Available online at www.ijpab.com

ISSN: 2320 – 7051
Int. J. Pure App. Biosci. **1 (4):** 42-50 (2013)
Research Article

International Journal of Pure & Applied Bioscience

Detection of Autoantibodies in Lada patients and their siblings and HLA Typing of these patients

PAVAN KUMAR D*[1], D. SANKARI[2]
Department of Biotechnology, SRM University, Kattankulathur 603203, Kancheepuram, Tamil Nadu, India
*Corresponding Author Email: dhara14101987@rediffmail.com

ABSTRACT

The present study is to determine the prevalence of auto antibodies in sera of identified LADA (late auto immune disease in adults) patients and their siblings by ELISA technique and HLA typing of these patients by PCR specifically Sequence specific priming (SSP-PCR) method. The 22 diabetic individual blood samples were collected for present study. Lastly, antibody & allelic levels of siblings and LADA patients were compared.

KEYWORDS: *LADA (late autoimmune disease in adults) patients, auto antibodies, ELISA, PCR, HLA typing.*

INTRODUCTION

LADA (LATENT AUTOIMMUNE DIABETES IN ADULTS) :

Latent Autoimmune Diabetes in Adults is a form of autoimmune (type I diabetes which is diagnosed in individuals who are older than the usual age of onset of type I diabetes (that is over 30 years of age at diagnosis) [1].Alternate terms that have been used for LADA include Late Onset Autoimmune Diabetes of Adulthood. Patients with LADA do not have insulin resistance, as do people with Type 2[2.]

Treatment for LADA is different from treatment for type 2 diabetes because the primary problem is insulin deficiency caused by failing beta cells rather than insulin resistance. This means that oral drugs given to people with Type 2 diabetes often will have very little impact on the blood sugar of a person with LADA.

Also several of the oral drugs used to treat type 2 diabetes stimulate the beta cells to produce insulin and because LADA involves an autoimmune attack which is stimulated by the production of insulin at the beta cells. Stimulating insulin production by the beta cells with drugs may increase the ferocity of the attack killing more beta cells.

So it is very important to get a correct diagnosis so you canavoid the drugs that stimulate insulin production by the beta cells. These drugs include the sulfonylurea's like amaryl and gilpzide and may also include in cretin drugs. Autoimmunity is the failure of an organism to recognize its own parts as self which allows an immune response against bits own cells and tissues. Any disease that results from such an aberrant immune response is termed as an autoimmune disease .Autoimmunity is often caused by a lack of germ development of a target body and as such the immune response acts against its own cells and tissues.

Genetic Factors

Certain individuals are genetically susceptible to developing autoimmune diseases. This susceptibility is associated with multiple genes plus other risk factors. Genetically predisposed individuals do not always develop autoimmune diseases. Three main sets of genes are suspected in many autoimmune diseases, they are Immunoglobulin's, T cell receptors, The Major Histocompatibility complexes.

AUTOANTIBODIES:

Group of antibodies 'immune proteins' that mistakenly target and damage specific tissue/organs of body. One /more auto antibodies may be produced by a person's immune system when it fails to distinguish between self and non-self proteins. Usually immune system is able to discriminate by recognizing foreign substances 'non-self' and ignoring body's own cells 'self', yet not over reacting to non-threatening substances such as foods, dust, pollen/beneficial microorganisms' .It creates antibodies only when it perceives what it has been exposed to as a threat 'non-self'. When immune system ceases to recognize one/more of body's normal constituents as 'self', it may produce autoantibody that its own cells, tissues or organs causing inflammation and damage. Causes of this inappropriate action in a chronic autoimmune disorder. Type of autoimmune disorder / disease that occurs and amount of destruction done to body depends on which system/ organs are targeted by auto antibodies are-

Glutamic Acid Decarboxylase (GAD)[3], Pancreatic Islet Cell Auto antibodies (PICA)[4],Insulin Autoantibody (IAA)[3], Insulin Autoantibody -2A (IA-2A)[3].

GAD (GLUTAMIC ACID DECARBOXYLASE):

Major islet auto antigen in IDDM i.e. 'GAD' isoform Gad65 enzyme catalyses neurotransmitter GABA from glutamate. GAD65[5,6] antibodies are shown in prediabetic individuals. GADA positively was observed in 70-80% of onset IDDM in several population and 1-2% of healthy controls. GAD auto antibodies appear heterogenous and it's of interest i.e. GAD65 antibodies predict IDDM better than others.GAD, an enzymes catalyses decarboxylation of glutamate to GABA & CO2. GAD[3] has two isoforms encoded by two different genes GAD 1 & GAD2, they are isoforms GAD67 & GAD65 with molecular weight 67 & 65 KDa. Both these are targets of auto antibodies in people who later develop type 1 DM or late auto immune diabetes.

