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HLA-DQB1 Genotyping in Infertile Iraqi Patients

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Abstract :

A total of 116 males with primary infertility and 32 fertile males (controls) were genotyped for HLA-DQB1 alleles by the reverse hybridization method (INNO-LiPA). Based on WHO criteria of 2010 for general seminal fluid analysis, the patients were distributed into three groups: 32 azoospermic (AZO), 40 oligozoospermic (OLI) and 44 asthenozoospermic (AST) patients. Four HLA-DQB1 alleles (DQB1*0204, DQB1*0301, DQB1*0302 and DQB1*0601) showed differences between AZO patients and controls, but none of them attended a significant level. In OLI patients, two alleles (DQB1*0301, DQB1*0501) showed increased frequencies as compared with controls, but the difference was not significant for DQB1*0301 allele, while it was significant (P=0.03) for DQB1*0501 allele (25.0 vs. 6.3%). For AST patients, DQB1*0301 (22.7 vs. 6.3%) and DQB1*0501 (27.3 vs. 6.3%) alleles showed increased frequencies as compared with controls, but both differences attended a significant level (P = 0.05 and 0.02, respectively). With respect to the impact of these alleles on anti-spermatozoa antibody (ASA) production, DQB1*0301 allele was probably associated with predisposition to develop ASAs in OLI patients, while DQB1*0501 was probably associated with a protection in AST patients. These results suggest that HLA-DQ locus may harbour genes that are important in the predisposition to develop infertility in Iraqis.

Key Words: *HLA-DQB1, Azoospermia, Oligozoospermia and Asthenozoospermia.*

Introduction:

The World Health Organisation (WHO) has defined infertility as a period of two years without conception, although many couples actually seek a medical opinion after one year of infertility (1). Infertility can results from female disorders (anovulation, tubal obstruction, or other pathologies) in approximately 30%, a male disorder in 30%, and disorders in both partners in 30%, while no abnormalities have been found in approximately 10% (2).

Approximately, half of male infertility cases are due to defined reasons; including varicocele, infection, hormone imbalances, environmental exposures (for instance; drugs or medications, x-rays and tobacco use), blockage of the reproductive tract ducts, and previous surgery; but also, an estimated 40-90% of male infertility is due to a deficiency in spermatozoa production of indefinable origin (3). A large proportion of infertile men fail to impregnate their female counterpart due to a lack of spermatozoa (azoospermia; AZO) or low spermatozoa concentration (oligozoospermia; OLI); and in addition, infertility may also be due to abnormal spermatozoa morphology (tetratozoospermia) or insufficient spermatozoa motility (asthenozoospermia, AST) (4).

A further important cause of male infertility is genetics. Genetic factors have been demonstrated to influence all stages of development and functioning of reproductive system. Genetic factors that may disrupt reproductive function include chromosome abnormalities (numerical and/or structural), mutations of genes controlling reproductive functions (Y-linked, X-linked, autosomal and mitochondrial), mosaicism, different DNA and chromosome polymorphisms, as well as epigenetic factors (5). It should be also considered that a large part of the endocrine dysfunction (defects in the synthesis of hormones) and immunological disorders (inadequate immune response) can be attributed to the influence of genetic factors that may be their primary cause or may represent a favorable background for their development (6).

The genetic factors can be further extended to involve genes located within the major histocompatibility complex (MHC), which is known in human as human leukocyte

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antigen (HLA) system that is mapped to chromosome 6 at the region 6p21.1-21.3 (7). Such genes have been shown to be highly polymorphic, and up to July 2011, 1698, 2271, 1213, 1074, 158 and 149 alleles have been molecularly recognized at HLA-A, -B, -C, -DRB, -DQB1 and DPB1 loci, respectively, while, the total number of alleles is currently 6810 HLA and related alleles that are described by the HLA nomenclature committee (8). HLA alleles have been demonstrated to be associated with a variety of diseases, especially autoimmune diseases, but have also been proposed to regulate reproductive processes in animals and humans (9). As HLA alleles are immunogenetic markers; therefore the immunological profile of male infertility has also to be targeted, and such target has been monitored through the evaluation of anti-spermatozoa antibodies (ASAs).

