World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers
Available online at: http://www.wjpsonline.org/
Original Article

The Study of Frequencies of HLA-DR3 and DR4 Among Type 1 Diabetes Mellitus Patients

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Received: 15-11-2014 / Revised: 28-11-2014 / Accepted: 29-11-2014

ABSTRACT

Type 1 diabetes mellitus (T1DM) is so far thought to be an auto-immune disease. This disease has revealed characteristics regarding predisposing factors. One of the genetic predisposing factors is thought to be HLA-DR3 and DR4 genes. The present study aimed at investigating the frequencies of (Human Leukocyte Antigen) HLA-DR3&DR4 genes in a group of 64 Iraqi patients, in whom the T1DM has been already diagnosed and compared to a group of apparently healthy (control group N= 32) individuals. The Real-time PCR was used for the HLA-genes in all members of the study. On the context of genotyping of HLA- allele, DR3 and DR4 were found to be different in their frequencies significantly among T1DM patients that creating high etiological fraction of 0.404 and 0.707 respectively compared to healthy controls, with odd ratio (OR) 4.61 for DR3 and high OR for DR4 26.73. This allele, occurred in high frequencies of the two genes in T1DM patients, was high significantly different between patients group for DR3 compared to healthy group which 51.6% and 18.8% respectively, and very high significantly different between patients group for DR4 compared to healthy group which 73.4% and 9.4% respectively, and both DR3&DR4 also occurred in high significantly difference which 43.8% and 6.3% respectively in T1DM and healthy control group.

Key words: T1DM, HLA, DR3, DR4, Gene, Alleles, Real-time PCR.

INTRODUCTION

For at least 20 years, diabetes rate has been increasing substantially in the world. In 2013, there were about 382 million persons who have diabetes all over the world. More than 34.6 million persons can be found in the Middle East and North Africa regions (MENA). By 2035, this ratio will increase to 67.9 million. The diabetic patients in Iraq alone increased from 668,000 cases in 2000 to became 1.2 million in 2013 [1, 2]. Type 1 Diabetes Mellitus (T1DM) is immune-mediated. It is one of the most common severe chronic illnesses today, affecting approximately 1 in 300 children and probably a greater number of adults [3]. Mean incidence rates of T1DM varied considerably, depending on the geographic region [4]. The worldwide incidence of T1DM is described to differ by at least 100- to 350 fold among different countries [5]. The highest incidence rates are found in Finland and Sardinia (Italy) and the lowest in the countries of South American region, e.g. Venezuela and Brazil, and Asian countries, e.g. China or Thailand [5, 6, 7]. The incidence seems to be increasing in countries around the world and is predicted to be approximately 40% higher in 2010 than in 1997 [8]. High incidence rate is now reported from a number of non-European population. Kuwait has the seventh highest rate in the world [5]. Egypt is on top of all the countries in the (MENA), with a prevalence of diabetes reported as 15.27% in adults [9]. In 2010, it has been shown that almost a quarter of all the diabetic children in the MENA region under 15 years old were in Egypt with a prevalence of 12.6%. The disease is characterized by irreversible autoimmune selective destruction of insulin-producing beta cells in the pancreatic islets [10]. Genomic studies have confirmed that the main locus, which determines the genetic susceptibility to T1DM, is encoded within the HLA (Human Leukocyte Antigen) region on human chromosome 6p21.3 [11]. The HLA is classically divided into three regions known as: class I genes (HLA-A, HLA-B, HLA-C), class II genes (HLA-DR, HLA-DQ, HLA-DP) [12]. There are two major classes of class II genes, the DR and DQ genes. It has been estimated that 60% of the genetic susceptibility to T1DM is conferred by the HLA

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[13]. The highest risk DR haplotype for T1DM are DR3 and DR4; these alleles account for 30%-50% of genetic T1DM risk ([14]. More than 60 genes have been identified to affect the risk of T1DM, with the HLA loci having the greatest impact on susceptibility [15, 16]. The strongest genetic association for T1DM is with the HLA class II genes. It is estimated that 30–50% of the genetic risk for T1DM can be attributed to the HLA region [14]. T1DM susceptible HLA alleles are very common in the general population. The HLA genotype, which is the combination of HLA alleles inherited from both parents, is the key for the development of T1DM. The major genetic determinants of T1DM are polymorphisms of class II HLA genes encoding DR, DQ, and to a lesser extent, DP [17]. A strong HLA association with T1DM was reported as early as 1973. By early 1980s, several associations between juvenile diabetes and various HLA antigens in case-control studies had been reported [18], and by 1990 it had become clear that a heterodimer, formed by class II molecules, showed the strongest risk [19]. In later studies, the extensive heterogeneity of the HLA component to T1DM emerged.

