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Detection of nDNA antibodies in rheumatoid arthritis patients by an immunofluorescent technique

Hussain, Z. and Hasnain, N.*

Department of Microbiology and Molecular Genetics, Quaid-e-Azam Campus, University of the Punjab, Lahore-Pakistan.

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The main objective of this study was to focus on the relationship of nDNA antibodies with rheumatoid arthritis (RA) and to determine the specificity, sensitivity, positive predictive value and negative predictive value of nDNA for the clinical diagnosis of rheumatoid arthritis. The study included a total of 40 rheumatoid arthritis cases that fulfilled the American College of Rheumatology (ACR) diagnosis criteria for rheumatoid arthritis as well as 40 age and sex matched controls. Agglutination technique was used for qualitative and semi-quantitative measure of rheumatoid factor (RF) and indirect immunofluorescence assay was employed for the determination of anti-nDNA antibodies. RF latex agglutination test was carried out to confirm RA cases, out of which four (10%) turned out to be negative, so only 36 RA cases were further investigated and analyzed through indirect immunofluorescence assay. Out of 36 individuals, 31 (86%) were negative, three (8.3%) were strong positive and two (5.5%) were weak positive. No significant association was found between nDNA antibodies and rheumatoid arthritis disease.

Key words: Rheumatoid arthritis, anti-native DNA antibodies, immunofluorescence assay, sensitivity, positive predictive value, negative predictive value.

INTRODUCTION

Joint disorders involving inflammation of one or more joints are collectively called arthritis. These conditions are classified as over hundred different types. Most important types of arthritis are immune-mediated, for example psoriatic arthritis and rheumatoid arthritis (RA). The word “rheumatoid” originated from Greek rheuma meaning "flow or current" and -oid "resembling" that translates as joint inflammation that is similar to rheumatic fever. It is also known as chronic inflammatory polyarthritis. It is a progressive and degenerative autoimmune disorder in which defense mechanism of the body starts to invade the articular system of the body. In this long-term disease, the joints become inflamed and swelled, resulting in pain and stiffness throughout the body. This disease can lead to chronic inflammation and irreversible destruction of bone and cartilage. It can also contribute to the occurrence of clinically significant severe comorbid conditions if not properly treated (Kalreskog et al., 2009). The risk of mortality for patients with rheumatoid arthritis is 38% greater than the general population throughout

*Corresponding author. E-mail: nageen1704@hotmail.com.

Abbreviations: RA, Rheumatoid arthritis; **ACR,** American College of Rheumatology; **RF**, rheumatoid factor; **IFA,** immunofluorescence assay.

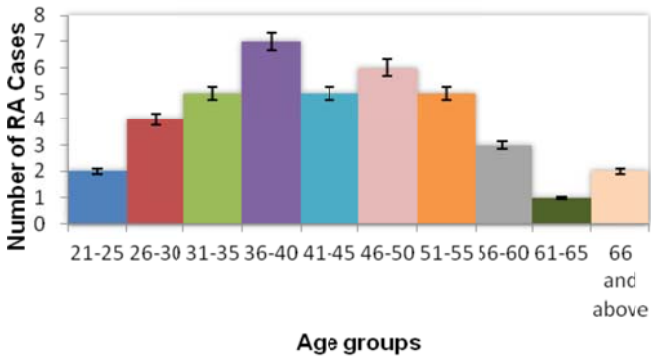

Figure 1. Age distribution in RA cases.

