HLA-DRB1 typing among Type I Diabetic Patients and their First Degree relatives in a Comparative Study

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Background: The genetic susceptibility to IDDM maps to the MHC class II region, thus one approach to investigating the immunopathogenesis of diabetes is to study first degree relatives of affected individuals.
**Objectives:** To detect the inherited relationship between (HLA-DRB1) alleles and type one Diabetic mellitus.

**Material and Method:** A case-control study was performed on 90 persons, they divided into three group which are diabetic, siblings and control (30 persons in each group), who attended to Al Zahraa Teaching Hospital /Diabetic Center in Al Kut/Iraq between the period from April; 2012 till April; 2013. Data was analyzed by using SPSS 16th version. Distribution of HLA-DRB1 loci among the study groups (type 1 Diabetic patients, Siblings and Control group) were performed using MR.SPOT ROBOTING system. Results: results showed that the frequencies of HLA-DRB1*3,*4 {25 (69.4%)} among Diabetic group were Significant (P value = 0.002), in compare to the corresponding frequencies among control group {11 (30.6%)}, in compare to the frequencies of other HLA-DRB1 loci in both Diabetic group and Control group { 15 (34.1%), 29 (65.9%)} respectively. Meanwhile results have showed that there were no Significant (P value = 0.116), in the frequencies of HLA-DRB1*3,*4 {25 (58.1%)} among Diabetic group in compare to the frequencies of HLA-DRB1*3,*4 {18 (41.9%)} among Siblings group, in compare to the frequencies of other HLA-DRB1 loci among Diabetic group and Sibling group {15 (40.5%), 22 (59.5%)} respectively, also results have showed that there were no Significant (P value = 0.104), in the frequencies of HLA-DRB1*3,*4 {18 (62.1%)} among Sibling group in compare to the frequencies of other HLA-DRB1 loci among Sibling group and Control group {22 (43.1%), 29 (56.9%)} respectively. Conclusion: In conclusion there were a genetic predisposition of diabetic Siblings for development of Diabetes since, both Diabetic group and Sibling group showing the highest frequencies of HLA-DRB1 *3,*4, in compare to Control group. Recommendation: larger sample group are required to include other alleles.

