----------> ABXYαβγδ

If A acquires extra characteristics by combining with B forming AB & similarly if AB acquires extra characteristics by combining with XY forming ABXY & similarly if ABXY acquires extra characteristic by combining with αβ forming ABXYαβ and if ABXYαβ with extra characteristic combine with γδ and proceeds further in a sequential way, then AB, ABXY, ABXYαβ, ABXYαβγδ and in this way more complex form will be produced.

Example 2

AB+ XY ----------> ABXY + γδ ----------->ABXYγδ ----------->Ÿ

Here AB acquires extra characteristics by combining with XY but ABXY does not acquire extra characteristic after combining with γδ. So, it proceeds to form AB, ABXY & ABXYγδ. But as it does not acquire extra functional characteristics after combining with γδ, so it does not proceed further from ABXYγδ. In each step, specific way of electron sharing or electron transfer, different type of bond formation (covalent bond, vander - waal bond, disulfide bond, hydrogen bond etc.) played a crucial role towards the formation of more complex form which needs a lot of further researches in this field.

So in this way, following the nature's rule it can be explained that bio-molecules by random collision, then combination and then acquisition of some functional capability, proceeded towards the more complex form with extra functional capability through the process of well organized, highly efficient, directional and specific electron transfer processes in donor-accepter sequences and ultimately led to the formation of living form with complete functional autonomy with information storage, retrieval, transfer and processing of information.

Step by step by random association, selection and rejection, it acquired the step by step functional capability. From the structures of viruses it can be depicted that before the origin of fully developed unicellular organisms only the RNA and then DNA core was developed with limited functional capability of producing few structural proteins, enzymes and in presence of water later the nucleic acid core evolved to form more complex form to produce all the necessary components of cytoplasm, cell membrane and other necessary enzymes to evolve into an independent existing cell. Here the Darwin's theory of evolution helps in explaining the development of higher living forms from the primitive cell or protocell.

Discussion

With the help of this theory- Random Collision, the Association, Selection, Rejection with the Acquisition of Added characteristic Hypothesis, Oparin's primitive soup, Miller-Urey's experiment, Muller's "naked gene" and Orgel and Walter Gilbert's "RNA world" can be connected.

However, it is worth mentioning here that the problem arose with the "RNA world" hypothesis in between 1980 to 2011 when

Jack Szostak, David Bartel, [15] Philipp Holliger, Gerald Joyce and Tracey Lincoln in separate experiments in their laboratory created RNA enzymes (R18, tC19Z) that replicates itself but can copy sequences up to 6 to 48% of its own length, but not necessary 100%. So, RNA has proved to be enormously difficult to make and the problem is the sugar and the base that make up each nucleotide. It is possible to make each of them individually, but the two stubbornly refuse to link together.

So, it was thought that there might be some other type of molecule on the early Earth: something simpler than RNA, which really could assemble itself out of the primordial soup and start self-replicating. This might have come first, and then led to RNA, DNA and the rest. In 1991, Peter Nielsen [17] of the University of Copenhagen in Denmark came up with a candidate for the primordial replicator. It was Polyamide nucleic acid, or PNA where the backbone of the molecule was made up of Polyamide instead of Ribose sugar. PNA can coil up into a double helix, just like DNA and Stanley Miller also thought that PNA was a more plausible candidate for the first genetic material. In 2000, Albert Eschenmoser [18] made another nucleic acid named Threose nucleic acid (TNA) which is basically like DNA, but with a different sugar in its backbone and can pair up to form a double helix, and information can be copied back and forth between RNA and TNA and in 2005 Eric Meggers [19] made glycol nucleic acid, which can form helical structures.Though each of these alternative nucleic acids has its supporters, but as there is no trace of them in nature, so it is very difficult to say whether the first life did use them or not at all.

However, it can be said that studies of more specific conditions, including the laboratory simulation of localized environments such as volcanic islands, tidal zones and micro-environments, including liposomes, clays and mineral surfaces, montmorillonite (James Ferris, 1986) and volcanic ponds, which could have been prevalent in the primitive environment, may yield promising results [20].

Now if come to the currently described theory which is based on nature's rule- Random Collision, the Selection, Rejection with the Acquisition of Added characteristic Hypothesis, it can be said that it can explain all the three theories like "Genetic first", "Metabolic first" or the "Compartmentalization first" theory. However, one thing that strikes the mind is what is meant by the acquisition of new functional capability. Bio-molecules in a living cell are interconnected in such a way that as an example it can be said if one interconnecting pathway is linked with the signal transduction, the effector molecule produced will result in a cleavage of another molecule and that cleaved product may be responsible for stimulating a particular gene to produce a final end product by transcription and translation. So, it can be said that all the bio-molecules are interconnected with one another in a finely tuned way or as a finely programmed way which is responsible for several functional outcomes. Here with the help of this selection, rejection with the acquisition of new functional capability hypothesis, it can be said that overall functional capability & structural organization was achieved step by step with the formation of new molecules. In each step the terminal product produced was one step ahead in functional capability from the preceding one, whether it was the formation of trans-membrane receptor or the formation of cAMP or cGMP molecules or the creation of signal transduction pathway or the creation of photosynthetic pigment like chlorophyll. Might be an unique way of electron sharing or electron transfer and different types of bond formation (covalent bond, vander-waal bond, disulfide bond, hydrogen bond etc.) played a crucial role towards the formation of more complex form with information storage, retrieval, transfer and processing of information.