PICA (PANCREATIC ISLET CELL AUTOANTIBODIES) :

Pancreatic islet cell auto antibodies were first disease specific auto antibodies to be described in patients with type 1 diabetes reactive against islet antigens. It provides strong evidence for autoimmune etiology and pathogenesis type 1 DM and was detected by indirect immunoflorescence [4].

IAA (INSULIN AUTOANTIBODY):

Insulin autoantibody[3] first islet auto antigen and B cell specific auto antigens. Auto antibodies that are presence of IAA in diabetes are positively associated with expression of HLA DR4 in patients, which provides a link with genes conferring susceptibility to disease.

HLA TYPING:

It stands for 'Human leukocyte Antigen' forms part of major histocompatibility complex (MHC), found on short arm of chromosome 6 and its nomenclature alleles were evaluated using the nomenclature of the World Health Organization Nomenclature Committee for factors of the HLA system [7]. MHC antigens are integrated to normal functioning of immune response. Essential role of HLA antigens lies in control of self recognition and defense against microorganisms and surveillance HLA comprises of two classes- CLASS I & CLASS II.

Class I, A B C most significant than remaining E F G H ….which are expressed on most nucleated cells. These are soluble in plasma and are adsorbed onto platelets, RBC will absorb some class 1 antigens E.g: blood group system B7, A28. HLA B most polymorphic system and studies have shown is most significant followed by A & C. 45Kd glycoprotein comprising three heavy chain domains, non-covalently associated with B2 micro globulin which plays an important role in structural support of heavy chains. Class I molecules assembled with cell and ultimately sit in cell surface with a section inserted into lipid bi-layer of cell membrane and a short cytoplasmic form of peptide to cytotoxic T ($CD8^+$) cells.

HLA class II, five loci 'DR, DQ, DP, DM & DO' HLA DR, DQ, DP are most significant, expressed on B-lymphocytes, activated T lymphocytes, macrophages, endothelial cells i.e. immune competent cells. Comprises, 2 chains encoded by HLA α, β and each with 2 domains. Hyper variable region is in B-1 domain. HLA class 11 present peptide in cleft to helper T ($CD4^+$) cells. Thus class II presentations

involve helper function of setting up a general immune reaction involving cytokine, cellular and humoral defense. Role of class II in initiating a general immune response is why only needed to be present on immunologically active cells.

HLA association and studies in type 1 diabetic patients have been focused on children [18-20.] IDDM prediction can be performed using genetic and immunological markers. Among genetic markers, most is known about HLA genes, DR and DQ genes being most informative markers for genetic susceptibility HLA-DR and DQ types [8-10], which is consistent with sensitivity of HLA screening in IDDM. However upto 60% of healthy individuals may carry susceptible HLA-DR and DQ Haplotypes [8], resulting in low specificity of HLA screening.

MATERIALS AND METHODS

ELISA

PREPARATION AND STABILITY OF THE REAGENTS

COATED WELLS: Ready to use. Tear open the protective wrapping of the micro plate. Do not open until the micro plate has reached room temperature to prevent the individual strips from moistening. Immediately replace the remaining wells of a partly used micro plate in the protective wrapping and tightly seal with the integrated grip seam.

ALIBRATORS AND CONTROLS: Ready for use. The reagents must be mixed thoroughly before use.

GAD/IA2 LYOPHILIZED: Reconstitute the contents of one vial with 5.5 ml GAD buffer. If more than one vial is going to be used, pool the contents of each vial after reconstitution and mix gently before use. The reconstituted GAD is stable for a maximum of 3 days at +2°c to +8°c.

SAMPLE BUFFER: Ready to use.

ENZYME CONJUGATE: The enzyme is 20x concentrate. The quantity required should be removed from the vial using a clean pipette and diluted 1:20 with conjugate buffer (1 part reagent + 19 parts conjugate buffer) mix gently before use. The diluted enzyme conjugate is stable for a maximum of 16 weeks at +2 °c to +8°c.