**Key words:** type 1 diabetes mellitus, siblings, HLA-DRB1

**INTRODUCTION**

It is clear that there is a genetic factors to the development of type one diabetes mellitus (T1DM) although the association is much less strong than in type 2 diabetes mellitus. Diabetes UK estimates that if a mother has the condition, the risk of the child developing T1DM is about 2% whereas if the father is diabetic then the risk to the child is estimated to be 8%. If both parents suffer from T1DM then the offspring will have a 30% chance of developing the condition [1]. For T1DM, the concordance rate for monozygotic twins from a number of studies has been estimated as 21-53%, with most estimates being between 30-50% [2]. Candidate gene studies and genome wide analysis have revealed that the most important loci are in the HLA class II region on chromosome 6p21. This region accounts for 50% of familial aggregation through protective and detrimental effects [3]. HLA class II antigens are responsible for self-versus non-self-recognition and antigen presentation to CD4+ T cells. The DR and DQ genes have the greatest influence with certain combinations being of particular importance, for example DR4-DQ8 or DR3-DQ2 which are present in 90% of diabetic patients. Alternatively DR15-DQ6 are protective haplotypes which occur in only 1% of type 1 diabetic patients but in 20% of the general population [4]. Recently a weak association has also been described between the ubiquitously expressed class I HLA antigens A and B [5]. The proposed mechanism by which these haplotypes confer susceptibility is either by poor binding of auto antigens in the case of HLA class II or through endogenous antigens synthesized in response to a viral trigger in the case of HLA class I. Both mechanisms may lead to compromised adaptive T cell self-tolerance. Use of the transgenic mouse model has confirmed that mice expressing DR3 and DQ8 are susceptible to diabetes, shown by loss of
immune tolerance to glutamic acid decarboxylase. Those mice expressing DQ6 were protected from this effect thus confirming its protective function [6]. To date, candidate genes have been identified, which manifest their effects through defects in T cell maturation, activation and self-tolerance leading to increased autoimmunity. In addition, genes controlling defects in antigen presentation and response to viral triggers leading to alterations in self/non self-recognition have been described. It is possible that genetic defects in the developing immune system could account for other host mediated tissue damage including periodontal disease [2]. One approach to investigating the immunopathogenesis of diabetes is to study first degree relatives of affected individuals. An important advantage of such studies is that they eliminate the confounding effects of chronic hyperglycaemia and insulin therapy upon the immune system [7,8]. Although at relatively low risk of progression to IDDM, these individuals exhibit an increased prevalence of both autoantibodies and T cells reactive against islet cell autoantigens [9, 10, 11, and 12]. In addition, they have demonstrated a range of lymphocyte subset abnormalities in peripheral blood of healthy siblings and parents of diabetic children [13]. They have extended these investigations by measuring circulating and mitogen- stimulated cytokine production in a series of diabetic children and their non-diabetic parents and siblings. Since much of the genetic susceptibility to IDDM maps to the MHC class II region, they also studied the association between cytokine levels in these individuals and HLA DR types. A portion of estimable genetic risk can be quantified from family history information and the presence of particular alleles of the genes that contribute to the familial risk. This risk estimate can already be determined at or close to birth, both for individuals with and without a family history of disease. Approximately 400 in every 100,000 U.S. children will be born into an already affected family [14]. These 400 children will have a T1D risk that exceeds 5%, as compared to around 0.4% in the remaining children. Risk in the 400 children can be further stratified on the basis of which affected family member has T1D (3%, 5%, and 8% if they have an affected mother, father, or sibling, respectively) [15, 16]. Moreover, a minority of such children will have two affected first-degree relatives and will have a 20% risk. Much of the familial risk is provided by human leukocyte antigen (HLA) genotypes. In view of the multiple roles of HLA genes in T cell selection, antigen presentation, and immune response, there are many opportunities for HLA-mediated influences on disease risk and progression. HLA genetic susceptibility clearly influences the types of specificities recognized by autoreactive T cells, which can be considered the first checkpoint in the selection and activation of autoimmunity. Alleles at the HLA DR and HLA DQ class II loci are the most useful determinants of inherited risk. T1D risk in a child who has a T1D sibling can be stratified from 0.3% up to 30% depending on his or her HLA class II genotype [17, 18]. Importantly, T1D risk in the children without a family history of T1D can be stratified from ~ 0.01% to more than 5% [19]. Risk can be estimated empirically on the basis of the frequency of the HLA genotype of the child in the nondiabetic population and in those who have T1D. For example, the HLA DRB1*03,*04; DQB1*0302 genotype, which confers the highest T1D risk, is present in 2.3% of U.S.-born Caucasian children and 39% of patients who develop T1D before age 20 (odds ratio 17), providing a T1D risk of 6.8% [20]. The same genotype is present in 7% of the 400 children born with an affected family member, and therefore risk in the 40 children who have a T1D relative and have the HLA DRB1*03,*04; DQB1*0302 genotype will be ~ 25%. Extreme T1D risk (up to 50%) will be present in
children with the HLA DRB1*03,*04; DQB1*0302 genotype born into a family with two or more affected family members\textsuperscript{[21]}. Similar extreme risks were reported for children who are HLA DRB1*03,*04; DQB1*0302 and are identical by descent to their affected sibling at these loci \textsuperscript{[18]}. Finally, T1D is special with respect to genetic susceptibility in that there are HLA genotypes that confer extreme protection \textsuperscript{[22]}. Thus, T1D risk in a child with a T1D family history and with protective HLA-DQB alleles, such as HLA DQB1*0602, is reduced to \~ 1\% of the risk in children with similar family history but without this allele. Alleles at HLA DP class II loci and class I loci, such as HLA A*24, B*38, and B*39, also contribute to T1D risk, but they have not been incorporated into risk prediction models.

**PATIENTS AND METHOD**

This study was performed on 120 persons, selected randomly with matched age and sex, with male to female ratio \( \frac{1}{2} \), who were attended to Al Zahraa Teaching Hospital /Diabetic Center in Al Kut/Iraq between the periods from April, 2012 till April, 2013.

- Three study groups were investigated which included:
  - **First group:** Forty patients with IDDM, fulfilling inclusion criteria for IDDM (1.younger age less than 35yrs., 2.Positive family history. 3. Positive HLA association. 4. Autoimmune association and Positive antibody to B-cells (ICA). 5. Low BMI less than 18.5, 6.FBS >120mg/dl , RBS >180-200mg/dl and HbA1c > 6 plus clinical signs and symptoms) \textsuperscript{[23]}.
  - **Second group:** Forty relatives (Sibling) of IDDM patients had no history or clinical evidence of IDDM or any autoimmune disease.
  - **Third group:** Forty healthy control group who had no history or clinical evidence of IDDM or any autoimmune disease.