Also, it can be said that as the primitive earth's environment was reduced and probably that time primitive microbes were heterotrophic in nature and only after the generation of photosynthetic pigments and the generation of autotrophic organisms, gaseous oxygen appeared in earth's atmosphere, so in the primitive unknown reduced atmosphere of earth, biochemical reactions might have occurred through different unknown pathways to perform the nano-scale organization.

Conclusion

Though Günter Wächtershäuser's [21] idea of the process of harnessing energy is utterly essential but the metabolic-first approaches requires the confirmation that metabolic (or protometabolic) routes can replicate and evolve and so far there are no indications that this is the case and on the contrary the form in which the energy is stored in biological world (ATP) is made up of nucleotide, so it can be said that the polymerization of nucleotide with the genetic first description of life is more pragmatic and initially the energy source was external from the surrounding environment and later on compartmentalization with the creation of enzyme on the membrane surface to harness energy from the proton gradient across the membrane in the form of ATP is more plausible to believe and one interesting thing is the theory that have proposed following the natures rule - Random Collision, the Selection, Rejection with the Acquisition of Added characteristic Hypothesis, can explain all the three theories like "Genetic first" , "Metabolic first" or the "Compartmentalization first" theory.

Lastly it can be said that though several researchers tried to explain the origin of life supported by the newly developed knowledge of cellular biology, for example Thomas Cech and his colleagues (1982) first discovery of RNA as an enzyme made the Leslie Orgel and Francis Crick's assumption of RNA hypothesis more plausible , and Günter Wächtershäuser's [21] metabolism first hypothesis also acquired reasonable support with the fact that energy is required for survival and Peter Mitchell's [22] clear delineation of proton gradient across a membrane and the stream of protons passing through give the enzyme residing on the membrane the energy it needed to make ATP, so until and unless more discoveries of cellular working pathways at the sub-cellular level come to our knowledge, it will be hard to delineate the exact pathway of nano-scale organization of the first protocell and the lack of exact knowledge of the environmental conditions of earth four billion years ago is another major problem to prove this nano-scale organization at the laboratory set-up [23- 37].

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Research Articles

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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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 (Guermazi *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 antiperineuclear factors and antikeratin antibodies combine with the same antigen identified as filaggrin (filament aggregating protein).

Science Publications

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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, Schellenkens 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 losses 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 p<0.0001	31

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 p=0.0002	31

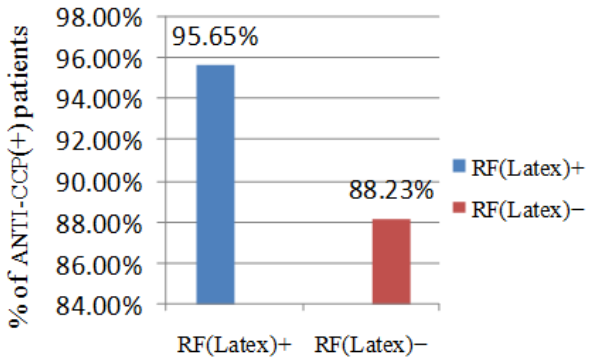

Fig. 1. Percentage of anti-CCP positive(+) patients in Latex test + and – patient

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).

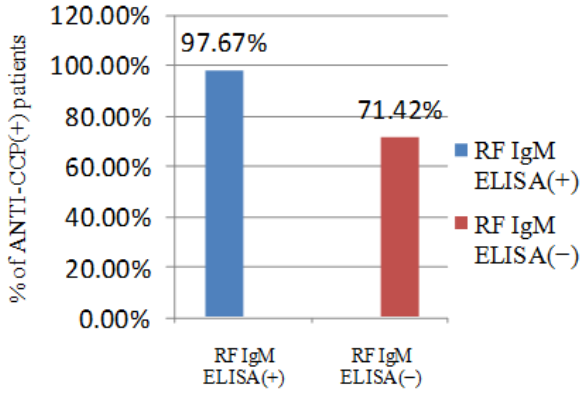

Fig. 2. Percentage of anti-CCP positive(+) patients in RF IgM ELISA + and - patients

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:

Specificity of combined test = 1−
(1−specificity of test 1) × (1−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:

Specificity of three combined tests
= 1 − (1 − specificity of test 1) × (1− specificity of test 2) × (1− 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.

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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.