WASH BUFFER: The wash buffer is a 10x concentrate. If crystallization occurs in the concentrated buffer, warm it to 37°c and mix well before dilution. The quantity required should be removed from the bottle using a clean pipette and diluted 1:10 with deionised or distilled water (1 part reagent + 9 parts water)

CHROMOGEN / SUBSTRATE SOLUTION: Ready to use. Close the bottle immediately after use, as the contents are sensitive to light.

STOP SOLUTION: Ready to use.

GLUTAMIC ACID DECARBOXYLASE (GAD) ANTIBODIES TETS

Micro plate ELISA, Anti GAD, Anti IA2,Anti GAD/IA2 Pool-In addition to being useful in making an early diagnosis for type 1 diabetes mellitus ,GAD antibodies tests are used in differential diagnosis between LADA and type 2 diabetes and may also be used for the differential diagnosis of gestational diabetes ,risk prediction in immediate family members for type 1 as well as a tool to monitor prognosis of the clinical progression of type 1 diabetes[11].

INSULIN ANTIBODIES (IAA) TESTS

Anti GAD, Anti IA2, Anti insulin. Insulin Antibodies-These tests are used in early diagnosis of type 1 diabetes mellitus and for differential diagnosis between LADA and type 2 diabetes as well as for the differential diagnosis of gestational diabetes, risk prediction in immediate family members for type 1 and to monitor prognosis of the clinical progression of type 1 diabetes .Persons with LADA may test positive for insulin antibodies.

ANTI-GAD ELISA (IgG) TEST:

PRINCIPLE:

The assay is based on antigen-antibody interaction and subsequent formation of divalent bridges between them. In the first reaction step, sera obtained from donors are incubated in the antigen coated wells. If the

samples are positive specific antibodies bind to the GAD antigen. Bound antibodies act divalent forming chemical bridges between GAD on reagent wells and biotin labeled GAD added in the second incubation step is carried out using enzyme labeled avidin (enzyme conjugate) catalyzing a colour reaction the intensity of the colour formed is proportional to the concentration of antibodies against GAD.

PROCEDURE:

Transfer 25 µl of the calibrators, negative, positive controls or patient samples into the individual microplate wells according to the pipetting protocol. Cover the frame and incubate for 1 hour at room temperature (+18°c to +25°c) on a microplate shaker set at 500 rpm. Empty the wells and subsequently wash 3 times using 350 µl of working strength wash buffer for each wash. Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, and then empty the wells. After manual washing, thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffers. Pipette 100 µl of GAD (biotin-labeled GAD) into each of the microplate wells. Cover the frame and incubate for 1 hour at room temperature (+18°c to +25°c) on a microplate shaker set at 500 rpm. Empty the wells. Wash as described above. Pipette 100 µl of enzyme conjugate (peroxidase labeled avidin) into each of the microplate wells. Cover the frame and incubate for 20 minutes at room temperature on a microplate shaker set at 500 rpm. Empty the wells. Wash as described above. Pipette 100 µl of chromogen /substrate solution into each of the microplate wells. Incubate for 20 minutes at room temperature. (Protect from direct sunlight). Pipette 100 µl of stop solution into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced. Photometric measurement of the colour intensity should be made at a wavelength of 450 nm and then 405 nm using a microplate readers wihin 5 minutes of adding the stop solution.

ANTI-IA2 ELISA (Ig G) TEST

PRINCIPLE:

The assay is based on antigen-antibody interaction and subsequent formation of divalent bridges between them. In the first reaction step, sera obtained from donors are incubated in the antigen coated wells. If samples are positive specific antibodies bind to the IA2 antigen. Bound antibodies act divalently forming chemical bridges between IA2 on reagent wells and biotin labeled IA2 added in the second incubation step is carried out using enzyme labeled avidin (enzyme conjugate) catalyzing a colour reaction. The intensity of the colour formed is proportional to the concentration of antibodies against GAD.

