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THE ASSOCIATION OF HLA-*DRB1& DQB1* ALLELES AND HAPLOTYPE FREQUENCY IN IRAQI PATIENT WITH PULMONARY TUBERCULOSIS IN THI-QAR PROVINCE, IRAQ

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ABSTRACT

Tuberculosis (TB) is infectious disease that usually affects the lung. It is the second greatest killer due to a single infection agent worldwide (*Mycobacterium tuberculosis*), about one-third of the world, population is believed to have latent TB. There is 10% chance of latent becoming active TB. This study conducted in Thi-Qar province, included 210 samples (70 patients, 70 household contacts and 70 controls) with ages ≤10 to ≥70 years who were referred to AL-Nasiriya Center of Tuberculosis and Chest Diseases from the period September 2012—July 2013. Regarding the collected samples were both sputum and blood were obtained from 210 samples then conduct several test to diagnosis the infection with *Mycobacterium tuberculosis* as Tuberculin Skin Test (TST), and Gene X-pert. The results of TST were positive for total patients, positive for 25 cases of the HHCs and negative for total control group. While AFB examination were positive for total patients group, positive for 22 cases of HHCs group and negative for control group. The results of x-ray screening were positive for all patients group, in HHCs were positive for 20 cases and negative for all control group. While the results of the last diagnostic test Gene X-pert showed positive for all patients' cases, negative for control group and positive for 24 cases of HHCs.

To evaluation of immunogenic risk factors of tuberculosis infection by HLA alleles (DRB1*1051and DQB1*0601). The HLA antigens of the patients and HHCs groups showed significant increased frequencies compared with controls. In patients group, HLA DRB1*1501 allele (54.3 vs. 20%), and in HHCs group, HLA DRB1*1501 allele (30 vs. 20%), while HLA DQB1*1601 allele in patients showed (31.5 vs. 8.5%), and in HHCs group, HLA DQB1*1601 allele (35.7 vs. 8.5%).

KEYWORDS: Tuberculosis, HLA, Drb1*1501, Dqb1*1601

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by the bacterium, namely *Mycobacterium tuberculosis* and responsible for taking away so many lives all over the world. According to World Health Organization (WHO), TB is considered the second only to HIV/AIDS as the greatest killer worldwide (WHO, 2012).Primary infections with *Mycobacterium tuberculosis* produce active disease in approximately 10 % of those infected (Ahmad, 2011). *M. tuberculosis* TB-antigen specific immunity usually successfully controls the pathogen, although viable bacteria do remain

in granulomas for extend periods, this is known as latent tuberculosis infection (LTBI) (Huynh, 2011). Also, depend on many criteria a patient was considered as active pulmonary TB case if he has symptoms for 3 weeks or more with one of the following: fever, night sweating, loss of weight, persistent cough, tiredness, loss of appetite and fatigue. (Khatri, 2002; CDC, 2010). In 2011, 8.7 million people became infected and suffered from active TB and 1.4 million died due to this disease (WHO, 2012). WHO survey found that over 95 % of TB death occurs in low and middle incoming countries (WHO, 2012). Reactivation of latent tuberculosis (TB) may be induced by the administration of cytotoxic chemotherapy and high dose corticosteroid therapy (Adzic, 2004).