Chitralekha 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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A Study on the Importance of Anti Cyclic Cytrullinated Peptide Antibodies (Anti-CCP) and Rheumatoid Factor (RF) Assay in Detection of Patients with Rheumatoid Arthritis in North Eastern Part of India

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Abstract: *Objective:* Rheumatoid arthritis (RA) is a polyarticular and systemic chronic inflammatory autoimmune disease, occurring throughout the world. The aim of the present study is to observe the role of anti-CCP antibody and Rheumatoid Factor (RF) assay in diagnosing patients of rheumatoid arthritis in north-eastern part of India, in the state of Assam and also to evaluate the individual and combined sensitivity and specificity of anti-CCP antibody and RF assay in diagnosing RA. *Methods:* A total of 88 cases presenting with history of polyarthritis were included in the study of which the study group comprised of 57 clinically diagnosed cases of rheumatoid arthritis and control group comprised of 31 patients of non-RA rheumatic diseases having joint pain. Individual sensitivity and specificity of the above tests, and also the combined specificity of the two tests and also the three tests were calculated and the results of the tests were compared with each other to examine the correlation between them. *Results:* Sensitivity of anti-CCP ELISA, RF IgM ELISA and RF Latex test was 91.23%, 75.44% and 40.35% with specificity of 70.97%, 67.74% and 90.32% respectively. If the three tests are combined then the combined specificity of the three tests is 99.59%. One important observation was that out of 9 anti-CCP positive cases in the control group(n = 31) 5 cases were weakly positive (titer 6.25 – 20 U/ml), considering cut-off value 6.25 U/ml as mentioned by the kit.If these weakly positive cases in the control group are considered as negative, then specificity of anti-CCP ELISA increases from 70.97% to 87.1% which is more acceptable. *Conclusion:* From this study it is observed that anti-CCP antibodies are highly sensitive and specific for diagnosing RA. If this test is combined with RF IgM ELISA and Latex test then combined specificity can be increased more than 99%. However, weakly positive titers of anti-CCP antibodies are more prevalent in other non-RA rheumatic diseases in North-Eastern part of India.

Keywords: Anti-CCP Antibody, Rheumatoid Factor, Latex Agglutination Test, Combined Specificity, Resultant Sensitivity, Enzyme Linked Immunosorbent Assay (ELISA), Cut-Off Value

1. Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease with a significant level of morbidity. [1] The morbidity and mortality it causes are the consequence of local and systemic inflammatory process that damage cartilage, bone and soft tissue, as well as blood vessels and viscera. [2]

Because early initiation of disease modifying treatments can significantly improve long term outcomes for patients with RA, there is a considerable motivation to accurately diagnose RA in patients with inflammatory arthritis, early in the course of the disease. [3] But if RA is not accurately diagnosed early in the course of the disease, then overenthusiastic initiation of treatment with disease modifying agents can cause a lot of harm to the patient because, these drugs have many side

effects. The ability to identify those patients who will have progressive, erosive disease also remains an objective, because this subset of patients may benefit most from early aggressive treatment. [4]

The first autoantibody in RA, rheumatoid factor (RF), was described by Norwegian Dr Erik Waaler in 1937 and re-described by Dr H.M. Rose and colleagues in 1948. It was not until the early 1960s that investigators in the field agreed that RFs are antibodies to Fc fragment of IgG. [5] They are probably the most studied antibody since their discovery by Waaler in 1937. [6] Although RFs can be detected in the sera of a majority of RA patients, RF has been observed in many other autoimmune diseases, such as, in systemic lupus erythematosus, mixed connective tissue disease and primary Sjogren syndrome, as well as in infectious diseases such as Mycobacterium tuberculosis, Lyme disease, and also 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. [7]

The need for a better laboratory marker with a higher disease-related specificity and sensitivity for RA was always evident and an ongoing research was going on over the past 30 years. A specific antibody for RA was first described by Nienhuis and Mandema (1964), which is called antiperinuclear factor (APF) as these antibodies combine with constituents of the keratohyaline granules which are located close to the nucleus of buccal mucosa cells of adult people. Hoet *et al.* in 1991 showed that APF had an acceptable sensitivity and much higher specificity in comparison to RF. However, the test was never used for routine testing due to practical inconvenience. The test actually required carefully selected buccal mucosal cells which were differentiated enough to contain the perinuclear factor. At the same time, experienced laboratory technicians were also required to perform the test based on indirect immunofluorescence and to recognize the different immunofluorescent patterns. [8]