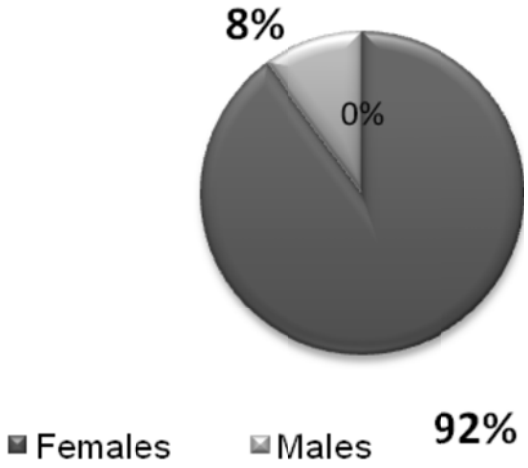

Figure 2. Gender ratio in RA case.

the world whereas this ratio for female patients is 55% more than the general population (Gabriel et al., 2003). Male gender, rheumatoid factor, extra-articular manifestations and co-morbidity have been implicated as major predictors for increased mortality (Rochmis et al., 1974).

Until 1973, no study was done to determine the prevalence of antibody to nDNA in a large group of patients with well-characterized rheumatoid arthritis. After decades of development in the field of laboratory techniques of diagnostics, more accurate and specific methods are now available. In the urban population of Southern Pakistan, the prevalence of RA is reported to be 0.142 per 100 inhabitants, whereas in northern Pakistan the estimated prevalence is 0.55 per 100 inhabitants (Hameed and Gibson, 1996; Farooqi and Gibson, 1998). According to a local survey, female to male ratio in Pakistani RA patients is 4:1 (Alam and Kidwai, 2011). This is the first study done for the detection of nDNA antibodies in the sera of rheumatoid arthritis patients by indirect immunofluoresce assay. The study focused on the relationship of nDNA antibodies with rheumatoid arthritis and to determine the specificity, sensitivity, positive predictive value and negative predictive value of nDNA for the clinical diagnosis of rheumatoid arthritis.

MATERIALS AND METHODS

The study included a total of 40 rheumatoid arthritis cases that fulfilled at least four of the seven the American College of Rheumatology (ACR) criteria. A total of 40 individuals were included in this study on initial clinical diagnosis as RA patients. Also, 40 healthy individuals that were age and gender matched with RA cases were included as controls. There were two (5%) RA cases from age group 21 to 25, four (10%) from age group 26 to 30, five (12.5%) from age group 31 to 35, seven (17.5%) from age group 36 to 40, five (12.5%) from age group 41 to 45, six (15%) from age group 46 to 50, five (12.5%) from age group 51 to 55, three (7.5%) from age group 56 to 60, one (2.5%) from age group 61 to 65 and two (5%) from age group 66 and above (Figure 1). Around 92% of the RA cases were female (Figure 2). Therefore, the female to male ratio was approximately (11:1). Average age of cases was 40.9 years.

Agglutination technique was used for qualitative and semi-quantitative measure of rheumatoid factor (RF) and indirect immunofluorescence assay was employed for the determination of anti-nDNA antibodies. For serum agglutination RF test, RF latex kit was used by Spectrum, an Egyptian Company for Biotechnology (catalog number: 518 002). It is the rapid latex agglutination test for the qualitative screening and semi-quantitative determination of RF in human serum. This test was employed to confirm RA cases included in this study. For indirect Immunofluorescence assay, anti-nDNA antibodies kit was used for the determination of anti-nDNA antibodies *in vitro* by Orgentec (catalog number: ORG 801). Statistical analysis like univariate analysis, Chi-square test and Fisher's exact test were carried out by SPSS (ver. 13). Specificity, sensitivity, negative predictor value and positive predictor value were calculated by using online software "Diagnostic Test Statistics" (Maceneaney and Malone, 2000).

RESULTS

Different parameters in this study were calculated and for further analysis of the findings, graphical formats were used. Pain levels were estimated in each RA case from clinical analysis. Patients were asked to score for their pain level from one to ten, according to the intensity of the pain. These scores were calculated into percentages. According to the standard in Figure 3, 10 levels of pain intensity were established in which 0 stands for no pain in the joints, whereas, 100 stands for severe pain intensity. Level 3 had one (2.5%) number of cases, level 4 had four (10%), level 5 had six (15%), level 6 had eight (20%), level 7 had 15 (37.5%) and level 8 had six (15%) number of RA cases (Table 1). RF latex agglutination test was carried out to confirm RA cases, out of which four (10%) turned out to be negative (Figure 4). As RF test is one of the important criteria in ACR guidelines, the negative cases of RF latex agglutination test were excluded from the study to ensure further standard results of indirect immunofluorescence assay (IFA). Semi-quantitative RF latex test was also carried out to estimate the titer of RF in blood samples of rheumatoid arthritis patients.