Significant levels of ASAs are detected in up to 12% of seminal fluid samples from infertile men and have been associated with specific male genital tract pathology (10). Anti-spermatozoa antibodies are believed to have an adverse impact on male fertility by directly interfering with spermatozoa surface interactions (e.g., fertilization) and indirectly by mediating the release of cytokines that can impair spermatozoa function through a mechanism that may disrupt DNA integrity (11).

Accordingly, the present investigation was designed to inspect the association between HLA-DQB1 alleles and three types of male infertility (AZO, OLI and AST), and equally important the impact of these alleles on the production of ASAs was also evaluated.

Subjects, Materials and Methods:

***Subjects*:**

A total of 116 males with primary infertility attending Kamal Al-Samaraie Hospital, Centre of Infertility and *in vitro* Fertilization (Baghdad) and Baghdad Teaching Hospital (Infertility Clinic) during the period March-August 2010 were enrolled in this study. They were clinically examined and evaluated by the consultant medical staff at the two hospitals. The patients were Iraqi Arabs and their age mean ± S.E. was 30.3 ± 0.7 years. In addition, 32 fertile males (controls), matched patients for ethnicity and age (31.4 ± 1.1 years), were also included in the study, and they were husbands of wives who had fertility complications. Based on seminal fluid analysis and according to the recommendations of WHO (12), patients were distributed into three groups: AZO (32 patients and their age was 28.2 ± 0.7 years), OLI (40 patients and their age was 31.3 ± 1.3 years) and AST (44 patients and their age was 33.4 ± 1.0 years).

***Seminal Fluid and Blood Collection*:**

Seminal fluid was obtained by masturbation after 3-5 days of sexual abstinence. The samples were collected in sterile, wide mouthed, non-toxic container and processed in the laboratory within an hour of ejaculation. All the semen samples underwent semen analysis in accordance with the WHO laboratory manual for the examination of human seminal fluid (12). After semen analysis, OLI and AST samples were centrifuged, and the deposits were frozen at -20°C for a maximum period of 10 weeks, and were used for DNA extraction. In the case of AZO, blood (2-3 ml) was also collected in EDTA tubes and stored in the freezer (-20°C), and it was used for DNA extraction because of lack of spermatozoa.

***DNA Extraction*:**

The genomic DNA was extracted from spermatozoa deposit (OLI, AST and controls) or EDTA blood (AZO) using the Invisorb® Spin Blood Mini Kit (Invitek, Germany), which is designed for manual isolation and purification of genomic DNA from fresh, frozen or old human blood with common anticoagulants (EDTA and Citrate), as well as from buffy coat, spermatozoa and bone marrow.

***Assessment of DNA Yield*:**

The DNA yield was spectrophotometrically assessed using Cecil E1021 spectrophotometer (England), in which the sample was read at two optical densities that were 260 and 280 nm. The second reading was divided by the first reading, and if the outcome was 1.8-2.0, the sample was considered as free of contamination and having a sufficient amount of DNA for a further analysis.

***Principles of HLA-DQB1 Genotyping (Inno-LiPA HLA Typing)*:**

The Inno-LiPA HLA typing test is based on the reverse hybridization principle, in which amplified biotinylated DNA material is chemically denatured, and the separated strands are hybridized with specific oligonucleotide probes immobilized as parallel lines on membrane-based strips. This is followed by a stringent wash step to remove any mismatched amplified material. After that, streptavidin conjugated with alkaline phosphatase is added and bound to any biotinylated hybrid previously formed. Incubation with a substrate solution containing a chromogen results in a purple/brown precipitate. The reaction is stopped by a wash step, and the reactivity pattern of the probes is recorded.

***DNA Amplification of HLA-DQB1 Gene*:**

The DNA amplification was carried out using the Inno-LiPA HLA-DQB1 Multiplex kit (Innogenetics, Belgium), which is intended for nucleic acid amplification of the second and the third exons of HLA-DQB1 locus. The reaction is performed by means of polymerase chain reaction (PCR) in a multiplex format

***Molecular Typing of HLA-DQB1 alleles by Inno-LiPA*:**

As summarized in the principles of test, the amplification products were subsequently hybridized using one HLA-DQB1 typing strip (Innogenetics, Belgium) for each sample, on which 37 sequence-specific probe lines and 2 control lines are fixed. (Figure 1). The HLA-DQB1 genotype was determined using the computer package Interpretation software LiRAS™.