In 1979, Young *et al*. reported that sera of RA patients contained another group of antibodies, named antikeratin antibodies (AKA) which bind to keratin-like structures in the cornified layer of stratum corneum. Hoet and van Venrooij in 1992 mentioned that specificity of AKA was comparable to APF. Kurki *et al.* in 1992 demonstrated that AKA also precede the onset of RA. [8] Sebbag *et al*. in 1995 showed that APF and AKA target the same antigen identified as the epithelial protein filaggrin (filament aggregating protein), which is actually involved in the organization of cytoskeletal structures. [9] During differentiation of epithelial cells several filaggrin subunits result from proteolytic cleavage of profilaggrin. In profilaggrin, about 20% of the basic arginine residues are converted into neutral citrulline residues by the enzyme peptidyl-arginine deiminase (PADI). [8] Four isoforms of PADI have been identified, known as PADI1, PADI2, PADI3 and PADI4. Schellekens *et al*. in 1998 documented that, citrullination is essential for the auto-antigenicity of filaggrin. Citrullinated fillaggrin is the antigen targeted by APF and AKA. Based on that finding, Schellenkens *et al.* developed an enzyme linked immunosorbent assay (ELISA) derived from the sequence of human filaggrin as substrate.[10]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 and controls. [21]

The usefulness of anti-CCP antibodies to identify rheumatoid arthritis from other rheumatic diseases, presenting with joint pain has not yet been studied in patients of North-Eastern part of India. As we know that the incidence and prevalence of RA varies based on geographic location, both globally and among certain ethnic groups in a country, and as genetic factors play some role in causation of the disease, [29] the aim of the present study is to see the role of anti-CCP antibody and rheumatoid factor assay in diagnosing patients of rheumatoid arthritis in North-Eastern part of India, and also to evaluate the combined sensitivity and specificity of both anti-CCP antibody and rheumatoid factor assay in diagnosing RA.

2. Materials and Methods

2.1. Study Design

The present study was a hospital based observational/case control study, carried out over a period of one year from August 2014 to July 2015, that was conducted on a total of 88 patients presenting with history of polyarthritis in the Rheumatology OPD of the Gauhati Medical College and Hospital (GMCH), which caters to a large outpatient population in the North Eastern part of India. The study was conducted in two groups of which the study group was composed of 57 patients who attended the Rheumatology OPD for treatment and were diagnosed as having rheumatoid arthritis by the attending physician. The diagnosis was based on clinical features and "The New 2010American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) criteria" for the diagnosis of rheumatoid arthritis and finally on the expert opinion of attending physician of Rheumatology OPD. The control group was composed of 31 patients attending the Rheumatology OPD of GMCH, and was diagnosed as having non-RA rheumatic diseases with joint pain like systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), ankylosing spondylitis (AS) and spondyloarthropathy, gouty arthritis, osteoarthritis (OA), reactive arthritis and other non-specific polyarthritis. The study group consisted of 42 (73.68%) females and 15 (26.32%) males, while the control group consisted of 12 (38.71%) females and 19 (61.29%) males. The female to male ratio in the study group was 2.8: 1, whereas in the control group males were more than the females, with a male to female ratio of 1.6: 1.

2.2. Ethical Considerations

Ethical clearance was obtained from Institutional Ethics Committee (IEC), Gauhati Medical College and Hospital,

Guwahati, India (reg. no. MC/02/2015/100). Participation in the study was voluntary and a signed consent form was obtained from all the patients. The study results were returned to the patients and incorporated into their medical records.

2.3. Sample Collection

About 5 ml of venous blood was collected aseptically in a sterile vial from each patient of both study and control groups. The vial was left at room temperature and the blood was allowed to clot for separation of serum. Then serum was separated by centrifuging the sample vials in a centrifuge machine at 3000 revolutions per minute (rpm) for 5 minutes in Microbiology laboratory. The separated serums were then transferred to sterile vials and, labeled properly with serial numbers to identify them properly later on. The serum samples were stored at −80ºC till the assay was done.

2.4. Laboratory Methods

Anti-CCP antibodies were detected by ELISA, using a commercial kit (Omega Genesis EDRA bioMerieux, Cambridgeshire,UK) and according to the manufacturer's instructions, expressed in optical density (OD) along y-axis and corresponding antibody titer of the standards, plotted along x-axis of the curve, as shown in Fig.1. Rheumatoid Factor (RF) assay was done by Latex Agglutination Slide Test with RHELAX-RF reagent Kit,(Tulip Diagnostics (P) Ltd. India) and subsequently the sera were examined for IgM Rheumatoid Factor by Autostat^{TM}II Rheumatoid Factor IgM ELISA Kit (Hycor Biomedical, Garden Grove, California, USA). The tests were done according to manufacturer's instructions.

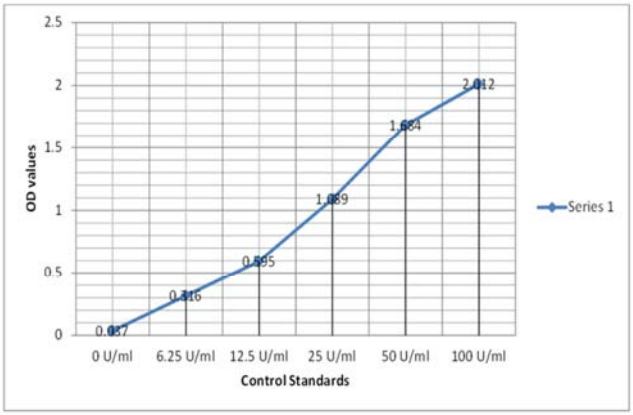

Fig. 1. *Titer of Control Standards and their OD value producing the curve*

2.5. Statistical Analysis

Then statistical analyses of the test results were done by using the software GraphPadinStat and online Med Calc. statistical calculator. The distribution of laboratory test results were compared between study group and control group using the chi- square test or the Fisher's exact test,where necessary. Two-sided $p < 0.05$ was considered significant. Diagnostic value 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 Spearman's correlation coefficient and Pearson correlation.