After confirmation through RF latex agglutination test, only 36 RA cases were further investigated and analyzed through indirect immunofluorescence assay. Out of 36

None 0 ----------`----------`----------`----------`----------`----------`----------`----------`----------`100 Severe

Figure 3. Standard used for the calculation of pain intensity.

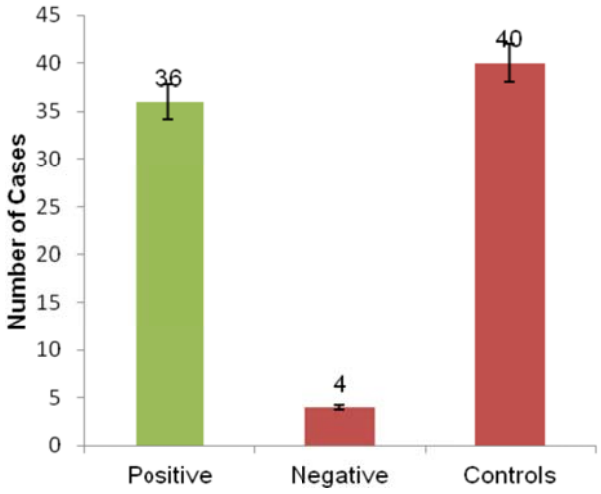

Figure 4. Bivariates of RF in RA patients (n = 40) and control groups (n = 40).

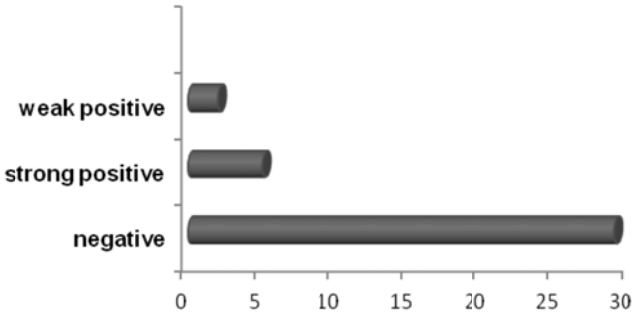

Figure 5. IFA for nDNA antibodies results.

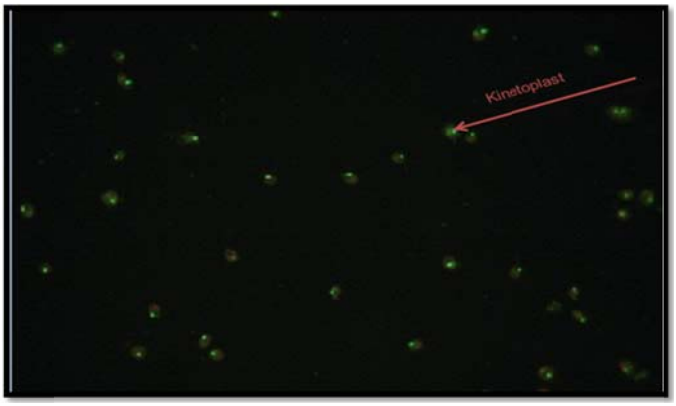

Figure 6. IFA for nDNA antibodies positive result (100× magnification power).