The human Major Histocompatibility Complex (MHC) is considered within about 4 Mbp of DNA on the short arm of chromosome 6 at 6p 21.3 (Marsh, 2007). A number of PCR-based methods for HLA class II typing have been emerged in recent year in order to provide a clear picture of the molecular basis of allelic polymorphism; although, they have their own limitations (Moalic and Ferec, 2005 ; Itoh *et al*., 2006). More recently a new methodology based on DNA microarray has developed to identify single Nucleotide Polymorphism (SNPs) encoded within the highly polymorphic MHC genes and allows simultaneous analysis of many SNPs in DNA from a large number of individuals, in a single experiment (Palmisano *et al*., 2005 ; Jinag *et al*., 2006) . PCR- Sequence Specific Priming (PCR-SSP) are the most common techniques that are being used for molecular HLA typing (Buhler *et al*., 2002; Downing *et al*., 2004). Methods such as PCR-SSP can be used but these procedures require large number of primers and probes. Many evidence support a significant role for host genetic variation in susceptibility to TB, and a complex interaction of genetic and environmental factors cause the development of clinical TB (Stead, 2001). This technique is being used more frequently because of its simplicity and time frame (Rudwaleit *et al*., 1995). A number of genes are thought have a role in the pathogenesis of TB (Hoal, 2002). The aim of study to evaluation of immunogenic risk factors of tuberculosis infection by HLA alleles (*DRB1*1051and DQB1*0601*)

MATERIALS AND METHODS

This study was conducted in AL-Nasiriya Center of Tuberculosis and Chest Diseases, Thi-Qar province, Iraq from the period (September 2012—July 2013). The samples were obtained from 70 patients with active pulmonary tuberculosis, 70 household contacts (HHCs) and 70 controls. The patients and their HHCs were investigated for the presence of LTBI and active TB.

Samples Collection

The patients and their HHCs and controls (n =70 of each) were interviewed using a structured questionnaire to collect information on socio demographic and clinical features and history of TB. In addition, they were clinically examined.

Blood Samples

Five ml of blood were collected by vein puncture into test tube containing anticoagulant EDTA until using in PCR test for HLA typing.

SSP- PCR TECHNIQUE

Genomic DNA Extraction (Vogelstein and Gilles pine, 1979)

Genomic DNA from blood samples were extracted by using Genomic DNA mini kit extraction kit (Frozen Blood) Gene aid, and done according to company instructions as following steps:

- A 200µl of frozen blood was transferred to sterile 1.5ml micro centrifuge tube, and then added 30µl of proteinase K and mixed by vortex. And incubated at 60℃for 15 minutes.
- After that, 200µl of lysis buffer was added to each tube and mixed by vortex vigorously, and then all tubes were incubated at 70℃for 15 minutes, and inverted every 3 minutes through incubation periods.
- 200µl absolute ethanol were added to lysate and immediately mixed by shaking vigorously.
- DNA filter column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to column. Then centrifuged at 10000rpm for 5 minutes. And the 2 ml collection tubes containing the flow-through were discarded and placed the column in a new 2 ml collection tube.
- 400µl W1 buffer were added to the DNA filter column, then centrifuge at 10000rpm for 30 seconds. The flow-through was discarded and placed the column back in the 2 ml collection tube.
- 600µl Wash Buffer (ethanol) was added to each column. Then centrifuged at 10000rpm for 30 seconds. The flow-through was discarded and placed the column back in the 2 ml collection tube.
- All the tubes were centrifuged again for 3 minutes at 10000 rpm to dry the column matrix.
- The dried DNA filter column transferred to a clean 1.5 ml micro centrifuge tube and 50 µl of pre-heated elution buffer were added to the center of the column matrix.
- The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

Genomic DNA Profiling

The extracted genomic DNA from blood samples was checked by using Nanodrop spectrophotometer (THERMO.USA), that check and measurement the purity of DNA through reading the absorbance in at in at (260 /280 nm) as following steps:

- After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
- A dry Chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet 1µl of ddH2O onto the surface of the lower measurement pedestal.
- The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of the appropriate.
- Blanking solution was added as black solution, which is same elution buffer of RNA samples.
- After that, the pedestals are cleaned and pipet 1µl of DNA sample for measurement.