3. 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 in the study group. In case of control group the picture was different as anti-CCP antibody was positive in 9(29.03%) out of 31cases in this group. One important observation was that, out of 9 anti-CCP positive cases in the control group, 5 cases were weakly positive (titer < 3 × cut-off value) for anti-CCP antibody. Anti-CCP antibody was negative in 22 out of 31(70.97%) cases in the control group. When the serological profile of anti-CCP antibody in the study group and control group was compared it was found to be significant.($p < 0.0001$)The sensitivity, specificity, positive predictive value and negative predictive value of the test was 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.

One important thing that is observed in this study, that if 5 weakly positive cases (titer < 3× cut-off value) in the control group are excluded from the 9 positive cases in the control group and considered as negative, then False Positive cases become 4. In that case, 52 patients are True Positive for anti-CCP antibody, 5 patients are False Negative for anti-CCP antibody, 4 patients are False Positive for anti-CCP antibody and 22 + 5 = 27 patients are True Negative for anti-CCP antibody, which has been shown in Table1. When the serological profile of anti-CCP antibody is modified by considering weakly positive cases as negative and the study group and control group is compared, it is found to be significant. ($p< 0.0001$).

Table 1. *Modification of serological results, considering weakly positive cases as negative.*

Test	Study group	Control group
anti-CCP positive	52(true positive)	(positive – weakly positive =false positive)(9 – 5 = 4)
anti-CCP negative	5(false negative)	22 + 5 = 27(true negative)
Total (n = 88)	57	31
χ^2= 49.902, degree of freedom = 1, p < 0.0001		

Here, due to this modification, the sensitivity and specificity of test are showing more accurate results, which are 91.23% and 87.10% respectively. Though according to the kit, cut-off value is 6.25U/ml, in the control group significant numbers of patients are becoming weakly positive (titer between 6.25 – 20 U/ml) for anti-CCP antibody test. So if the 5 weakly positive (6.25 – 20 U/ml) cases in the control group are considered as negative, then specificity of the test is increasing significantly from 70.97% to 87.10%.

Modification of the results can be done in another way by increasing the cut-off value from 6.25 U/ml to 20 U/ml, in order to exclude the weakly positive cases from the positive cases of the control group who are having non-RA other rheumatic disorders. So after increasing cut-off value to 20U/ml, 51patients are True Positive for anti-CCP antibody,6 patients are False Negative for anti-CCP antibody, 4 patients are False Positive for anti-CCP antibody and 27 patients are True Negative for anti-CCP antibody which is shown in Table 2.

Table 2. *Modification of serological results by increasing cutoff value from 6.25U/ml to 20U/ml.*

Test	Study group	Control group
anti-CCP positive	51(true positive)	4(false positive)
anti-CCP negative	6(false negative)	27(true negative)
Total (n = 88)	57	31
χ^2= 47.016, degree of freedom = 1, p < 0.0001		

Then sensitivity, specificity, positive predictive value and negative predictive value of the test is 89.47%, 87.1%, 87.39% and 89.21% respectively. Therefore, if the cut-off value is increased to 20 U/ml (3 x previous cut-off), then specificity of the test is increasing significantly from 70.97% to 87.1% although, the sensitivity is reduced slightly from 90.23% to 89.47%.

In the control group, distribution of anti-CCP positive (titer>20U/ml), anti-CCP weakly positive (titer 6.25 - 20 U/ml) and anti-CCP negative (titer < 6.25U/ml) cases in non-RA other rheumatic disease patients, like Ankylosing spondylitis, SLE, Spondyloarthropathy, mixed connective tissue disease(MCTD), Osteoarthritis(OA), Reactive arthritis, Gout + Osteoarthritis and Non specific arthritis are shown in Table 3.

Table 3. *Number of Anti-CCP (+), Anti-CCP weakly (+), Anti-CCP (−) cases in different non-RA rheumatic diseases.*

Disease	Total	anti-ccp(+)	anti-ccp weakly(+)	anti-ccp (−)
Ankylosing spondylitis	8	1	1	6
Systemic lupus erythematosus (SLE)	6	2	0	4
Spondyloarthropathy	2	0	1	1
mixed connective tissue disease (MCTD)	2	0	0	2
Osteoarthritis (OA)	2	0	0	2
Reactive arthritis	2	0	0	2
Gout + Osteoarthritis	2	0	1	1
Non specific arthritis	7	1	2	4
Total	31	4	5	22

The sensitivity and specificity of Rheumatoid Factor Latex agglutination test is 40.35% and 90.32% respectively. Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of the test is 80.65% and 60.22% respectively. Here sensitivity of RF Latex agglutination test is 40.35%, but the sensitivity of anti-CCP antibody test is 91.23% and 89.47% (after increasing cut-off value). The difference between the sensitivity of the two tests is statistically extremely significant with p value of < 0.0001 (using Fisher's exact test).