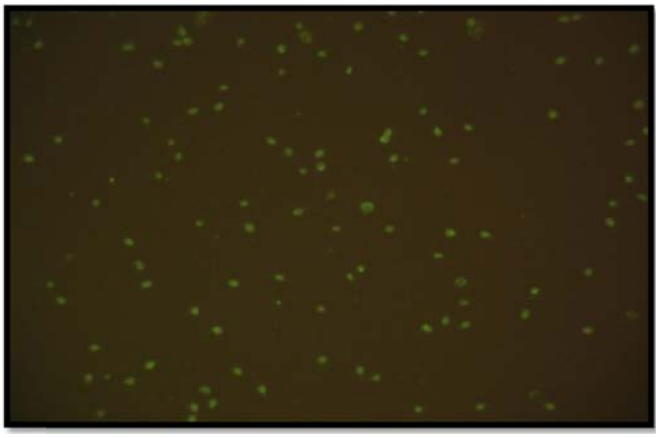

Figure 7. IFA negative result (100× magnification power).

individuals, 31 (86%) were negative, three (8.3%) were strong positive and two (5.5%) were weak positive (Figure 5). The slides used in IFA for nDNA antibodies kit contained hemoflagellates Crithidia luciliae. In these pear-shaped microorganisms, the helical nDNA is present in the large mitochondrion, part of which is called kinetoplast. It appears to be slightly concave disc-shaped structure found between the central nucleus and the basal body of the flagellum. In positive samples,
formation of a stable three-part complex consisting of fluorescent antibody bound to human anti-nDNA antibody, which is bound to nDNA antigen (Figure 6). This binding was visualized using fluorescent microscope with required filters, under which the kinetoplast appears to be of bright apple green color. While in negative samples, kinetoplast showed no fluorescence (Figure 7).

Statistical analysis

Mean and standard deviations were also calculated for both techniques (Table 2). Mean values for RF test was 38.2 ± 2 for positive results whereas for negative results, it was 22 ± 18. Mean values for IFA for nDNA antibodies for positive results was 20.5 ± 15.5 whereas for negative results, was 33.5 ± 2.5. A number of statistical analysis measures were applied (Table 3). Chi-square test and

Table 1. Pain intensity in RA cases.

Pain level	Pain level in percentage (%)	Number of RA cases	RA cases in percentage (%)
1	10	0	0
2	20	0	0
3	30	1	2.5
4	40	4	10
5	50	6	15
6	60	8	20
7	70	15	37.5
8	80	6	15
9	90	0	0
10	100	0	0

Table 2. Univariant analysis of RF and IFA test results.

Analysis applied	Mean	
	Positive	Negative
RF	38.2±2	22±18
IFA	20.5±15.5	33.5±2.5

Table 3. Statistical analysis of RF test results and IFA for nDNA antibodies test results.

Status	Case	Control	Fisher's exact test value (p)	Chi square test value (p)
RF				
Positive	36	0		
Negative	4	40	*<0.0001	*<0.0001
Total	40	40		
IFA test				
Positive	5	0		
Negative	31	36	**0.0539	**0.0637
Total	36	36		

*Extremely statistically significant, ** not statistically significant.

Table 4. Sensitivity, specificity, positive and negative predictor value of nDNA for the clinical diagnosis of RA.

Antibody	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	PPV (%)	NPV (%)
nDNA	13.88 (4-29)	100 (90-100)	100 (47-100)	43.73 (41-66)

PPV, Positive predictive value; NPV, negative predictive value; CI, confidence interval.

Fisher's exact tests were employed to determine association between nDNA antibodies and rheumatoid arthritis. As Chi-square test is used for larger population whereas Fisher's exact test is used for small number of population, both tests were used for comparison of statistical analysis.

Both tests showed that there was no significant association of nDNA with RA, while RF was found to be significantly associated with RA. In this study, the IFA nDNA antibodies test was 100% specific and 13.88% sensitive for rheumatoid arthritis. Table 4 represents specificity, sensitivity, negative predictive value and positive predictive value calculated by diagnostic test statistics tool for the clinical diagnosis of rheumatoid arthritis. Furthermore, 95% confidence interval was calculated by binomial expansion.