Primers of HLA-Alleles

MHC HLA allele's specific primers that used in this study were design by using HLA-alleles specific sequence from NCBI-GenBank database and Batch primer design online, where provided from Bioneer Company, Korea as in (Table 1)

Table 1: The Primers and Their Sequence and Size

Primer		Sequence	Product Size
DRB1*1501	F	`5-ATCCAGGCAGCATTGAAGTC-3`	698bp
	R	`5-CAGCCTTGATGTAAGGCACA-3`	
DRB1*1501 Allele	F	`5-CAGGAAGAGAAGGCTGGGAT-3`	
	R		
DQB1*0601	F	`5-ACCCATTCCTTCCCATCTCT-3`	697bp
	R	`5-TCCTCCCATCTTGTCCTGTC-3`	
DQB1*0601 allele	F	`5-GGCTGGGCCTTATCATCC-3`	
	R	`5-CTCACCTTTCTGACTCCTTTGA-3`	

SSP- PCR Master Mix Preparation

SSP PCR master mix was prepared for detection DRB1501, DQB10601 gene by using (AccuPower PCR PreMix Kit) and this master mix done according to company instructions as following table: After that, these PCR master mix component that mentioned above placed in standard AccuPower PCR Pre-Mix Kit that containing all other recompo-nentswhich needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl2, stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000-rpm for3 minutes. Then placed in PCR Thermo cycler,(Table 2).

Table 2: Reaction Master Mix of PCR and Their Volume

PCR Master mix	Volume
DNA template	5ul
Forward primer	1.5ul
Reverse primer	1.5ul
Allele primer	1.5
PCR water	12ul
Total volume	20ul

PCR Thermo Cycler Conditions

PCR Thermo cycler conditions were done by using conventional PCR thermocycler as following (Table 3).

Table 3: Thermo Cycler Conditions of PCR

PCR step	Temp	Time	Repeat
Initial Denaturation	95C	5min	1
Denaturation	95C	30sec.	30 cycle
Annealing	58C	30sec	
Extension	72C	45sec	
Final extension	72C	7min	1
Hold	4C	Forever	-

SSP-PCR Product Analysis

The PCR products of 16S rRNA gene of Ana plasma spp. was analyzed by agarose gel electrophoresis following steps:

- A 1.5% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.
- Then 3µ of ethidium bromide stain were added into agarose gel solution.
- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperet-ure, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 5ul of (100bp Ladder) in one well. 4- The gel tray was fixed in electrophoresis chamber and fill by 1X T TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour.
- PCR products (577bp) as specific for 16S rRNA gene were visualize by using UV Trans illuminator.

RESULTS

Dignostic Tests for Tuberculosis

Our results revealed many tests for all study groups (patients, household contacts and controls) that included Tuberculin Skin Test (TST), Gen X-pert screening. The results of TST were positive for all patients group, positive for 25 cases of HHCs and negative for all control group. .Finally Gene X-pert results were positive for all patients group, positive for 24 cases of HHCs group and negative for 46 cases, as in (Table 4).

Table 4: The Diagnostic Tests of Tuberculosis Disease for All the Study Groups

Age Years	Patients				Household Contacts				Controls				Total
	TST		Gen X Pert		TST		Gen X Pert		TST		Gen X Pert		
	-	+	-	+	-	+	-	+	-	+	-	+	
≤ 10	Zero	10	Zero	10	7	3	7	3	10	Zero	10	Zero	10
11-20	Zero	10	Zero	10	6	4	6	4	10	Zero	10	Zero	10
21-30	Zero	10	Zero	10	5	5	5	5	10	Zero	10	Zero	10
31-40	Zero	10	Zero	10	7	3	7	3	10	Zero	10	Zero	10
41-50	Zero	10	Zero	10	8	2	6	4	10	Zero	10	Zero	10
51-60	Zero	10	Zero	10	8	2	8	2	10	Zero	10	Zero	10
≥ 70	Zero	10	Zero	10	7	3	7	3	10	Zero	10	Zero	10
Total	**zero**	**70**	**zero**	**70**	**45**	**25**	**46**	**24**	**70**	**zero**	**70**	**zero**	**70**
	70				**70**				**70**				**210**