PROCEDURE:

Transfer 50 µl of the calibrators, negative and positive controls or patient samples into the individual microplate wells according to the pipetting protocol. Add 25µl of sample buffer into each of the microplate wells used. Cover the frame and shake for 5 seconds at 500 rpm. Incubate for 16-20 hours at +4° c to +8 °c. Empty the wells and subsequently wash 3 times using 350 µl of working strength wash buffer for each wash. Leave the wash buffer in each well for 30-60 seconds per washing cycle, then empty the wells. After manual washing, thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the opening facing downwards to remove all residual wash buffer. Pipette 100 µl of IA2 (biotin-labeled IA2) into each of the microplate wells. Cover the frame and incubate for 1 hour at +4°c to +8°c. Empty the wells. Wash as described above. Pipette 100 µl of enzyme conjugate into each of the microplate wells. Cover the frame and incubate for 20 minutes at room temperature on a microplate shaker set at 500 rpm. Empty the wells. Wash as described above. Pipette 100 µl of chromogen / substrate solution into each of the microplate wells. Incubate for 20 minutes at room temperature (protect from direct sunlight). Pipette 100 µl of stop solution into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced. Photometric measurement of the colour intensity should be made at a wavelength of 450nm and then 405 nm using a microplate reader within 5 minutes of adding the stop solution.

PCR

PRINCIPLE:

PCR is used to amplify a specific region of a DNA strand. Most PCR methods typically amplify DNA fragments. Typically PCR consists of a series of 20-40 repeated temperature changes, called cycles with each cycle commonly consisting of 2-3 discrete temperature steps usually three. The cycling is often preceded by a single temperature step called hold at a high temperature and followed by one hold at the end for the final product extension on brief storage.

INITIALIZATION STEP

The step consists of heating the reaction to a temperature of 94-96ºC (or 98ºC if extremely thermostable polymerases are used) which is held for 1-9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.

DENATURATION STEP:

This step is the first regular cycling event and consists of heating the reaction to 94-98ºC for 20-30 seconds .It causes DNA melting of DNA template by disrupting the hydrogen bonds between complementary bases yielding single stranded DNA molecules.

ANNEALING STEP:

The reaction temperature is lowered to 50-65ºC for 20-40 seconds allowing annealing of the primers to the single stranded DNA template.DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

EXTENSION/ELONGATION STEP

The temperature at this step depends on the DNA polymerases used. Taq polymerase has its optimal activity temperature at 75-80ºC and commonly at a temperature of 72ºC is used with this enzyme. At this step DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTP‘s that are complementary to the DNA template in 5’ to 3’ direction condensing the 5’ phosphate group of the dNTP’s with the 3’ hydroxyl group at the end of the nascent DNA strand. The extension time depends both on the DNA polymerases used and the length of the DNA fragment to be amplified.

FINAL ELONGATION

This single step is occasionally performed at a temperature of 70-74ºC for 5-15 minutes after the last PCR cycle to ensure that remaining single stranded DNA is fully extended.

FINAL HOLD

This step at 4-15ºC for an indefinite time may be employed for short time storage of the reaction.

PCR SSP (SEQUENCE SPECIFIC PRIMING):

This method can be used for HLA class 1 & 11 typing using a panel of primer pairs either for low to medium resolution whereby primers amplify groups of alleles or high resolution whereby primers pairs amplify specific alleles. Each PCR reaction takes place in a separate tube therefore number of tubes also contains a pair of primers for part of human growth harmone gene as an internal control. These are at a much lower concentration thus do not compete with specific primers. Electrophoresis is used following amplification PCR product is runout on an agarose gel containing Ethidium Bromide. Each product moves according to its size and is compared to a molecular weight marker. Interpetetion every tubes should produce an identical sized product as anternal control and either a specific band or not dependent on whether allele (s) is / are present or not. Results are visualized using 312nm UV transilluminator and recorded either by video imaging/Polaroid photography.

PCR SSOP (SEQUENCE SPECIFIC OLIGONUCLEOTIDE PROBES)

‘Dot blot’ in house method usually whereby one labels ones own probes with Digoxegenin. ‘Reverse dot blot’ normally commercial where specific nucleotide probes are attached to a nylon membrane.

AMPLIFICATION: DNA of interest is amplified by a single pair of biotinylated primers which flank whole of exon.PCR amplifies all the alleles in exon.

HYBRIDISATION: PCR product is denatured and then added to a 'well' containing nylon membrane with bound probes and incubated with hybridization buffer. PCR product hybridizes to probes with complementary sequences. Excess product is washed away during a series of wash steps. Temperature is very important during these stages. Visualization of results is achieved by incubating with a conjugate and enzyme often streptadivin and HRP which binds to biotin of PCR product and then adding a substrate. Band with PCR product turns blue. Strips will have internal control bands to slow test have worked. Interpetation is usually achieved by entering band pattern into a computer programme. This is an excellent method for low resolution batch testing and can be semi automated.