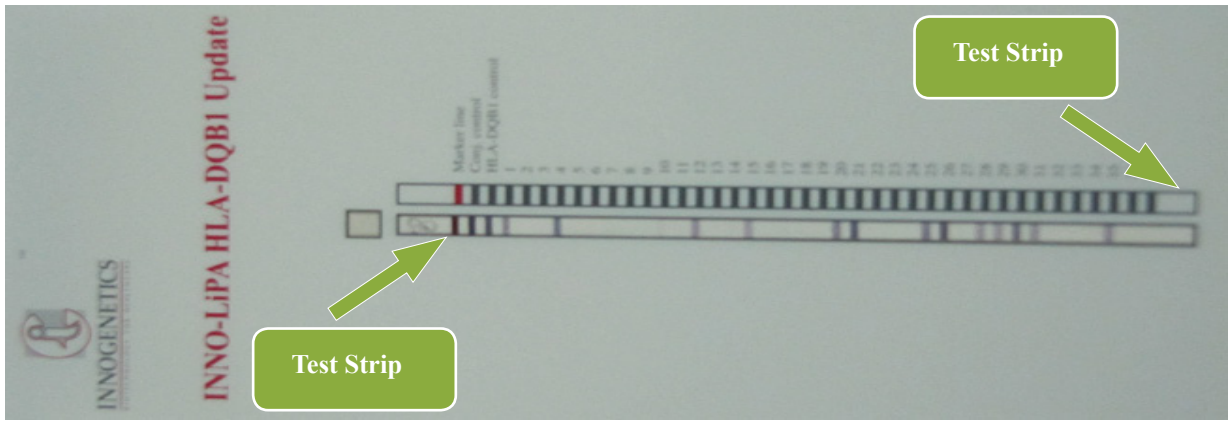

Figure 1: HLA-DQB1 strip with positive bands.

Seminal Plasma Level of ASAs:

The ASA level was assessed in the seminal plasma by means of ELISA method using a commercially available kit (DRG, Germany), which is a solid-phase sandwich enzyme-immunoassay for the quantitative determination of anti-spermatozoa antibodies in human seminal fluid. The ELISA-plate is coated with a mix of spermatozoa proteins which are recognized by anti-spermatozoa antibodies. The samples and standards were pipetted into the wells and then incubated for 60 minutes at 37°C in a humid chamber. During this incubation, anti-spermatozoa antibodies bind to the spermatozoa proteins and are thus immobilised on the plate. After washing, the enzyme conjugate (consisting of anti-human globulin antibodies covalently coupled to horseradish peroxidase) was added. After removal of the unbound conjugate by washing the horseradish peroxidase oxidizes, the then added TMB (3,3',5,5'-tetramethylbenzidine) substrate yielded a colour reaction which is stopped with 0.25 M sulphuric acid (H2SO4). The absorbance is measured at a wavelength of 450 nm with a microplate reader, and the sample results were calculated by interpolation from a standard curve that was performed in the same assay as that for the samples by using a standard curve fitting equation.

Statistical Analysis:

The association between HLA alleles and male infertility was expressed in terms of relative risk (RR), etiological fraction (EF) and preventive fraction (PF). The RR value can range from less than one (negative association) to more than one (positive association). If the association was positive, the EF was calculated, while if it was negative, the PF was given. The significance of such association (positive or negative) was assessed by Fisher's exact probability (P) (13). The calculations of such parameters were carried out using the computer Programmes for Epidemiologists (PEPI) version 4.0. For ASAs, the seminal plasma level was analyzed using the computer programme SPSS (Statistical Package for Social Sciences) version 13. Their data were presented in terms of means ± standard errors (S.E.), and differences between means were assessed by F-test.