HLA-ALLELES RESULTS

HLA- Alleles (DRB1*1501 and DQB1*1601) Of Patients and Control

The present study showed an increased association between the HLA-alleles (DRB1*1501, DQB1*1601) and pulmonary tuberculosis infection. DRB1*1501 allele revealed the highest association when compared with controls (54.3 vs. 20%), followed by HLA-DQB1*1601 allele (31.5 vs. 8.5%) (Table 5)

Table 5: The HLA Alleles Present in Patients with Active Pulmonary Tuberculosis

Type of Antigen of Class Ii HLA System	HLA Drb1 And HLA Dqb1 Alleles	Controls (N=70) Frequency		PTB Patients (N=70) Frequency		RR	X2
		Positive	%	Positive	%		
DRB1	DRB1*1501	14	20	38	54.3	4.75	18.4*
DQB1	DQB1*1601	6	8.5	22	31.5	4.9	41.92*

*significant difference (P≤0.05) between the patients and control groups

Degrees of Freedom =1; Pα 0.05=3.84

Positive =Persons possessing the particular allele.

RR-relative risk = $\frac{\text{Numberofantigenpositivepatientsxnumberofantigennegativecontrols}}{\text{Numberofantigenpositivecontrolsxnumberofantigennegativepatiens}}$

On the other hand we studies these alleles in household contacts (HHCs), the results showed an increased association between the HLA-alleles (DRB1*1501, DQB1*1601) and pulmonary tuberculosis infection.

DQB1*1601 allele revealed highest association in the HHCs when compared with controls (35.7% vs 8.5%), followed by HLA-DRB1*1501 allele (30% vs. 20%),(table:6)

Table 6: The HLA Alleles Present in Household Contacts Compared with Controls

Type of Antigen of Class Ii HLA System	HLA Drb1 And HLA Dqb1 Alleles	Controls (N=70) Frequency		Contacts (N=70) Household Frequency		RR	X2
		Positive	%	Positive	%		
DRB1	DRB1*1501	14	20	21	30	1.7	26*
DQB1	DQB1*1601	6	8.5	25	35.7	5.9	38.65*

*significant difference (P≤0.05) between the patients and HHCs groups

Degrees of freedom =1; Pα 0.05 =3.84

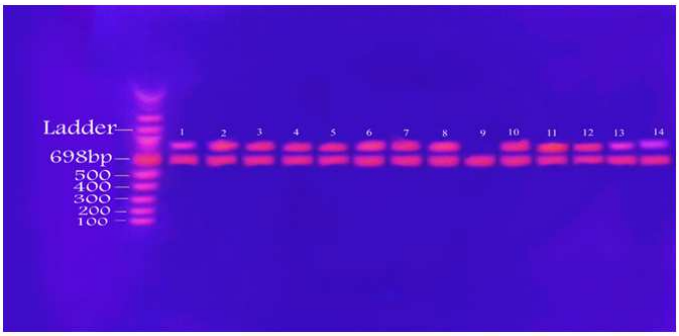

Figure 1: Bands of Extracted DNA on Agarose Gel Electrophoresis of DRB1*1501 Allele Obtained by SSP-PCR

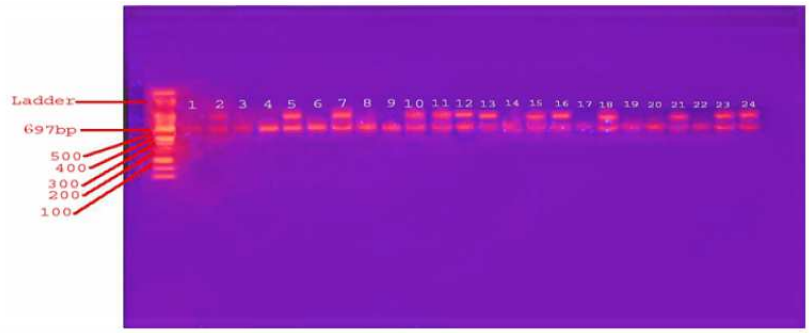

Figure 2: Bands of Extracted DNA on Agarose Gel Electrophoresis of DQB1*1601 Allele Obtained by SSP-PCR