SBT- SEQUENCING BASED TYPING:

DNA sequencing is determination of sequence of a gene and thus is highest resolution possible .SBT involves PCR amplification of gene of interest.

Ex:-HLA DRB1 followed by determination of base sequence. Sequence is then compared with database of DRB1 gene sequences o find comparable sequences and assign alleles. This method allows for detection on new alleles.

This assay is based on the polymerase chain reaction (PCR) which enables an enrichment of defined DNA sequences. After successful amplification the sample contains the target DNA sequence in quantities sufficient for detection. Sequence specific priming (SSP) describes a specific type of PCR in which amplification occurs only if the allele is present; samples lacking the target for an allele specific primer set do not produce that particular PCR product [12]. PCR-SSP analysis requires a number of amplifications to be carried out in parallel. In the "touch down" program described in the test procedure, an initial denaturing period is followed by 10 two-temperature PCR cycles at a high annealing temperature (65®c) that guarantees specific amplification of all the SSP reactions. This is followed by 20 cycles of a three-temperature PCR with a lower annealing temperature at which further amplification of PCR products is favoured. Amplification of internal control primers targeted to the gene for human growth hormone (HGH) demonstrates acceptable reaction conditions for each PCR tube. Negative control tube detects exogenous DNA contamination if present. PCR products are separated by agarose gel electrophoresis on pre cast E-Gel, during which the PCR products are then viewed on an UV transilluminator. A gel image may be captured by film or digital photography as a record of the assay. Determination of alleles is performed by the recording of allele specific bands from the gel image on the enclosed Recording sheet. The pattern of allele specific bands is then compared to the band patterns on the Interpretation Tables. Alternatively, the positive reactions can be entered into the EZ Typer software corresponding to the kit lot; the software will then determine the HLA alleles present.

SPECIMEN COLLECTION:

Isolate DNA using a publication method or a commercial kit manufactured for that purpose. Resuspend the DNA in sterile water or 10mMtris. Ph 8-9 Sample should not be rehydrated in solutions containing greater than 0.5 Mm EDTA or other chelating agents. DNA samples may be assayed immediately after isolation or stored in anon- defrosting freezer at or below -20c for an extended period without affecting results. DNA samples should be in the range of 12.5- 100 mg/µl and a 260nm/280 nm ratio > 1.60 Presence of excess contaminating protein, RNA, heparin, EDTA, or other chelating agents may interfere with PCR amplification of the purified.

MATERIALS:

Eight 96 tube colour- coded PCR trays sealed with marked foil covers, HLA PCR reagent, Thermo-sealing foils, Taq polymerase, Programmable thermal cycler either block sized to accept 96* 0.2 ml PCR tube, Adjustable micro pipette to deliver 1-1000µl and sterile barrier tips, Molecular biology grade water (DNA and DNAse free), PCR (0.2 ml) tube rack, Vortex mixer, Ice bath or cold blocks to fit 0.6 ml or 1.5 ml tubes.

PREPARATION OF MASTER MIX:

Molecular biology grade water – 638 µl

HLA PCR Reagent -319 µL
TAQ Polymerase -8.5 µl

DNA Extraction from whole blood [13]

Protease: When using QIAamp DNA blood mini kit (50) pipette 1.2ml protease solvent into the vial containing 1.3ml of lyophilized Qiagen protease

Buffer AL: Mix thoroughly by shaking before use.

Buffer AW1: To the 19ml of AW1 concentrate, add 25ml of 96-100% of ethanol.

Buffer AW2: To the 13ml of AW2 concentrate, add 30ml of 96-100%of ethanol.

Buffer AE (Elution buffer): Mix thoroughly by shaking before use.

PROCEDURE:

Pipette 20µl protease (or proteinase K) into the bottle of a 1.5ml microcentrifuge tube. Add 200µl of sample to the microcentrifuge tube. Mix well. If the sample volume is less than 200µl, add the appropriate volume of PBS. Add 200µl Buffer AL to the sample. Mix by pulse-vortexing for 15 seconds. Incubate at 56®c for 10 minutes. Centrifuge the 1.5ml microcentrifuge tube to remove drops from the inside of the lid. Add 200µl ethanol (96-100%) to the sample and mix by vortexing for 15 seconds. Centrifuge the 1.5ml microcentrifuge tube to remove drops from the inside of the lid. Add this above mixture to the QIAamp mini spin column (in a 2ml collection tube) without wetting the rim. Close the cap and centrifuge at 8000rpm for 1 minute. Place the column in a collection tube (provided) and discard the filtrate. Carefully open the column and add 500µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at8000rpm for 1 minute. Place the column in a clean 2ml collection tube containing the filtrate. Carefully open the column and add 500µl Buffer AW2 without wetting the rim. Close cap and centrifuge at 8000 for 1 minute and 14000 rpm for 3 minutes. Place the QIAamp mini spin column in a new 2ml collection tube and discard the old collection tube with the filtrate .Centrifuge at full speed (8000 rpm for 1min). Place the column in a 1.5ml microcentrifuge tube and discard the filtrate. Carefully open the column and add 200µl buffer AE or distilled water and kept at room temperature for 10 minutes. Incubate at 6000*g(8000rpm) for 1 minute.

Take PCR plate. Mark in negative control in PCR tube no 01, 04, and 10. Add master mix in negative control 10µl. Remain master mix of about 100µl is added to DNA extracted sample. Add 3µ of elution buffer and mix well. Add 10µl of master mix (with sample) to all PCR tubes except negative control. Cover the PCR plate. RUN PCR DNA ENGINE. Take E gel plate Add 16µl of deionized water to all except M well. Add 15µl to 20µl of DNA ladder in M well. Add 6µl of PCR sample to all wel except M well. Run E gel machine for 9 min. Readings are taken from gel document machine.

RESULTS AND DISCUSSION

IDENTIFICATION OF PATIENTS

The 22 serum samples were collected from diabetic individuals proved by blood sugar values to determine the autoantibody GAD/ IA2 level.

DETECTION OF AUTOANTIBODIES

With the aid of ELISA reader the OD values of the collected serum samples (including siblings) were observed for the Anti-GAD and IA2 test [2].

The samples having the IgG value corresponding to its OD value less than 10 IU/ml were considered to low intensity for IgG and hence called as negative for the test, whereas the samples having the IgG values corresponding to its OD value either equal to or more than 10 IU/ml was considered as positive for the test. From the results obtained out of 22 serum samples 8 serum samples were positive for either GAD/ IA2 antibody. Patients with LADA often had single positive islet cell specific auto antibodies in contrast to those with adult onset type 1 Diabetes with rapid progression [11].

HLA TYPING IN LADA PATIENTS:

HLA test was performed for 8 patients for whom we had previously performed Anti- GAD and Anti-IA2 using DNA extraction and HLA kit. The result is based on polymerase chain reaction which enriches the

particular DNA sequence. PCR typing showed high accuracy in HLA loci and especially in DRB1 loci [14]. The 8 serum samples which were detected as LADA was subjected to HLA typing. After the amplification step by PCR technique the samples were loaded in the agarose gel plate and the allele specific bands were identified. The allele specific bands were viewed by using internally integrated software in a personal computer. The position of alleles & sub alleles of the individual patients were noted.

Table 1

Sample No	GAD	IA2	HLA Typing result
1	positive	negative	A10501,DQB1,DQA10301
2	positive	positive	B10602,A10102,DQA*0301
3	positive	positive	DRB*10401,A0203,B0405
4	positive	Negative	A01021,DQ*0506.DRB*10312
5	positive	positive	A12010,DB*0107,DR*10312
6	positive	negative	B0406.DRB*10402,A12011

HLA TYPING FOR SIBLINGS OF THE IDENTIFIED PATIENTS:
Out of 22 samples, sample no. 7 & 8 belongs to the identified patient siblings. The level of auto antibody was measured at 405 nm & 450 nm which were found to be less than 10 IU/ml. It indicated the absence of both GAD / IA2 antibody. To determine the allele which are specific for autoimmune disease: HLA typing was performed and the results were determined by using internally integrated software in a personal computer [15,16].

Table 2

Sample No	GAD	IA2	HLA typing result
07	negative	Negative	A2B46,DRB1*0401,DQB*0301
08	negative	Negative	B15,A2,DRB1

The result demonstrates that 3 patients who were positive for both GAD/ IA2 had DRB1*0401, DQA*0301, DRB1*0312 were the main susceptible haplotypes in LADA. Whereas in case of sample no.7 the presence of DQ*0301 indicates the presence of susceptible haplotypes of LADA [17].

CONCLUSION

The siblings were identified as different from LADA patients because in the sera samples of siblings it is indicated that both autoantibodies GAD / IA2 are absent / negative, but the allele (susceptible haplotype) DRB1* which is specific for autoimmune disorder were observed .