Results:

The observed numbers and percentage frequencies of HLA-DQB1 alleles in infertile patients (AZO, OLI and AST) and controls are presented in table 1, while alleles showing variations between patients and controls are given in table 2. In AZO patients, four alleles (DQB1*0204, *0301, *0302 and *0601) showed differences between patients and controls, but none of them attended a significant level. However, the highest RR was recorded for the allele DQB1*0301 (RR = 6.30), which showed an increased percentage frequency in patients as compared with controls (18.8 vs. 6.3%). For OLI patients, Two alleles (DQB1*0301 and *0501) showed increased frequencies in patients as compared with controls, but the difference was not significant for DQB1*0301 allele, while it was significant (P=0.03) for DQB1*0501 allele (25.0 vs. 6.3%). The latter difference scored an RR value of 5.0. As in OLI patients, DQB1*0301 (22.7 vs. 6.3%) and DQB1*0501 (27.3 vs. 6.3%) alleles showed increased frequencies in AST patients as compared with controls, but both differences attended a significant level (P = 0.05 and 0.02, respectively), and the scored EF values were 4.41 and 5.63, respectively.

Table 1: Observed numbers and percentage frequencies of HLA-DQB1 alleles in infertile patients (azoospermia, oligozoospermia and asthenozoospermia) and controls.

HLA-DQB1 alleles	Azoospermia (No. = 32)		Oligozoospermia (No. = 40)		Asthenozoospermia (No. = 44)		Controls (No. = 32)	
	No.	%	No.	%	No.	%	No.	%
DQB1*0201	2	6.3	4	10.0	ND	ND	ND	ND
DQB1*0204	4	12.5	6	15.0	10	22.7	8	25.0
DQB1*0301	6	18.8	4	10.0	10	22.7	2	6.3
DQB1*0302	6	18.8	ND	ND	2	4.5	4	13.0
DQB1*0303	ND	ND	ND	ND	ND	ND	2	6.3
DQB1*0305	2	6.3	ND	ND	ND	ND	ND	ND
DQB1*0310	ND	ND	ND	ND	ND	ND	4	13.0
DQB1*0312	ND	ND	2	5.0	ND	ND	2	6.3
DQB1*0316	ND	ND	4	10.0	ND	ND	ND	ND
DQB1*0402	ND	ND	ND	ND	2	4.5	ND	ND
DQB1*0501	2	6.3	10	25.0	12	27.3	2	6.3
DQB1*0502	ND	ND	ND	ND	2	4.5	ND	ND
DQB1*0503	ND	ND	2	5.0	2	4.5	ND	ND
DQB1*0601	6	18.8	2	5.0	ND	ND	2	6.3
DQB1*0602	ND	ND	2	5.0	2	4.5	4	13.0
DQB1*0603	2	6.3	ND	ND	2	4.5	2	6.3
DQB1*0604	2	6.3	2	5.0	ND	ND	ND	ND
DQB1*0609	ND	ND	2	5.0	ND	ND	ND	ND

ND: Not detected.

Table 4-2: HLA-DQB1 alleles showing variations between infertile patients (azoospermia, oligozoospermia and asthenozoospermia) and controls.

HLA Antigens	No.	%	No.	%	RR	EF	PF	P
Azoospermia Patients *versus* Controls								
DQB1*0204	2	6.3	8	25.0	0.43	-	0.14	N.S.
DQB1*0301	6	18.8	2	6.3	6.30	0.13	-	N.S.
DQB1*0302	6	18.8	4	13.0	1.62	0.07	-	N.S.
DQB1*0601	6	18.8	2	6.3	3.46	0.13	-	N.S.
Oligozoospermia Patients *versus* Controls								
DQB1*0301	4	10.0	2	6.3	1.67	0.04	-	N.S.
DQB1*0501	10	25.0	2	6.3	5.0	0.20	-	0.03
Asthenozoospermia Patients *versus* Controls								
DQB1*0301	10	22.7	2	6.3	4.41	0.18	-	0.05
DQB1*0501	12	27.3	2	6.3	5.63	0.22	-	0.02

RR: Relative risk; EF: Etiological fraction; PF: Preventive fraction; P: Fisher›s exact probability.

To assess the impact of HLA-DQB1 alleles on the level of ASAs, the patients and controls were distributed as positive or negative for DQB1*0301 or DQB1*0501 alleles. Comparing positive and negative patients for the two alleles revealed two significant differences. In the first, DQB1*0301+ve OLI patients showed a significant increased seminal plasma level of ASAs as compared with DQB1*0301-ve patients (41.3 vs. 35.2 U/ml). In the second, the level was significantly decreased (43.9 vs. 58.3 U/ml) in AST patient positive for DQB1*0501 allele as compared with patients negative for the allele (Table 3).