DISCUSSIONS

Diagnostic Tests for Tuberculosis

There is no gold standard for the diagnosis of latent tuberculosis (TB) infection (LTBI), the Tuberculin skin test (TST) is used as a standard diagnostic tool to assess TB infection in Iraq.Our results showed that tuberculin skin test was positive for all patients group. In addition, the tuberculin skin test was positive in 25:70 (35.7%) household contacts (HHCs).Our finding match with study by Comstock *et Al* (1974) which they reported that only about 5 to 10% of persons with a reactive TST develop post primary TB or progress to reactivation TB during their lifetime, providing evidence of protective innate and adaptive immunity in the majority of persons .This rate is lower than reported from Pakistan (49.4%) (Marks *et al*.,2000;Rathi*et al*.,2000), and higher than report of Druszczynska *et al*. (2013)which he mentioned that the prevalence rate of TB(27%) was showed in HHCs TB contacts, and higher than report from Iraq (33.4%) among HHCs of skin-test positivehousehold members(Al-Kubaisy *et al*., 2003). Our finding approximately the same increment of report from USA(36%) among close contacts of skin-test positive household members (Rathi *et al*., 2000). However, this discrepancy in skin test results is most probably related to the characteristics of the studied population and the type of skin test solution used as well as the cut-off point used for positive results (Fernandez, 1994 ;Vidal,1997) .Our finding disagree with report of Hussain *et al*. (1995) who reported that 87% of household contacts were TST positive.

Recently, a new sensitive and more specific test that has been developed to diagnose the infection is known as Gene X pert, is more sensitive than sputum smear microscopy in detecting TB, and it has similar accuracy as culture (Boehme *et al*., 2010). Examination of sputum patients by Gene Xpert were positive for all (100%). This finding match with (Boehme *et al*., 2010) which he mentioned that, X pert MTB/RIF test among patients with sputum smear-negative, TB increased sensitivity by reach to 90.2%.Also agreement with reports of Boehme *et al*. (2010) and Zeka *et al*. (2011) which they mentioned that X pert MTB/RIFdetected rifampicin resistance with 99.1% sensitivity and excluded resistance with 100% specificity. The X-pert MTB/RIF assay identified correctly 97.6% (200 out of 205) of patients harboring rifampicin-resistant strains and 98.1% (504 out of 514) of those with rifampicin-susceptible strains. Also the results of our study showed that a sputum examination of HHCs group by gen Xpert MTB/RIF found that *Mycobacterium tuberculosis* bacteria are positive in 24:70 (34.30%) cases, while negative in 46:70 (65.72%) cases. There was study confirmed our results subjected by Boehme *et al*.(2010) which he reported that the x-pert assay detected pulmonary TB in all TB patients, including over 90% of smear-negative patients, with a high sensitivity of over 97%. Sensitivity for rifampicin resistance by

X-pert assay was 94.4% and specificity was 98.3%. Clinical validation trials done in four distinctly diversesettings showed that 92.2% of culture-positive patients were detected by a single direct x-pert MTB/RIF test (in comparison to the sensitivity of a single direct smear of 59.5%) (Rachow *et al*., 2011).