REFERENCES

1. Bottazzo GF, Florin-Christensen A, Doniach D: Islet cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies.Lancet **2:**1279 –1283 (1974)
2. ELJI, Kawaski, Hiro kazu yama guchi.v, Journal of immunology in diabetes. Autoantibodies to IA2 in type 2 diabetes.
3. Leslie RDG, Atkinson MA, Notkins AL. Autoantigens IA2 and GAD in Type 1 (insulin-dependent) diabetes. Diabetologia **42:** 3–14 (1999)
4. Olsson ML, Sundkvist G, Lernmark Å. Prolonged incubation in the two-colour immunofluorescence test increases the preva-lence and titres of islet cell antibodies in type 1 (insulin-depen-dent) diabetes mellitus. Diabetologia **30:**327–32 (1987)

5. Hoppu S, Ronkainen MS, Kulmala P, Akerblom HK, Knip M: GAD65 antibody isotypes and epitope recognition during the prediabetic process in siblings of children with type I diabetes. Clin Exp Immunol**136:**120 –128, (2004)
6. Achenbach P, Warncke K, Reiter J, Naserke HE, Williams AJ, Bingley PJ, Bonifacio E, Ziegler AG: Stratification of type 1 diabetes risk on the basis of islet autoantibody characteristics.Diabetes, **53:**384 –392, (2004)
7. T E H. Rokmens, M.G.Netea, P.M. Netten, Prevelance and clinical characteristics of insulin treated, anti GAD positive type 2 diabetic subjects in an outpatient clinical department of a Dutch teaching hospital, The journal of medicine, **64:** (2006)
8. Elin Pettersen, Frank Skorpen, Genetic heterogeneity in Latent autoimmune diabetes is linked to various degrees of autoimmune activity, Diabetes, **59:** (2010)
9. Sarika Arora, Latent autoimmune diabetes in adults: A distinct but heterogenous clinical entity, World journal of Diabetes, **1:** (2010)
10. Johnny Ludviggson, Rosaura Casas, GAD-Alum treatment seems effective in slowing residual Beta-cell function loss, Review of Endocrinology, (2009)
11. Bodmer JG, Marsh SG, Albert ED, et al. Nomenclature for fac-tors of the HLA system, 1994. Hum Immunol, **41:**1–20 (1994)
12. Fernandez-Vina MA, Bignon JD. Primers and oligonucleotide probes (SSOP) used for DNA typing of HLA class II alleles. In: Charron D, ed. Genetic diversity of HLA: functional and medi-cal implications. Paris, France: EDK Press, 596–632 (1997)
13. Miller SA, Dykes DD, Polesky HF. A simple salting out proce-dure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16:1215
14. V A Horton, M Bunce, D R Davies, R C Turner, HLA Typing for DR3 and DR4 using artificial restriction fragment length polymorphism PCR from DNA, Journal of clinical pathology, **48:** 33-36 (1995)
15. Ebitssam Msalah et.al, Prevalence of diabetes assisted antibodies & impaired insulin response to glucose in first degree relatives of diabetic patients, **6(2):** 139-148.
16. Jennifer Listgarten, Zabrina Brumme, Mary Carrington, Stastical resolution of ambiguous HLA typing data, Plos computational biology, **4:** (2008)
17. M. Desai.E.Zeggini, V A Horton, K. R Owen, An association analysis of the HLA gene region in latent autoimmune diabetes in adults, Diabetologia, **50:** 68-73 (2007)
18. Svejgaard A, Jakobsen BK, Platz P, et al. HLA associations in insulin - dependent diabetes: search for heterogeneity in differ-ent groups of patients from a homogeneous population. Tissue Antigens; **28:**237–44 (1986)
19. Heimberg H, Nagy ZP, Somers G, et al. Complementation of HLA-DQAand -DQBgenes confers susceptibility and protec-tion to insulin-dependent diabetes mellitus. Hum Immunol, **33:**10–17 (1992)
20. Vandewalle CL, Decraene T, Schuit FC, et al. Insulin autoanti-bodies and high titre islet cell antibodies are preferentially asso-ciated with the HLA DQA1*0301-DQB1*0302haplotype at clinical type 1 (insulin-dependent) diabetes mellitus before age 10 years, but not at onset between age 10 and 40 years. The Bel-gian Diabetes Registry. Diabetologia **36:**1155–62 (1993)