Ten milliliters (ml) of venous blood were collected from patients as well as controls by venipuncture. A volume of 10 ml of blood were drawn aseptically using the following procedure: gloves were worn and extraction sites scrubbed in an expanding circular motion first with 70\% isopropyl alcohol which was allowed to dry, then with iodine tincture (iodine in alcohol), allowed to dry before needle insertion \textsuperscript{[24]} . Blood was divided into 2 aliquots, one sample of 6 ml and other 4 ml each. **Sample A1** was kept for few minutes to allow the clot to form, followed by separation of serum from the clot. Serum was separated by centrifugation at 1500 rpm and divided into small (200 μl) aliquots and kept at deep freeze (\(-20 \text{\degree C}\)) to be used for different investigations.**Sample A2** was added to EDTA tubes for HLA genotyping.

**DNA isolation:**
Six ml. of fresh whole blood were collected in EDTA tube as mentioned before, 3 ml. of whole fresh blood have been used for DNA extraction by Genomic DNA Mini Kit (Geneaid-International UKAS quality management).

**Kit contents:**
- GT Buffer
GB Buffer  
W1 Buffer  
Wash Buffer (Absolute ethanol needs to be added prior to initial use).  
Ellution Buffer (10 mM Tris-HCL, ph 8.5 at room temperature)  
GD column  
2 ml. Collection Tube

**DNA Extraction Protocol:**

RBC lysis Buffer is provided to remove non-nucleated red blood cells and reduce hemoglobin contamination, as in the following steps:

**Step (1) RBC Lysis:**
1. Collect fresh blood in EDTA-NA2 treated collection tubes (or other anticoagulant mixtures).
2. Transfer up to 300 μl. of fresh blood to a 1.5 ml microcentrifuge tube. If the blood sample is more than 300 ML (up to 1 ml), add the sample to a sterile 15 ml centrifuge tube.
3. Add 3X the sample volume of RBC lysis Buffer and mix by inversion. Do not vortex.
4. Incubate the tube for 10 minutes at room temperature.
5. Centrifuge for 5 minutes at 3,000 xg and remove the supernatant completely.
6. Add 10 μl of RBC lysis buffer to re-suspend the cell pellet.

**Step (2) Cell Lysis:**
1. Add 200 μl of GB Buffer to the 1.5 ml microcentrifuge tube and mix by shaking vigorously.
2. Incubate at 60 centigrade for at least 10 minutes to ensure the sample lysate is clear, during incubation, invert the tube each 3 minutes.
3. At this time, pre-heat the required Elution Buffer (200 μl per sample) to 60 centigrade (for step 5 DNA elution).

**Step (3) DNA Binding:**
1. Add 200 μl of absolute ethanol to the clear lysate and immediately mix by shaking vigorously for 10 seconds. If precipitate appear, break it up by pipetting.
2. Place a GD Column in a 2 ml Collection Tube.
3. Transfer all of the mixture (including any precipitate) to the GD Column.
4. Centrifuge at 14,000-16,000 xg for 5 minutes.
5. Discard the 2 ml collection tube containing the flow-through and place the GD Column in a new 2 ml Collection tube.

**Step (4) Wash:**
1. Add 400 μl of W1 Buffer to the GD Column.
2. Centrifuge at 14,000-16,000 xg for 30 seconds.
3. Discard the flow-through and place the GD Column back in the 2 ml Collection Tube.
4. Add 600 μl of Wash Buffer (ethanol added) to the GD Column.
5. Centrifuge at 14,000-16,000 xg for 30 seconds.
6. Discard the flow-through and place the GD Column back in the 2 ml Collection Tube.
7. Centrifuge again for 3 minutes at 14,000-16,000 xg to dry the column matrix.

**Step (5) DNA Elution:**
1. Standard elution volume is 100 μl if less sample volume is used, reduce the elution volume (30-50 μl) to increase DNA concentration. If higher DNA yield is required, repeat the DNA Elution step to increase DNA recovery and the total elution volume to approximately 200 μl.
2. Transfer the dried GD Column to a clean 1.5 ml microcentrifuge Tube.
3. Add 100 μl of pre-heated Elution Buffer or TE to the center of column matrix.
4. Let stand for at least 3 minutes to ensure the Elution Buffer or TE is absorbed by the matrix.
5. Centrifuge at 14,000-16,000 xg for 30 seconds to elute the purified DNA.