Genetic heterogeneity was definitively established by a demonstration that the HLA class II DR3 and DR4 serological associations at the DRB1 locus showed an increased risk of DR3/DR4 heterozygotes [20]. Furthermore, no current molecular model is fully explained because of the various effects that can be noticed in protective HLA alleles in T1DM, genotype, and haplotype [21]. The study aimed to understand the role of HLA-DR3 & DR4 genes in the initiation of T1DM. In Iraq, the diabetes mellitus prevalence is highly increasing in children compared to the two decades ago. Thus, this study is adopted to evaluate immunogenetics of diabetic Iraqi patients under 18^{th}, by the detection of HLA-DR3 and DR4 genes in this group of patients compared to the normal population by using RT-PCR.

MATERIALS AND METHODS

Subjects: Sixty-four patient comprising of (32) males (50%) and (32) females (50%) clinically diagnosed as T1DM at the Diabetes Center/ Al-Diwaniya Teaching Hospital, with a range of (4) to (18) years, mean age is (12.6 ± sd 4.3) years whereas the healthy control group comprising of (16) males (50%) and (16) females (50%), with age range of (6-18) years, mean age is (13.0 ± sd 3.8) years. All patients were subject to a detailed history and chemical examination.

Blood Collection and DNA Extraction: Blood samples were collected by vein puncture three milliliters (ml) of venous blood, taken from each patient and controlled by vein puncture, using disposable syringes under aseptic condition. Three milliliters of each sample were transferred to tube with K_2-EDTA (ethylene demine tetra acetic acid) (2.5 mg / ml), kept at –20 ºC for the genes of HLA class II DR3 and DR4.

Real-Time PCR: Real-Time PCR technique was performed to detect HLA-DR3 and HLA-DR4 genes in blood samples of diabetes mellitus of patients group, as wells as in healthy control group. This technique was carried out according to the method described by [22] and Genomic DNA from blood samples, extracted by using Accupower®Genomic DNA extraction kit (Whole Blood. Bioneer, Korea).

Genomic DNA profiling: The extracted genomic DNA from blood samples was examined by using Nanodrop spectrophotometer (THERMO. USA); it was an examination and a measurement of the concentration and purity of DNA through reading the absorbance in at (260 /280 nm).

Genotyping: The genotypes of the DR3 & DR4 genes were determined by using HLA-alleles specific sequence from NCBI-Gen Bank database (HLA-DR3: Gen bank code. M17379.1 and HLA-DR4: Gen bank code. L79973.1) and primer3 plus design online and were provided by (Bioneer Company, Korea) Table (1). The RT-PCR products were prepared by using each HLA gene and each AccuPrep® GreenStar qPCR PreMix kit (Bioneer. Korea), and accomplished according to the instructions of the company.

Real-Time PCR Thermocycler conditions: qPCR Thermocycler conditions were designed to all alleles specific primers according to primer annealing temperature and qPCR Syber green kit instructions (Bioneer. Korea).

Statistical analysis: Data were presented, summarized and analyzed by using two software programs. These programs were the Statistical Package for Social Science (SPSS) version 21 and Microsoft Office Excel 2010. Associations between two categorical variables were explored by cross-tabulation. The statistical significance of such associations was assessed by Chi-square ($\chi 2$) test. To measure the strength of association between 2 categorical variables, such as the presence of certain HLA genotype and disease status, the odds ratio (OR) was used. For example, OR for the association between having a specific genotype and having type-I DM equals the ratio of odds having the specific genotype versus, lacking it among cases to the similar odds among controls [23, 24].

RESULTS AND DISCUSSION

The present study was designed to examine association between HLA-DR3, DR4 genes in T1DM of Iraqi patients under 18th compared to the normal population by using RT-PCR.

Demographic characteristics Profile: The demographic profiles of both T1DM patients and control group are shown in Table (2). There is a significant difference between the T1DM patients and healthy controls at parameters.