DISCUSSION

Rheumatoid arthritis is a disease caused by dysfunctional immune system of the human body. Throughout the

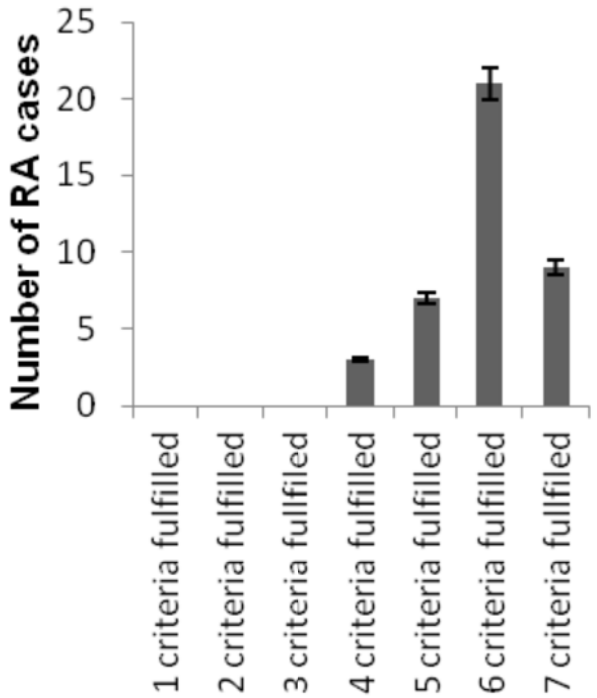

Figure 8. ACR criteria for diagnosis agreement in RA cases (n = 40).

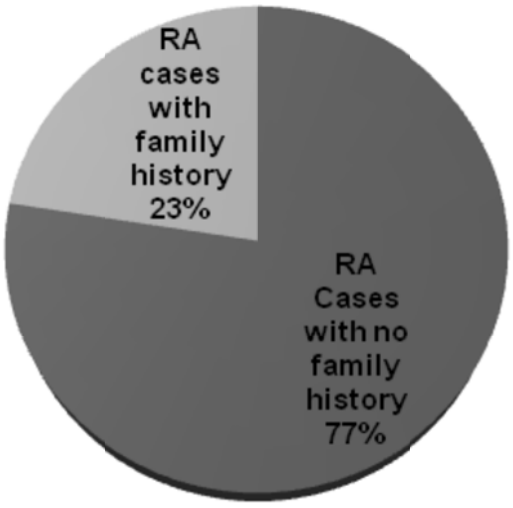

Figure 9. Family history in the studied RA cases**.**

history of medical advancements and discoveries, Rheumatology has remained a well sought field. Unfortunately, patients with rheumatoid arthritis disease have not experienced significant improvements in survival over the past four decades, despite dramatic downfall in the overall rates of mortality in the general population (Gonzalez et al., 2007). The present study was carried out for the evaluation of diagnostic measures of rheumatoid arthritis in routine laboratories. To establish this, two immunology-based techniques were employed and a number of other factors were studied among rheumatoid arthritis patients. Guidelines and criteria from ACR were considered to diagnose and select RA population from general population. Only three (7.5%) fulfilled four criteria out of seven, seven (17.5%) fulfilled five criteria, 21 (51%) fulfilled six criteria and nine (22.5%) fulfilled all seven criteria (Figure 8). The female to male ratio was 11:1, indicating higher number of females in RA cases. There can be several reasons for the unequal gender distribution in rheumatoid arthritis. Hormonal exposures, reproductive factors and live birth histories may have a contributing role in greater female ratio among RA cases. The distribution of RA cases in age groups was totally random and can be considered as the representative RA cases among general population of the same age groups. The distribution of age groups of RA cases indicate that the highest number of RA cases belonged to the age group 31 to 45 years (40%) whereas age 21 to 25 and above 65 had lowest number of RA cases (5%).

According to Orozco et al. (2007), there is a strong genetic linkage of rheumatoid arthritis among populations throughout the world. The pattern of rheumatoid arthritis hereditary is not simple, as there can be several genetic factors involved, that is the polygenic inheritance pattern (Deighton and Walker, 1991).

