

## HLA-DR TYPES IN RHEUMATIC, CONGENITAL AND DEGENERATIVE CARDIAC VALVE DISEASES IN SAUDI PATIENTS

Abdul-Islam Butt, Mphil; Zohair Halees, FRCS; Leticia Vencer, MSc;  
Nduna Dzimiri, PhD; Azadali Moorji, BSc; Syed S. Hussain, PhD

Subjects of Saudi origin were DNA typed for HLA-DR2, DR4 and DRw53 by amplification fragment-length polymorphism and amplification by sequence-specific primer techniques based on polymerase chain reaction. Of these subjects, 125 had sporadic heart valve disease (96 with rheumatic heart disease, 18 with degenerate and 11 with congenital degenerate valve disease) and 77 were healthy Saudi blood donors. While the frequency of individuals typed DR4 was about the same in the rheumatic heart disease as in the control category (30% versus 23%, respectively), it was found to be higher (55%;  $P < 0.02$ ), but below the level of marginal significance after correcting for the number of DR types, in the congenital degenerate valve category. No preferential association of any DR4 subtype could be detected. The incidence of DR2 was lower in the congenital cases compared to that in the controls (9% versus 21%) and remained about the same in the rheumatic heart disease patients as in the controls. The frequency of DRw53 in the degenerate valve categories was slightly lower than that in the controls, but the difference was not significant. The study failed to corroborate the association between HLA-DR4 and rheumatic heart disease shown in previous studies using the serotyping approach. *Ann Saudi Med* 1997;17(3):283-287.

The role of genetic factors in the etiology of rheumatic heart disease (RHD), an autoimmune disease, was documented many decades ago. As a result, the investigative efforts were focused on the genetic markers of susceptibility to this preventable disease. These studies have been influenced by the accumulation of data on the importance of major histocompatibility complex (MHC) in the immune response. In view of an abnormal autoimmune response exhibited by the rheumatic fever (RF)/RHD patients, the MHC region has been under scrutiny for markers of susceptibility.

The existing data, obtained by serotyping and mixed lymphocyte culture techniques (MLC), indicate the relevance of some specific HLA types to susceptibility to RHD: DR2 has been shown to be prevalent in American blacks;<sup>1</sup> DR4 in American Caucasians,<sup>1</sup> Turks,<sup>2</sup> Saudis and other Arabs;<sup>3</sup> DR3 has been associated with RHD in Indians;<sup>4</sup> and DR7 and DRw53 in Brazilians.<sup>5</sup> This association of HLA markers with RHD is rarely close enough to give a very high degree of assurance for preven-

tive intervention in the case of individuals possessing this marker. Furthermore, the validity of these associations for any other population has to be established. It also has to be considered that the serotypes are heterogeneous denominations without any specificities as regards haplotypes or the different alleles involved.<sup>6</sup> Obviously, improvements in the HLA-typing methodology are desired so that more specific relationships between the marker and the disease could be established. With this aim in view, HLA-DNA typing of RHD patients was undertaken.

HLA-DNA typing is expected to be a better alternative to serotyping. This has actually been tested for detecting DNA types for associations with pemphigus vulgaris,<sup>7</sup> insulin-dependent diabetes mellitus<sup>8</sup> and rheumatoid arthritis,<sup>9</sup> and subtypes in majority have been identified for each disease. Typing by this method has not been reported for RHD so far. Since the incidence of this disease is fairly high (2-7.5 per 1000) in Saudi Arabia and since a large number of cases of this disease are referred to KFSH&RC, it was considered to be of interest to look for the concurrence of DNA marker with the disease. As it was already established by serotyping that DR4 was associated with RHD,<sup>3</sup> the first step was deemed to be its confirmation by the DNA typing methodology. Subsequently, typing was done for DR2 and DRw53 as well.

This screening was also extended to congenital degenerate and degenerate cardiac valve disease cases.

### Subjects and Methods

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From the Departments of Biological and Medical Research and Cardiovascular Diseases, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia.

Address reprint requests and correspondence to Dr. Hussain: Department of Biological and Medical Research (MBC-03), King Faisal Specialist Hospital and Research Centre, P.O. Box 3354, Riyadh 11211, Saudi Arabia.

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The study involved 96 randomly selected Saudi patients, who fulfilled Jones's modified criteria<sup>10</sup> for the diagnosis of rheumatic fever, and were admitted for valve repair and replacement. The patients' age range was 4-76 years and showed an overrepresentation of males (56 males and 40 females). Although different regions of Saudi Arabia were represented, the number of patients from four cities—Riyadh, Jeddah, Mecca and Medina—represented about 30% of the total.

The initial diagnosis with echocardiography and Doppler ultrasonography was confirmed by surgical diagnosis. The subjects included 12 with mitral regurgitation, 12 with mitral stenosis, 8 with aortic regurgitation, 6 with aortic stenosis and 58 with mixed lesions.

In addition to the RHD patients, 29 cases of degenerate (35-76y) and congenital degenerate valve patients (age range 2-26y) were screened. A total of 77 healthy Saudi donors of comparable sex and age distribution served as controls.