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

In the present study, the correlation coefficient for anti-CCP ELISA and RF 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 correlation coefficient for anti-CCP ELISA and RF 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 the present study, anti-CCP antibody test is positive in 95.65% RF Latex positive patients versus 88.23% RF Latex negative patients.(Fig. 2)

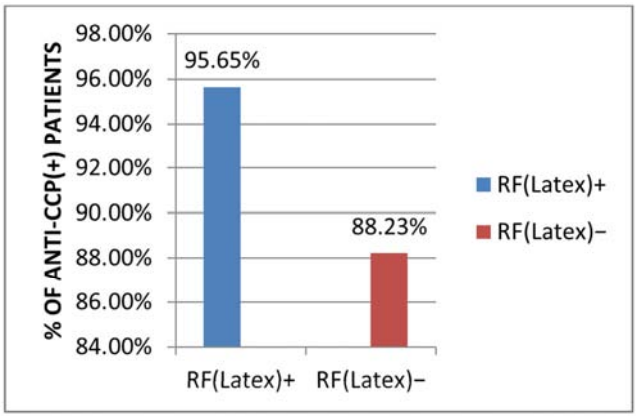

Fig. 2. *Percentage of Anti-CCP(+) patients in RF Latex test positive and negative patient.*

Similarly anti-CCP antibody is positive in 97.67% RF IgM ELISA positive patients versus 71.42% RF IgM ELISA negative patients. (Fig. 3) So we can say that anti-CCP antibody can be found more often in RF Latex test positive and RF IgM ELISA positive patients.

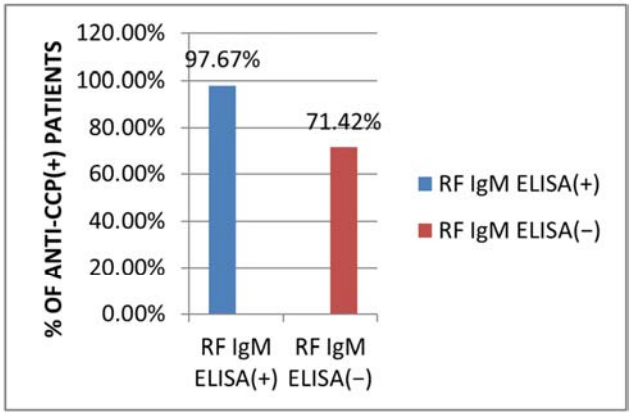

Fig. 3. *Percentage of Anti-CCP(+) patients in RF IgM ELISA positive and negative patients.*

Similarly Latex test is positive in 42.30% anti-CCP positive patients versus 20% of anti-CCP negative patients. (Fig. 4) So we can also say that RF Latex test can be found more often in anti-CCP positive patients.

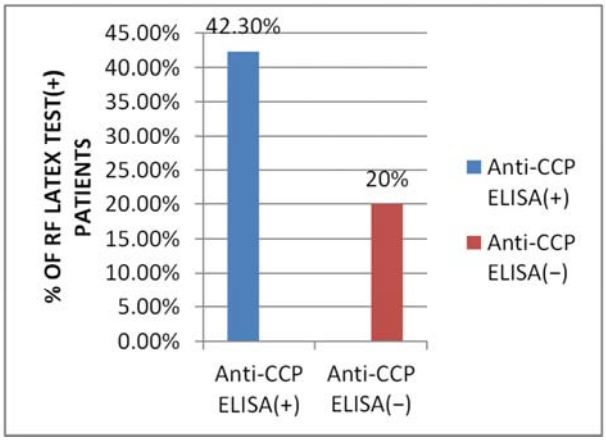

Fig. 4. *Percentage of RF Latex test (+) patients in Anti-CCP positive and negative patients.*

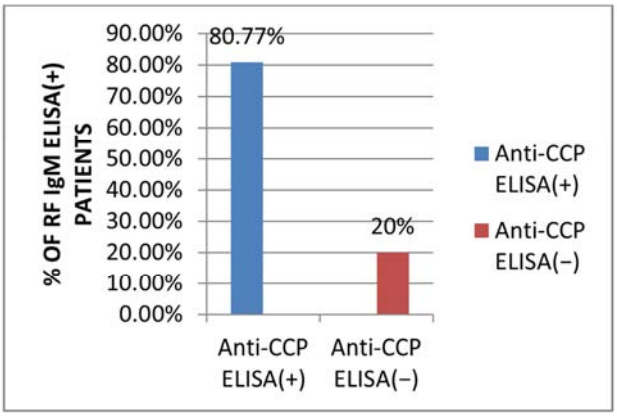

Fig. 5. *Percentage of RF IgM ELISA(+) patients in Anti-CCP positive and negative patients.*

RF IgM ELISA is positive in 80.77% anti-CCP positive patients versus 20% of anti-CCP negative patients. (Fig. 5) So we can also say that RF IgM ELISA can be found more often in anti-CCP positive patients.