*Table 3: Seminal plasma level of anti-spermatozoa antibodies in infertile patients (azoospermia, oligozoospermia and asthenozoospermia) and controls positive and negative for the alleles HLA-DQB1*0301 and HLA-DQB1*0501.*

Groups	Seminal Plasma Level Mean of Anti-spermatozoa antibodies ± S.E. (U/ml)					
	HLA-DQB1*0301		P ≤	HLA-DQB1*0501		P ≤
	Positive	Negative		Positive	Negative	
Azoospermia	45.1±12.0	40.2±7.2	N.S.	70.8	37.9±6.3	-
Oligozoospermia	41.3±1.9	35.2±2.3	0.05	32.1±5.2	38.3±1.5	N.S.
Asthenozoospermia	43.6±10.6	58.6±2.9	N.S.	43.9±4.2	58.3±8.6	0.05
Controls	20.7	47.1±3.9	-	42.8	43.9±4.7	-

P: Probability of difference between means of positive and negative cases; N.S.: Not significant.

Discussion :

The results revealed that two alleles may be considered important in the immunogenetic background of human male infertility; HLA-DQB1*0301 and HLA-DQB1*0501 alleles. The first allele showed increased frequency in the three groups of infertility (AZO, OLI and AST), while the second was restricted to OLI and AST patients. Such findings may highlight the importance of HLA-DQ locus in conferring immunogenetic predisposition to develop infertility in human beings, especially if we consider RR value of approximately 5.0 and EF value of approximately 0.20; therefore, HLA-DQ locus may harbours a risk gene for infertility, or such gene is in a linkage disequilibrium with specific alleles (i.e. DQB1*0301 and DQB1*0501) controlled by such locus. In agreement with such findings, investigators agree about the possible effects of HLA genes on male reproduction, and a study by Martin-villa et al. (14) showed that HLA molecules are expressed on the spermatozoa's surface, following a cyclic pattern. Avendano et al. (15) found that HLA transcripts were demonstrated in ejaculated spermatozoa, and the fertile group showed significantly higher levels of HLA mRNA than the infertile group. However, population based studies reported different associations between male infertility and HLA alleles in different populations. Aleksovski et al. (16) demonstrated an increased frequency of HLA-A26, HLA-A28, and HLA-B18 alleles in idiopathic AZO and OLI Yugoslavian patients, while other studies reported further significant associations between idiopathic male infertility and HLA alleles, such as HLA-A33, HLA-B13, HLA-B44 and HLA-DR13, in Japanese male populations (17-20). Such studies were based on serological typing of HLA alleles; while present study employed a molecular method that was based on a reverse hybridization after PCR amplification of DNA (INNO-LiPA method). The development of molecular biology techniques based on PCR has greatly improved the accuracy and practicability of HLA typing, and there are several kinds of PCR-based HLA typing, such as, DNA amplification with sequence-specific primers (SSP), single-stranded conformation polymorphisms (SSCP), DNA chip technology and sequence-based typing (SBT) (21). Among these, INNO-LiPA has been reported to have an accuracy rate of 100%, and final observed resolution of 85.6% for HLA-DQB1 alleles; therefore, it is considered rapid and easy-to-perform assay and yielded results fully concordant with other DNA-based tissue typing tests (22).

Reviewing molecular typing of HLA-class II region in infertility revealed that DRB1*1302 allele is linked to susceptibility in non-obstructive AZO in Japanese men (18). Moreover, certain microsatellite markers in the HLA class II region and at DRB1 and DQB1 loci displayed strong associations with non-obstructive AZO, in particular, the frequency of DRB1*1302 and DQB1*0604 alleles was increased in Japanese men with non-obstructive AZO (23). In the present study, DQB1*0604 allele was observed in 6.3 and 5.0% of AZO and OLI patients, respectively, while it was not detected in AST patients or controls, and there was no significant difference between patients and controls. Further molecular HLA typing revealed that HLA-A and HLA-B alleles showed no significant variation in idiopathic male infertility of Chinese patients (24,25), while DQA1*0401 allele demonstrated a significant increased in immune infertility patients from China (26). Such discrepancies can be better understood in the ground of race differences, because the distributions of HLA alleles show high variation in different ethnic groups or even the same ethnic group living in different geographical areas (27), and Iraqi population share a similar interest (28). However, the geographical variation may also highlight the importance of environmental factors (i.e.

infectious agents) that can trigger the disease in individual carrying specific alleles and such alleles may be different in different populations (29). Accordingly, type of infectious agent and HLA alleles may have their different interactions in different populations.