Distribution of HLA-DR3 and DR4 genes in patients and control group: Distribution of HLA-DR3 & DR4 detected by RT-PCR technique and the frequency distribution of class II HLA-DR3 & DR4 alleles for patients, as compared to healthy control group in (%, OR, Chi, P, EF), are shown in (Table 3, Figure 1, Figure 2, Figure 3 and Figure 4). A survey of the distribution of HLA-DR3 and HLA-DR4 genes frequency yielded evidence of positive association between class II alleles and T1DM disease. For DR3, there was a high significant difference in the frequency of this gene, that is, 51.6% vs. 18.8%, with OR: 4.61, EF: 0.404 and Chi: 8.732 in comparison to healthy control; there was statically a difference (P 0.003). Moreover, DR4, the second gene examined in this study has also showed a very high significant difference as it was expressed in high frequency in T1DM disease patients compared to control group; 73.4 vs. 9.4% with OR: 26.73, Chi: 24.101, EF: 0.707 and P 0.001. Both genes (DR3 and DR4) are found in high frequencies in patients of T1DM disease compared to healthy control groups. The percentages of these genes among T1DM disease patients were 43.8% vs. 6.3% with OR 11.67, P value 0.001, Chi: 10.113 and etiological fraction 0.4. The results in this study agreed with [17], they revealed the major genetic determinants of T1DM, they are polymorphisms of class II HLA genes encoding DR and DQ. These results were compared to previous Iraqi studies using serotyping technique, which is reported by [25] high significant association of HLA-DR3, DR4, DQ2 and DQ3 with TIDM in Iraqi patients**.** A comparable study, published by [26], noticed the DR4 and DR3 (RR=7.0, 3.21 respectively). It was concluded that there was an association between these alleles and T1DM disease. These results compared to [14] revealed the highest risk DR haplotypes of T1DM are DR3 and DR4, and these alleles account for 30%-50% of genetic T1DM risk**.** Another study showed that 40% of whites in the US population have an HLA-DR3 or DR4 allele, at least 1 of these alleles was present in 95% of patients with T1DM, and estimated risk of developing T1DM for the general population in children, who have the HLA-DR3 & DR4 genes, which is approximately 1 in 15 to 1 in 25 vs. a risk of 1 in 300 in the general population [26].

Moreover, Japanese studies revealed that DRB1*0901-DQB1*0303 haplotypes conferred genetic susceptibility and the protective haplotypes DRB1*1501–DQB1*0602 and DRB1*1502–DQB1*0601 [27], while other study from France was reported DQB1*0302 allele which was a major risk and closely associated with disease than DR4 [28]. Moreover, the Turkish study reported by [29] observed DRB1*0402 DQB1*0302 (28.1% vs. 5.2%, OR: 7.1, $p < 0.0001$) and DRB1*0301 DQB1*02 (57% vs. 18.1%, OR: 6.1, $p < 0.0001$), 8.9%, OR: 0.1, $p < 0.0001$), DRB1*1502 DQB1*0601 (1.1% vs. 7.7%, OR: 0.1, $p = 0.0023$), and DRB1*1101 DQB1*0301 (3.9% vs. 12.1%, OR: 0.2, $p < 0.0001$). Furthermore, among the Slovak diabetic patients, genotyping reported by [30], the significantly associated alleles with the disease were (DRB1*0401 and DQB1*0302, OR: 4.9, 7.8 respectively) and (DRB1*0301 and DQB1*02 OR: 4.2, 2.2 respectively)**.** Among the Asian (Korea) DR4 and DQ8 (DRB1*0401-DQB1*0302) haplotypes, also (DRB1*0405-DQB1*0401) haplotypes, conferred susceptibility to T1DM which reported by [31].While study from Iran [32] indicated a positive association between HLADRB1* 01 and DRB1*03 alleles in T1DM. In Lebanon, 77% and 40% of TIDM patients were positive for DQ2 and DQ3 respectively [33].

Many studies have reported a variety of associations or absence of associations between T1DM and a particular HLA antigen. Different results regarding this association have reported various results in different populations. This fact could be explained by the variable ethnic backgrounds of studied patients; more likely the multiple etiologic bases for this disease, or might be attributed to gene drift, when some genes get associated together by chance or by gene flow which is the result of admixture between different populations [34].

CONCLUSION

There is significantly a higher concentration of HLA-DR4 gene compared to HLA-DR3 and both HLA-DR3 & DR4 genes among Iraqi T1DM patients. There is significantly a higher concentration of HLA-DR4 and DR3 genes compared to control group. This concept provided a strong evidence that HLA-DR3 and DR4 genes are common predisposing factor and play a major role in the pathogenesis for T1DM disease.

Table 1: The primers sequence with orientation and the RT-PCR product size.