In normal clinical practice, the results of two independent tests can be combined to be more confident of the diagnosis. The two tests can be combined to increase the specificity by using following formula:

Specificity of combined test = 1 - (1 - specificity of test 1) × (1 – specificity of test 2)

Thus by combining anti-CCP assay and Rheumatoid Factor Latex test, the combined specificity is increasing to 98.75% which indicates that, in a polyarthritis patient if both anti-CCP and RF(Latex test) is negative then there is 98.75% 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 calculated by the following formula is 36.81%, which is less than the individual sensitivity of anti-CCP and RF (Latex test) assay.

Resultant combined sensitivity = sensitivity of test 1 × sensitivity of test 2

Similarly if anti-CCP antibody test is combined with RF IgM ELISA then combined specificity of the two tests is 95.81% and the resultant sensitivity is 68.82%. Individual and combined sensitivity and specificity of all the tests are shown below (Table 4).

Table 4. *Individual & combined sensitivity & specificity of different tests in the present study.*

Test	Sensitivity	Specificity
Anti-CCP IgGELISA (after modification)	91.23%	87.10%
RF IgMELISA	75.44%	67.74%
RF Latex test	40.35%	90.32%
Combined anti-CCP ELISA + RF IgM ELISA	68.82%	95.81%
Combined anti-CCP ELISA + RF Latex test	36.81%	98.75%

After comparing all the results it is seen that combining these two results can more efficiently diagnose rheumatoid arthritis because, if a patient is negative for both anti-CCP and RF assay there is 95 – 98% chance of the patient not having rheumatoid arthritis though the resultant sensitivity is reduced.

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:

Specificity of three combined tests = 1 − (1 − specificity of test 1) × (1 – specificity of test 2)× (1 – specificity of test 3)

Combined specificity of the three tests is 99.59%, 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 99.59% sure that the patient is not having rheumatoid arthritis, though the resultant sensitivity becomes 27.77%.

4. Discussion

The present study was done to compare the diagnostic utility of anti-CCP antibody and RF assay in RA patients in comparison to those with non-RA other rheumatic diseases. Several studies have shown that anti-CCP antibody test is highly specific and sensitive for diagnosing RA, and could be detected early in the disease course.

In the present study, sensitivity and specificity of

Rheumatoid Factor (Latex agglutination test) is 40.3% and 90.32% respectively. In another study conducted by Saraux *et al.* sensitivity of RF Latex test was 45%. [11] In a different study conducted by Binesh *et al.* (2014), it was observed that the sensitivity of RF Latex test was 46.24% and the specificity was 90.29%. [12] Aflaki *et al.* reported similar values of RF Latex test. [13] In the present study, sensitivity and specificity of Rheumatoid Factor IgM ELISA is 75.44% and 67.74% respectively. In a separate study conducted by Swedler *et al.* sensitivity and specificity of RF IgM ELISA was 91% and 76% respectively. [14] In another study conducted by Bas *et al.* sensitivity and specificity of Rheumatoid Factor IgM ELISA was 73% and 82% respectively. [15]

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.* has also mentioned that sensitivity of RF IgM ELISA is better than the sensitivity of RF Latex test. [14] Niewold *et al.* 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 when the test was done at first presentation, although rheumatoid factor was one of the criteria for the diagnosis of RA in these studies. Some of the variability in the sensitivity and specificity between studies may relate to slightly different cut-off points for positivity, and differences in disease duration, severity and other clinical characteristics of the groups being tested. [16]

In the present study, sensitivity and specificity of anti-CCP antibody is 91.23% and 70.97% respectively. In another study conducted by Gupta R *et al.* (2009) sensitivity and specificity of anti-CCP antibody was 85% and 90.19% respectively. [17] In a study conducted by Schellekens *et al.* (2000), sensitivity and specificity of anti-CCP antibody was 68% and 98% respectively. [18] Goldbach-M. *et al.* (2000) reported that sensitivity and specificity of anti-CCP antibody was 50% and 90% respectively. [19] According to Bizzaro *et al.* (2001) sensitivity and specificity of anti-CCP antibody was 41% and 98% respectively. [20] Walther J. *et al.* has mentioned that accumulated data from 164 studies published between 2002 and 2010 showed that sensitivity of anti-CCP antibody was 61 - 75% and specificity was 94-99%. [21] Suzuki *et al.* (2003) reported that sensitivity and specificity of anti-CCP antibody was 88% and 89% respectively. [22] So it is quite obvious that the present study is showing reduced specificity of anti-CCP antibody test in comparison to the other studies, and it is mainly due to the large number of anti-CCP positive cases (29.03%) in the control group who are having non-RA other rheumatic diseases.