No significant and well established study has been carried out in Pakistan to investigate genetics of RA in this region. For this reason, the genetic linkage was determined by clinical history of each RA case. There were six RA cases (23%) out of 40, who had family history of rheumatoid arthritis disease (Figure 9). All of them were females and five out of those six cases might have inherited rheumatoid arthritis disease from their mothers.

For the estimation of the overall disease status and condition of RA cases, a number of clinical parameters were considered. This was done to estimate the disease prognosis of each RA case. Edwards et al. (2009) suggested that pain is the most common and most impairing stressing factor in rheumatoid arthritis patients. This is the reason pain intensity levels were estimated. The highest was level 7, which indicates 37.5% of RA patients suffer from very intense pain, thus have severe conditions. This data helps in determination of severity and stage of RA disease, and can also be used for monitoring the treatment therapy effectiveness.

Recently, Salaffi et al. (2010) reported that overall health is significantly affected in RA patients. Health conditions are important hallmarks of rheumatoid arthritis disease. During clinical analysis of RA cases, overall health was observed and recorded through a series of questions (Figure 10). Patients were given four health statuses among which they were asked to mark their current health condition. There were 16 (40%) out of 40

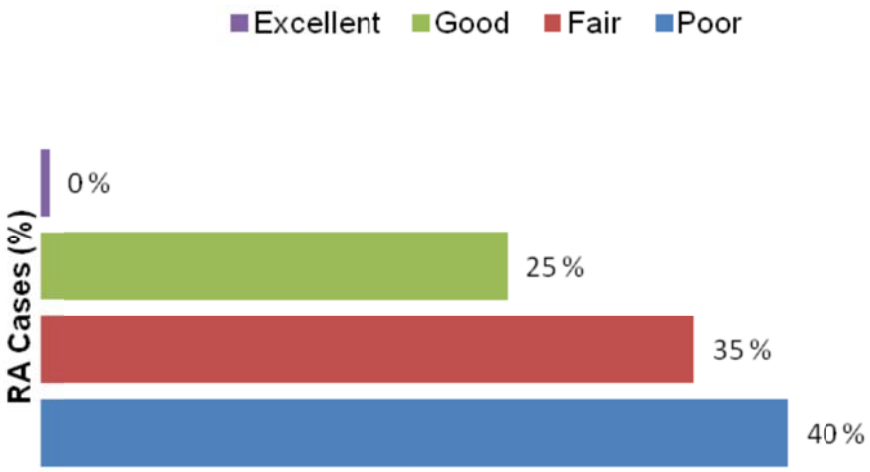

Figure 10. Overall health of RA cases.

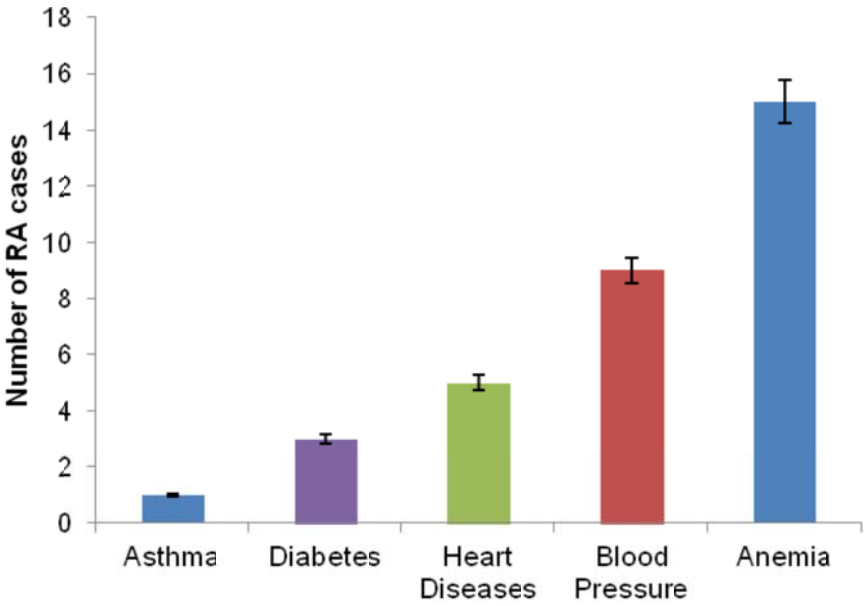

Figure 11. RA cases with other diseases.