HLA allele specific primer	Sequence		PCR Size
HLA-DR3	F	ATCCAGCCAGCATTGAAGTC	124bp
	R	ACTGTTTCCAGCATCACCAG	
HLA-DR4	F	AAGAGGAGTACGTGCGCTTC	147bp
	R	AGTTGTGTCTGCAGTAGGTGTC	

Table 2: The demographic characteristics of T1DM patients and controls group.

Parameters	Healthy controls		Diabetes Mellitus		P (t-test)
Age (years)					0.19 [NS]
Range	(6 to 18)		(4 to 18)		
Mean	13.0		12.6		
SD	3.8		4.3		
N$_{o}$	32		64		
Body weight (Kg)					0.9 [NS]
Range	(20 to 60)		(15 to 70)		
Mean	38.8		38.5		
SD	11.4		14.5		
N$_{o}$	32		64		
Gender	N	%	N	%	
Female	16	50.0	32	50.0	
Male	16	50.0	32	50.0	
Total	32	100.0	64	100.0	

SD: Standard deviation, N: number and NS: no significant.

Table 3: Distribution of HLA-DR3 and DR4 genes in patient and control group.

	Healthy controls (N= 32)		T1DM patients (N= 64)					
HLA gene	N	%	N	%	OR	Chi	EF	P
HLA-DR3	6	18.8	33	51.6	4.61	8.732	0.404	0.003
HLA-DR4	3	9.4	47	73.4	26.73	24.101	0.707	0.001
Both DR3 and DR4	2	6.3	28	43.8	11.67	10.113	0.4	0.001

Figure (1): Real-Time PCR amplification plot of gene HLA-DR3 in T1DM patients group.

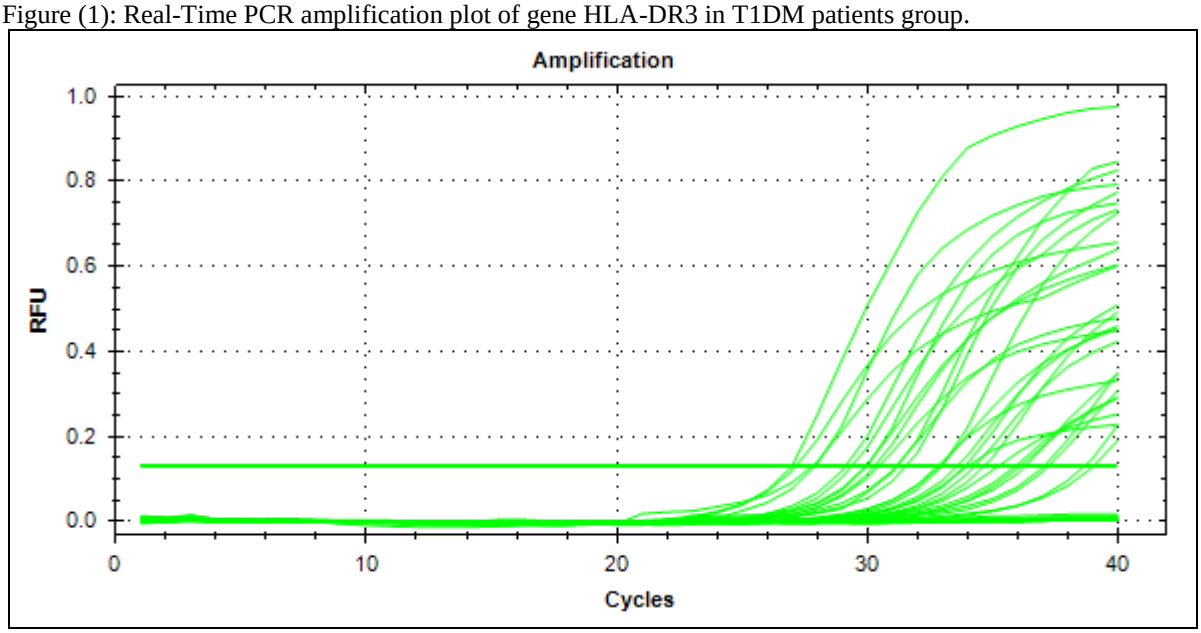


Figure (2): Real-Time PCR amplification plot of gene HLA-DR3 in controls group.