Human Leukocyte Antigen HLA-DRB1and DQB1

When genetic variations in pathogen polymorphism aresuperimposed on host genetic heterogeneity, considerable variation may occur in detectable allelic associations (Hill, 1998). The results showed that HLA-DRB1*1051 allele was found to be significantly more frequent inactive pulmonary tuberculosis patients compared with the controls group (54.3 *vs*. 20%) and decreased values of the relative risk (RR=4.75) compaction with RR of DQB1*0601 that was (4.9). In our study the same individuals has been used for all investigation such as PCR-SSP. We thought that, this result due to the role of HLA DRB1*1501 allele as primary risk factor in patients group. Also the increase in DQB1*0601 at patients group with active pulmonary tuberculosis (31.5 vs. 8.5%) and (RR=9.4). This result may be due to the role of DQB1*0601 as primary risk factor for increased susceptibility of pulmonary tuberculosis. Our results confirmed by many reports such as an association between tuberculosis and the HLA-DR2 antigen was shown by Singh in an Indian Hindu opulation (Singh *et al*., 1983). In addition, it was confirmed by the observation of (Khomenko*et al*., 1990) in Russian and Asian populations. The frequent haplotype in our study was HLA-DRB1*1501 and DQ1*0601 increased frequency in this haplotype in TB has been previously reported by (Ravikumar*et al*., 1999) in south India and by (Cox *et al*., 1988) in an Asian Indian population. Our study in agreement with (Singh *et al*., 1983) which he mention that there is association of tuberculosis with the HLA-DR2 antigen in an Indian Hindu populations.

Our results in agreement with (Teran*et al*., 1999) which he found increased frequencies of DRB1*1501 allele in Mexican patients with pulmonary tuberculosis. Our results disagree with (Goldfeld*et al*., 1998) which they reported that there is no specific HLA-DRB1 alleles were significantly increased in the patients with pulmonary tuberculosis.

The results in showed that the DRB1*1501 allele was detected more frequently in household contacts (HHCs) than controls, Its noted in 21 of HHCs (30 %) and disappear in 49, relative risk (RR=1.7). While DQB1*0601allele was detected more frequently in HHCs than controls It is noted in 25 (35.7%) and relative risk (RR=5.9). We thought this results due to the role of HLA-DRB1*1501 and DQB1*0601 in response immunity against *Mycobacterium tuberculosis* whereby appear of DRB1*1501 in some of HHCs consider primary risk factor of *Mycobacterium tuberculosis* infection, while disappear in negative HHCs, because it can act as protection factor against TB infection.

In addition, Appears of DRB1*1501 allele In some cases of controls group due to the role of this allele in immune response were act as risk factor so DQB1*0601 have the same role in the immune response against TB infection , whereby found it in the positive HHCs group consider indicate on its role as primary risk factor while disappearance in negative HHCs is indicate on its role as protection factor. Our results in agreement with (Ravikumar*et al*., 1999) which he mentioned that the most frequent haplotype was HLA-DRB1*1501 and DQB1*0601pulmonary tuberculosis infections. Also match with (Ravikumar*et al*., 1999 ; Sriram, 2001) which they reported that in India and china the marker of susceptibility to tuberculosis was the allele group DRB1*15. Study by Dubaniewicz and his colleagues, reported that the DRB1*15, especially the DRB1*1501 allele, is strongly associated with PTB susceptibility (Dubaniewicz*et al*., 2000). In addition, increased in HLA allele found association with susceptibility to pulmonary tuberculosis in other populations (mainly DRB1*15 and DQB1*0601) (Shi *et al*., 2011). DQB1*0601 were associated with PTB disease advancement in

Koreans while a strong association with resistance to recurrent PTB is observed (Kim *et al*., 2005, Yuliwulandari*et al*., 2010) with HLA-DRB1*12 in (Yuliwulandari*etal*., 2010).

CONCLUSIONS

Immunogenic risk factors of tuberculosis infection by HLA alleles (DRB1*1051and DQB1*0601). The HLA antigens of the patients and HHCs groups showed significant increased frequencies compared with controls. In patients group, HLA DRB1*1501 allele (54.3 vs. 20%), and in HHCs group, HLA DRB1*1501 allele (30 vs. 20%), while HLA DQB1*1601 allele in patients showed (31.5 vs. 8.5%), and in HHCs group, HLA DQB1*1601 allele (35.7 vs. 8.5%).

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