**Amplification:**
Use skirted PCR plates for the amplification, because they have to be held down at the skirt by a clamp in the MR.SPOT processor afterwards. For each sample to be amplified add the following reagents to each PCR tube:
- **10 μl** Mastermix
- **5 μl** MgCl2
- **5 μl** Sample DNA (15 ng/μl)
Total volume for each amplification reaction is 20 μl.
Seal the amplification tubes with lids or adhesive film, place in the thermal cycler and amplify under the following conditions:

<table>
<thead>
<tr>
<th>No. of Cycles</th>
<th>Time</th>
<th>Temperature</th>
<th>Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Cycle</td>
<td>2 Min</td>
<td>96°C</td>
<td>First Denaturation</td>
</tr>
<tr>
<td>10 Cycles</td>
<td>15 Sec</td>
<td>96°C</td>
<td>Denaturation</td>
</tr>
<tr>
<td></td>
<td>60 Sec</td>
<td>65°C</td>
<td>Annealing + Extension</td>
</tr>
<tr>
<td>20 Cycles</td>
<td>10 Sec</td>
<td>96°C</td>
<td>Denaturation</td>
</tr>
<tr>
<td></td>
<td>50 Sec</td>
<td>61°C</td>
<td>Annealing</td>
</tr>
<tr>
<td></td>
<td>30 Sec</td>
<td>72°C</td>
<td>Extension</td>
</tr>
<tr>
<td></td>
<td>∞</td>
<td>22°C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

**Automated hybridization assay on the MR.SPOT processor:**
**Reagent preparation:**
Take HISTO SPOT reagents and HISTO SPOT test wells out of the fridge and allow them to warm to
room temperature (25°C). Salt crystals may be observed in the hybridization buffer and in the stringent
wash solution. If crystals are present, warm reagents up to 30°C to dissolve. The whole content of the
bottle must be warmed, not an aliquot.
The conjugate has to be diluted 1:1666 in blocking buffer and the conjugate dilution must always be
prepared afresh for each test run. The required volumes of the reagents will vary depending on the
number of strips to be tested. MR.SPOT displays the required volumes for the chosen number of strips.
The required volumes of the reagents were put into the corresponding labeled reservoirs.
The test wells and the PCR plate were placed into the appropriate blocks of the MR.SPOT processor.
The correct arrangement of the PCR plate must be noted.

Setup of the MR.SPOT processor:
The MR.SPOT processor switched on, the internal PC and the touch screen. The startup screen will
appear. Follow the process as indicated on the screen, details are described in the User Manual for the
MR.SPOT processor.
Transfer of results to a PC for interpretation:
The data was transferred to the HISTO MATCH software via network or USB stick.
Interpretation of results:
Open the HISTO MATCH software (if this is not already installed, it could be installed from the CD
delivered with the MR.SPOT processor) and interpret the data.
Statistical Analysis:
Statistical analysis was done by using SPSS (statistical package for social science), version 16 in
which we use chi square test($X^2$) for categorical data. We set P value < 0.05 as Significant.

RESULTS

Table (1): Frequencies of HLA-DRB1 typing among the study groups:
Table (1), shows HLA-DRB1 distribution among the study groups (type 1 diabetes mellitus, siblings and control).
Table (2): The frequencies of HLA-DRB1*3,*4 in between Diabetic group and Control group:

<table>
<thead>
<tr>
<th>groups</th>
<th>HLA-DR</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DR3, DR4</td>
<td>Other DR loci</td>
</tr>
<tr>
<td>diabetic</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>69.4%</td>
<td>34.1%</td>
</tr>
<tr>
<td>control</td>
<td>11</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>30.6%</td>
<td>65.9%</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>100.0%</td>
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</tr>
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This Table shows the data that collected and interpreted. The Distribution of HLA-DRB1 loci among the study groups (type 1 Diabetic patient, Siblings and Control group), and the Frequencies have tested by chi square test(\(X^2\)), results have showed that the frequencies of HLA-DRB1*3,*4 \{25 (69.4\%)\} among Diabetic group were Significant (P value = 0.002), in compare to the corresponding frequencies among control group \{11 (30.6\%)\}, in compare to the frequencies of other HLA-DRB1 loci in both Diabetic group and Control group \{15 (34.1\%), 29 (65.9\%)\} respectively. Meanwhile results have showed that there were no Significant (P value = 0.116), in the frequencies of HLA-DRB1*3,*4 \{25 (58.1\%)\} among Diabetic group in compare to the frequencies of HLA-DRB1*3,*4 \{18 (41.9\%)\} among Siblings group, in compare to the frequencies of other HLA-DRB1 loci among Diabetic group and Sibling group \{15 (40.5\%), 22 (59.5\%)\} respectively. Also results have showed that there were no Significant (P value = 0.104), in the frequencies of HLA-DRB1*3,*4 \{18 (62.1\%)\} among Sibling group in compare to the frequencies of HLA-DRB1*3,*4 \{11 (37.9\%)\} among Control group, in compare to the frequencies of other HLA-DRB1 loci among Sibling group and Control group \{22 (43.1\%), 29 (56.9\%)\} respectively.