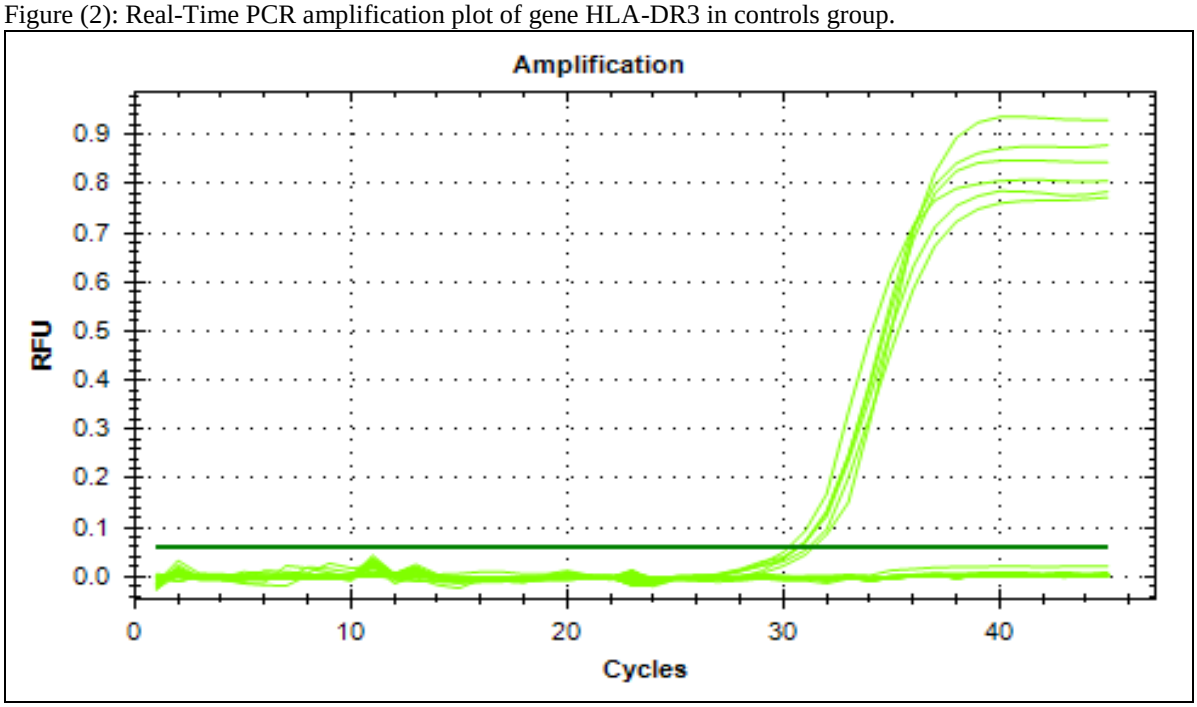


Figure (3): Real-Time PCR amplification plot of gene HLA-DR4 in T1DM patients group.