The association between HLA alleles and ASAs in male infertility has not been well-investigated, but class II HLA molecules, by presenting antigens to helper T cells, play a decisive role in induction of antibody production (30). Therefore, it might be expected to find an association between HLA-DQB1 alleles and production of ASAs in present patients, but such association was dependent on type of infertility (OLI or AST) and the deviated allele (DQB1*0301 and DQB1*0501). The first was probably associated with predisposition to develop ASAs in OLI patients, while the second was probably associated a protection in AST patients, because it down-regulated such production in positive cases for the allele DQB1*0501. Such theme has been recently investigated in cryptorchidism, which is one of the most frequent pathologies in early childhood in which one (unilateral) or both (bilateral) testes fail to descend into the ideal scrotal position, and it is also a major risk for male infertility (31). Many sperm proteins do not arise until spermatogenesis starts at puberty, nevertheless, ASAs in serum samples from prepubertal boys were reported (32), and earlier findings showed that cryptorchidism may play an important role in antibody formation at this age (33). Kurpisz et al. (31) reported that significant differences in DRB1*04 and DQB1*06 allele frequencies were seen between cryptorchid patients with (ASA+ve) and without ASA (ASA-ve). Such findings, together with the results of present study, suggest a role of HLA-DQ alleles in predisposition or protection against the development of ASAs, but certainly further investigations are required to validate such conclusion.

In conclusion, the present study results suggest that HLA-DQ locus may harbour genes that are important in the predisposition to develop infertility in Iraqis, and these genes may have their impact on the production of ASAs.

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التنميط الوراثي لمستضدات خلايا الدم البيض البشرية الجين DQB1 في مرضى عراقيين عقيمين

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الخلاصة:

نمط وراثيا 116 من الذكور العقيمين و32 من الذكور الخصبين (سيطرة) لأليلات مستضدات خلايا الدم البيض البشرية الجين DQB1 بطريقة التهجين العكسي (INNO-LiPA). وفي ضوء معايير تحليل السائل المنوي لمنظمة الصحة العالمية لعام 2010، وزع المرضى على ثلاث مجاميع وهي اللانطفية (32 مريض) وقلة النطف (40 مريض) ووهن النطف (44 مريض). أظهرت أربعة أليلات (DQB1*0204 و DQB1*0301و DQB1*0302 و DQB1*0601) اختلافات مابين مرضى اللانطفية والسيطرة، إلا إنها لم تكتسب الدلالة الإحصائية. في حين ظهرت زيادة في تكرار الأليلين DQB1*0301 و DQB1*0501في مرضى قلة النطف مقارنة مع السيطرة ولكن هذه الزيادة لم تكن معنوية للأليل DQB1*0301 بينما كانت معنوية (الاحتمالية = 0.03) للأليل DQB1*0501 (25.0 مقابل 6.3%). فيما يخص مرضى وهن النطف، ظهرت زيادة في تكرار الأليلين DQB1*0301(22.7 مقابل 6.3%) و DQB1*0501 (27.3 مقابل 6.3%) مقارنة مع السيطرة ولكن أكتسب كلا الاختلافين الدلالة الإحصائية. في حين ظهر تأثير لهذه الأليلات على إنتاج أضداد النطف، فالأليل DQB1*0301قد يترافق مع الاستعداد الوراثي لإنتاج أضداد النطف في مرضى قلة النطف بينما قد يكون الأليل DQB1*0501 عامل حماية ضد إنتاج أضداد النطف في مرضى وهن النطف. تبين من خلال هذه النتائج بأن الموقع HLA-DQ قد يحوي جينات مهمة تسهم في تطور العقم الأولي في العراقيين.