RA cases having poor health. These cases can be considered as severe cases of rheumatoid arthritis disease and various factors can be involved. Around 14 (35%) of the cases were of fair overall health and only 10 (25%) were of good health. No patient included in this study had excellent health. Extra-articular manifestations other than anemia have been reported in about 15 to 25% of individuals with rheumatoid arthritis (Turesson et al., 2003). Al-Ghamdi and Attar (2009) conducted a research to analyze the frequency and influence of other complicated diseases in RA population. According to this report, anemia is very consistently reported to be present (31%) in rheumatoid patients at RA care centers. In this present study, results are in accordance to Al-Ghamdi's work (Figure 11). One (2.5%) RA case was found to have asthma, three (7.5%) had diabetes, five (12.5%) had heart diseases, nine (22.5%) had blood pressure and 15 (37.5%) of the RA cases had anemia.

Radiological assessments are periodically done to the rheumatoid arthritis patients to observe the prevailing deformity and degeneracy of joint bones (Keelgren and Lawrence, 1957). X-ray radiographs were observed during clinical analysis from each RA case. These radiographs showed various degrees of deformity of joints. Age is an important factor and it is investigated to study the course of the rheumatoid arthritis disease. In

this study, age groups 31 to 35 and 36 to 40 both accounted 30% of total RA cases. Boey et al. (1987) reported similar results while studying patterns of disease in RA patients and found that age group 31 to 40 (27%) had most RA patients. Tobacco consumption is considered as a serious risk factor for many diseases including rheumatoid arthritis (Stolt et al., 2003). Although some recent studies show a high number of smokers in rheumatoid arthritis population, there was no smoker identified in this study. Several reasons can be accounted, such as there was much larger ratio of females in rheumatoid arthritis cases and comparatively very small ratio of males. Culturally in this part of the world, women do not smoke, and there was no smoker among male RA cases. Rheumatoid factor was found to be strongly associated with rheumatoid disease, although four (10%) out of 40 clinically diagnosed RA cases were found to be negative for RF test though they fulfilled the ACR criteria for rheumatoid arthritis diagnosis. There can be several factors involved. One of them might be the prolonged medication that accounted for RF seronegative rheumatoid arthritis patients.

It is very important to continue identification of new diagnostic elements such as autoantibodies, antigens and other immunogenic elements involved in the etiology of the disease. This provides new insight into pathogenesis of the disease and provides a better understanding of the disease process to ensure the future possibilities for the development of new therapeutic strategies to help RA patients to gain the quality of life. There are other autoimmunogenic elements having specificity with rheumatoid arthritis, such as anti CCP, anti-RA33, anti collagen and anti GP1 antibodies (Steiner and Smolen, 2002). Deegan (1980) suggested that anti nDNA antibodies were diagnostically important. The antinuclear antibodies for double stranded DNA were found to be present in some RA cases but there was no significant specificity found between nDNA antibodies for RA cases (Carolyn and Norman, 1979). Although it is considered to be a routine laboratory test for SLE for years, the association of nDNA antibodies and rheumatoid arthritis was estimated through indirect immunoflouresence assay (Juby et al., 1994). There were five (13.8%) positive rheumatoid arthritis cases in this study. Some studies showed the possibility that the antinuclear antibodies maybe present in both SLE and RA patients (22). Although there was no significant association found between nDNA antibodies and rheumatoid arthritis disease, indeed it provides an addition to scientific knowledge in the fields of immunology and rheumatology.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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