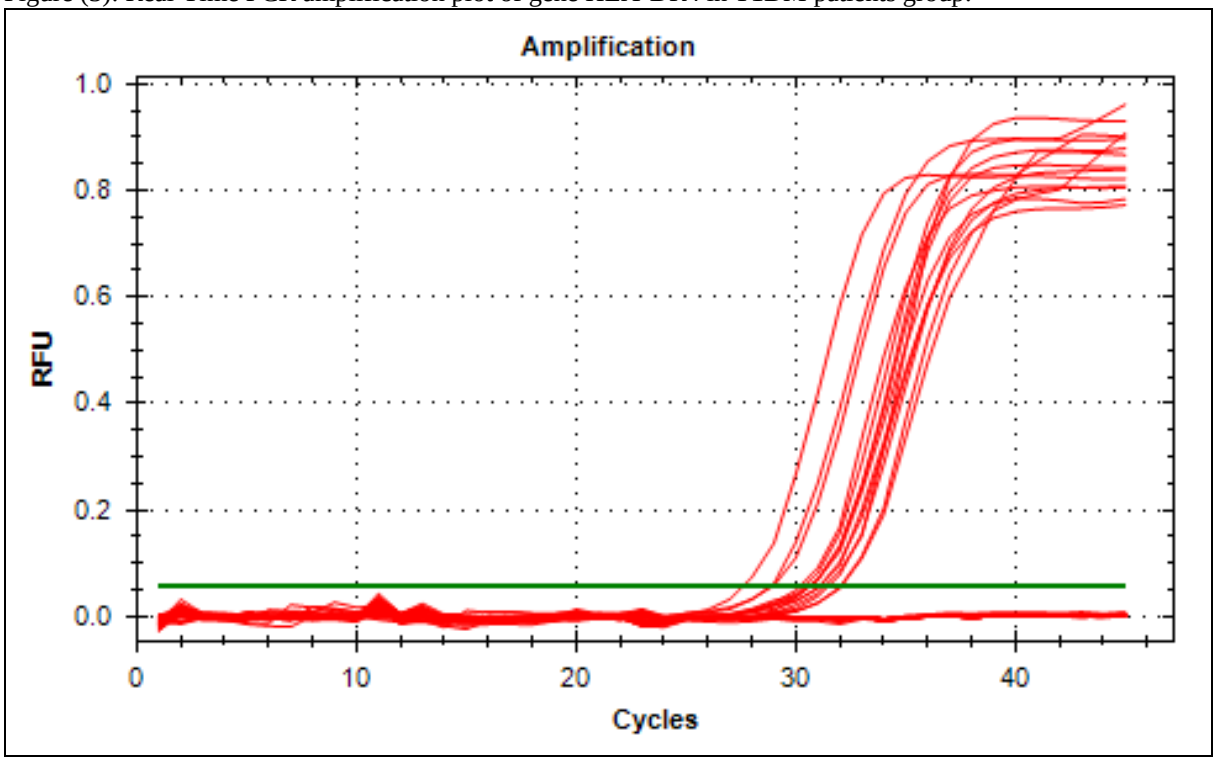
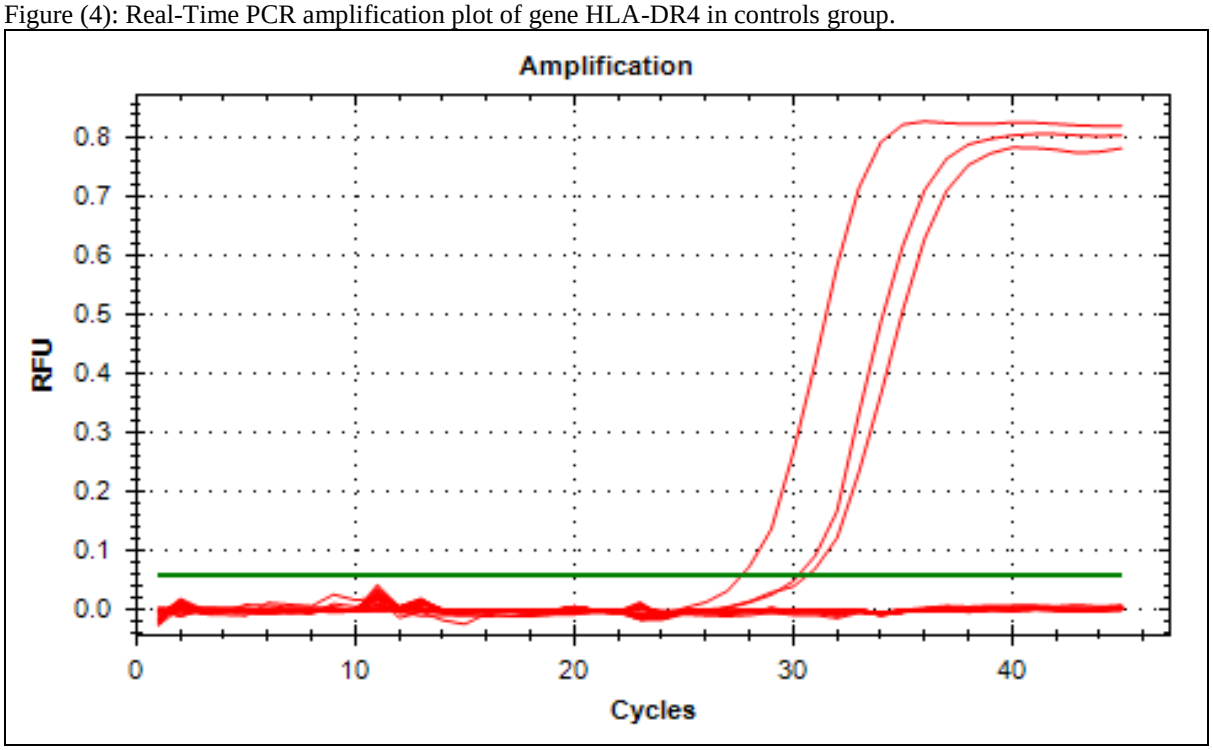


Figure (4): Real-Time PCR amplification plot of gene HLA-DR4 in controls group.



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