Sequence of primers GH46 and GH50 for exon 2 were obtained from Erlich and Bugawan,<sup>11</sup> primers for DR2 and DR4 from Yunis et al.,<sup>12</sup> and primer mixes for DR4 from Olerup and Zetterquist.<sup>13</sup> Sequences of probes GH78, GH100 and GH101 were taken from Scharf et al.<sup>7</sup> These probes can detect the different DR4 types.

Peripheral blood samples (10 mL) were drawn in tubes containing EDTA and lymphocytes were isolated by Ficoll-Hypaque method. DNA was extracted by the salting out procedure of Miller et al.,<sup>14</sup> involving cell lysis, digestion with proteinase K, salting out of proteins with NaCl and precipitation of DNA with ethanol.

Exon 2 of DR $\beta$ 1 was amplified for SSO typing. The amplification mixture was of the standard composition: Tris HCl pH 8.3 10 mM; KCl 50 mM; MgCl<sub>2</sub> 1.5 mM, gelatin 0.01%, dNTPs 0.2 mM, G46 and G50 primers 50 pmoles each, 1  $\mu$ g DNA and Taq polymerase 2.5 U per 100  $\mu$ L. After the first denaturation at 96°C for three minutes, the cyler was programmed as follows: denaturation at 96°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds for 30 cycles, followed by extension for three minutes at 72°C. Replicate slot blots were prepared according to the number of probes to be used. Fifteen  $\mu$ L of the amplification mixture was used for dot blotting on a Hybond N<sup>+</sup> membrane (according to the suppliers' protocol). The DNA was cross-linked to the membrane using a UV source (Stratalinker from Stratagene, La Jolla, USA). Allele-specific probe hybridization was done according to Scharf et al.<sup>7</sup> Briefly, prehybridization was done for 1.5 h at 50°C in 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin, 0.5% SDS and 3 x SSPE (1 x SSPE is 0.19 M NaCl, 10 mM sodium phosphate pH 7.4, 1 mM EDTA) for

GH78 or 5 x SSPE for GH100 and 101. Hybridization was carried out in the same solution at 16 h at 50°C. Washing of blots, hybridized to probe GH78, was done in 2 x SSPE at 60°C for 10 minutes. The blots hybridized to the other two probes (GH100 and GH101) were washed in 0.1 x SSPE at 60°C for 10 minutes. After washing, the blots were autoradiographed, using Hyperfilm<sup>MP</sup> (Amersham, Buckinghamshire, UK) at -70°C.

For AFLP, 2  $\mu$ g of genomic DNA was used for amplification in a volume of 100  $\mu$ L. The reaction mixture consisted of the following constituents: dNTPs 0.125 mM, Tris-HCl (pH 8.3) 10 mM, KCl 50 mM, MgCl<sub>2</sub> 1.5 mM, primers 50 pmoles each, 0.01% gelatin and Taq polymerase 1.25 U. The thermal cyler was programmed as follows for 30 cycles: denaturation at 96°C for 30 seconds, annealing at 61°C for 30 seconds and extension at 72°C for 1 minute. Fragments of 266 and 262 bp were amplified using primer groups DRG2 and DRG4,<sup>11</sup> respectively, for DR2 and DR4 types. The products were migrated in 2% Nusieve plus 1% Sea Kem GTG agarose.

Typing by SSP (sequence-specific primers) was done by amplifying the specific fragments corresponding to DR4 and DRw53 by using primer mixes according to Olerup and Zetterquist.<sup>13</sup>

Comparison of the frequencies of antigens in the controls and patients were done by the chi-squared method, using Yates correction in cases where the number of individuals was less than five.

## Results

The results obtained with SSO typing (Figure 1) and SSP amplification (Figure 2) were comparable for DR4 typing. The same results were obtained by adopting the amplification fragment-length polymorphism (AFLP)

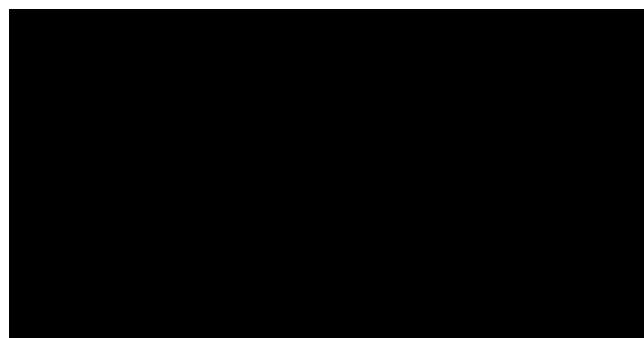


FIGURE 1. DRA4 typing by hybridization to sequence-specific oligonucleotides. The exon 2 of DR $\beta$ 1, amplified using polymerase chain reaction, was slot blotted on Hybond N<sup>+</sup> and hybridized to probes GH78, GH100 and GH101. The panels for GH101 are not shown, as only two positive slots were detected. A, B: patient panels hybridized to GH78; E, F: control panels hybridized to GH78; C, D: patient panels hybridized to GH100; G, H: control panels hybridized to GH 100.

approach suggested by Yunis et al.<sup>12</sup> (Figure 2A) and the SSP approach adopted by Olerup and Zetterquist<sup>13</sup> (Figure 2C).