Table (3): The frequencies of HLA-DRB1*3,*4 in between Diabetic group and Sibling group:

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<tr>
<td>diabetic</td>
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</tr>
<tr>
<td></td>
<td>58.1%</td>
<td>40.5%</td>
</tr>
<tr>
<td>Sibling</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>41.9%</td>
<td>59.5%</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>37</td>
</tr>
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Table (4): The frequencies of HLA-DRB1*3,*4 in between Sibling group and Control group:

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<td></td>
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<td>43.1%</td>
</tr>
<tr>
<td>control</td>
<td>11</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>37.9%</td>
<td>56.9%</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>51</td>
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Figure (1): Frequencies of HLA-DRB1*3,*4 and other HLA-DRB1 loci among the study groups:
This figure show Frequencies of HLA-DRB1*3,*4 and other HLA-DRB1 loci among the study groups.

DISCUSSION

Those results showing a step ladder decrement in the frequency of HLA-DRB1*3,*4 among the study groups i.e. Diabetic group showing the highest frequency, Control group showing the lowest frequency while Sibling group staying in a proximately equal distance from each them, Figure (1). Hence, explains the Significance between Diabetic group and Control group and the non-significance of Sibling group in either direction i.e. Diabetes group and Control group, consequently both Diabetic group and Sibling group showing the highest frequencies of HLA-DRB1 *3*4 in compare to Control group. So results that obtained in the current study are in agreement with Eman M.S. and Nidhal A. M. [25].
Whom stated that DR3 and DR4 were significantly increased in diabetic group in compared to control group. Also results of the current study agreed with Lambert \cite{20}, who stated that the risk of T1D can be estimated empirically on the basis of the frequency of the HLA genotype of the child in the non-diabetic population and in those who have T1D. For example, the HLA DRB1*03,*04; DQB1*0302 genotype, which confers the highest T1D risk, is present in 2.3% of U.S.- born Caucasian children and 39% of patients who develop T1D before age 20, providing a T1D risk of 6.8%. The agreement is continuous with Bonifacio et al \cite{21}, they said that The same genotype is present in 7% of the 400 children born with an affected family member, and therefore risk in the 40 children who have a T1D relative and have the HLA DRB1*03,*04; DQB1*0302 genotype will be ~ 25%. Extreme T1D risk (up to 50%) will be present in children with the HLA DRB1*03,*04; DQB1*0302 genotype born into a family with two or more affected family members. Similar extreme risks were reported for children who are HLA DRB1*03,*04; DQB1*0302 and are identical by descent to their affected sibling at these loci \cite{18}. That seems to be in agreement with the results that obtained in the current study. The agreement is continuous with Devendra D. and Eisenbarth G.S. \cite{4}, they stated that HLA DRB1*04-DQB1*0302 and / or HLA DRB1*03-DQB1*0201 are observed in > 90% of affected children with T1D and in only 40% of the general population. Also results in the current study agreed with Sabbah E. et al \cite{26}, whom published that presently it is accepted that type 1 DM in children is associated with HLA DR3, DQB1 0201 and DR4, DQB1 0302. Whereas results in the current study are disagree with Ciss et al \cite{27}, whom stated that there were no significant association between IDDM and any alleles of HLA- DRB1 locus. The disagreement is continuous with Vehik K. and Fourlanos S. \cite{28, 29}, they stated that interestingly, however, as T1D incidence increases, the percentage of those cases with the high-risk HLADR3/4 genotype seems to be decreasing. Also results in the current study are disagree with Andrea K. Steck and Marian J. Rewers \cite{30}, they published that these temporal changes in HLA genotypes association with T1D risk (high-risk HLADR3/4 genotype), suggest increased environmental pressure with higher disease progression rate in individuals with lower-risk HLA genotypes and/or contribution of other non-HLA class II alleles or non-MHC–related alleles to T1D risk.
CONCLUSION

1. The genetic predisposition of diabetic Siblings for development of Diabetes since, both Diabetic group and Sibling group showing the highest frequencies of HLA-DRB1 *3,*4 (risk factor), in compare to Control group.

RECOMMENDATION:

1. HLA-DRB1 typing should be done as a routine screening test in high risk population to detect HLA-DRB1*3,*4 alleles which are considered as a strong risk factor for development of diabetes.

2. Further studies with a large sample size are recommended.

3. HLA typing of other alleles should be considered in future studies.

REFERENCES:

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