In the present study it has been observed that in the non-RA control group of patients, anti-CCP antibody was positive in 9 out of 31(29.03%) cases. According to Walther J. *et al.* accumulated data from 164 studies published between 2002 and 2010 showed that 6.0% non-RA other rheumatic polyarthritis patients were positive for anti-CCP antibody. [21]

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. [23]

Another important observation in the present study was that, out of 9 anti-CCP positive cases in the control group 5 cases were weakly positive (titer $< 3 \times$ cut-off value) for anti-CCP antibody. Their anti-CCP titer is within the range of 6.25 − 20 U/ml. If 5 weakly positive cases (6.25 – 20 U/ml) in the control group are excluded from the 9 positive cases in the control group and considered as negative, then the specificity of the test becomes 87.10% which is quite acceptable.

Explanation in favor of this modification is that people of North-Eastern part of India are genetically related to Mongolian, Chinese and Japanese people. In a large cohort study in Japan, it has been observed by Suzuki A, *et al*. that single nucleotide polymorphism of Peptidyl-arginine deiminase (PADI) gene is closely related to rheumatoid arthritis patients. [24] This as sociation has been confirmed in other Asian populations, but not in Western Europe. [25] So single nucleotide polymorphism of PADI gene may be common in North-Eastern part of India. Vossenaar, E. R. *et al.* has mentioned that induction of PADI expression and citrullination of peptides are not specific to RA and can occur in many inflammatory settings. [26] So anti-CCP antibodies may be found in excess amount in other autoimmune inflammatory disorder. Although cross-reactivity of anti-CCP antibody with multiple citrullinated epitopes may occur, and could explain the recognition of multiple epitopes by a single antibody, the majority of anti-CCP antibodies seem to display distinct antigen-binding specificities. [21] Furthermore, as single nucleotide polymorphism may be common in this part of India, so weakly positive level of anti-CCP antibody may be found in other non-RA polyarthritis patients, and low positive level of anti-CCP antibody may vary from region to region in different ethnic groups of people. So in this part of India, if a patient has clinical findings suggestive of RA and anti-CCP titer at least three times higher than the cut-off value mentioned by the manufacturers, then we can be more confident about RA. But low positive titer of anti-CCP may be present in other type of arthritis patients.

In the present study anti-CCP antibody is positive in 97.67% RF IgM ELISA positive patients versus 71.42% RF IgM ELISA negative patients. So we can say that anti-CCP antibody can be found more often in RF IgM ELISA positive patients. Another study conducted by Saraux *et al.* also reported that anti-CCP antibody can be found more often in Rheumatoid Factor positive patients. [11] RF IgM ELISA is positive in 80.77% anti-CCP positive patients versus 20% of anti-CCP negative patients. So we can also say that RF can be found more often in anti-CCP positive patients. In a similar study Serdaroğlu *et al.* (2008) reported that RF is

positive in 90% of anti-CCP positive (18 out of 20) patients, and in 40% of anti-CCP negative (8 out of 20) patients. [27]

In the present study, if anti-CCP antibody test is combined with RF IgM ELISA, then combined specificity is 95.81%. In that case resultant sensitivity of the two tests is 68.82%. If anti-CCP antibody test is combined with RF Latex test, then combined specificity is 98.75%. In that case resultant sensitivity of the two tests is 36.81%. Similarly by combining the anti-CCP ELISA, RF IgM ELISA and RF Latex test, the combined specificity of the three tests is 99.59%. Parikh *et al.* has also shown that to make diagnosis of a disease more accurately, three diagnostic tests can be combined. [28]

5. Conclusion

This study showed that anti-CCP antibody assay can help significantly in diagnosis of RA. If anti-CCP antibody is combined with RF assay then RA can be more accurately diagnosed. However one problem of anti-CCP antibody test is determination of its cut-off value. The citrullination of arginine is under the control of PADI gene and in some studies genetic polymorphism of PADI gene has been found to be closely associated with rheumatoid arthritis in Asian people, particularly in Japanese people. The current study has been done in North Eastern part of India where people are genetically related to Mongolian, Chinese, Japanese people; so genetic polymorphism of PADI gene may be more prevalent in this region. In this study weakly positive titer (6.25−20U/ml) of anti-CCP antibody has been found in many other non-RA rheumatic diseases, and genetic polymorphism of PADI gene may be a cause behind this, which needs further studies. Correct determination of cut-off value is extremely important in predicting the accuracy of anti-CCP antibody assay. Cut-off value may vary from region to region and further study is needed to state this precisely. The combined anti-CCP and RF assay is more helpful than only anti-CCP assay in diagnosing RA accurately.

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