The frequency of DR4-positive controls was insignificantly lower than that of the RHD patients (23% in the controls versus 30%). Similarly, no difference was found between the frequencies of DR2 in the two categories of subjects (18% in the control versus 22% in the patients, Table 1, Figure 2B). The DR4-positive individuals did not represent any particular category of patients in the RHD group.

The DR4-positive individuals among the patients with congenital degenerative valve disease were found to be present at a higher frequency of 55%, compared to 23% of controls resulting in a significant difference ( $P=0.02$ ), which after correction for the number of DR antigens did not remain so. The frequency of DR4-positive cardiac valve disease patients was only slightly higher than that of the controls (Table 1).

The frequency of DR2-positive congenital degenerate valve patients was lower than that in the controls (the difference was not statistically significant, however), while that of the degenerative valve patients was slightly higher than the controls (28% versus 18% in the controls; Table 1).

The frequencies of individuals typed DRw53 remained comparable in the controls and RHD group (Table 1). There was a decrease in the degenerate and congenital degenerate valve cases but it was not statistically significant.

### Discussion

No increase in DR4 frequency has been detected in RHD patients by DNA typing in the present study, in contrast to the results obtained previously by serotyping by Rajapakse et al.<sup>3</sup> We examined the possibility of whether the prevalence of the antigen in the single valve lesions and the two valve lesions could explain the difference in the results of this and the previous study. On reviewing our cases with double lesions we detected no trend towards any increase in the frequency of DR4. Similarly, Jhinghan et al.<sup>4</sup> detected no difference in marker frequency between single and double valve lesions in the Indian population.

The discord with the previous results obtained by serotyping could be due to the following features of the present approach: 1) DNA typing focuses on a specific part of the genome, whereas serotyping is directed towards the recognition of epitopes, which are shared between DNA types. 2) While the DNA typing is more specific, serotyping is not—it is heterogeneous with regard to both haplotype and the alleles,<sup>6</sup> as mentioned already. 3) In contrast to the variability of the expression of the HLA antigen with age and health of an individual, the genetic

TABLE 1. Frequency of HLA-DR types in Saudi patients and controls.

Subjects	Total	DR4			DR2			DRw53		
		Pos	%	<i>P</i>	Pos	%	<i>P</i>	Pos	%	<i>P</i>
Control	77	18	23		14	18		43	56	
RHD	96	29	30	NS	21	22	NS	52	54	NS
Degenerative valve	18	6	33	NS	5	28	NS	7	39	NS
Congenital degenerative valve	11	6	55	0.02*	1	9	NS	5	45	NS

\*Non-significant after correcting for the number of DR types looked for; pos=positive; NS=not significant.

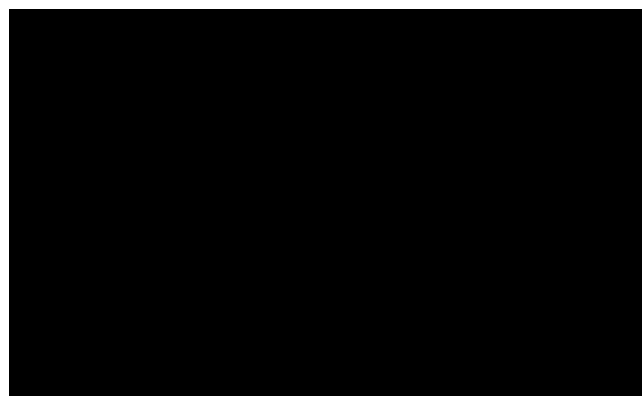


FIGURE 2A. DR4 typing by amplification fragment-length poly-morphism. A fragment of 266bp was amplified representing most of the DR4 alleles. Lane M,  $\phi$ X 174RF digested with the enzyme HaeIII.

make-up stays almost the same.

While the RHD patients and controls show almost the same frequencies of both DR2 and DR4, the situation appears slightly different for congenital degenerate and degenerate cases. The frequency of DR4 cases in the degenerate valve patients is ~33% and in the congenital degenerate valve patients it is 55%. The difference in the frequencies of DR4 in the congenital valve disease patients and the controls, however, is just below the marginal level of significance after adjusting for the multiple DR types and does not implicate this HLA-type in the pathogenesis of the valve disease. This observation warrants a study on a larger number of congenital valve disease patients. Considering that some subtypes within DR4 may be more closely associated with one or the other category of subjects of RHD, subtyping was done (data not presented) of a part of the samples and no overrepresentation of any DR4 subtype was detected.

In this study, a nonsignificant decrease in the frequency of DR2 has been observed in the congenital degenerate valve category, while in previous studies<sup>4,5</sup> such a decrease has been observed in the RF/RHD category. In view of the persistence of this observation (though insignificant) in connection with the congenital degenerate valve category, a protective role (attributed previously<sup>4</sup> to

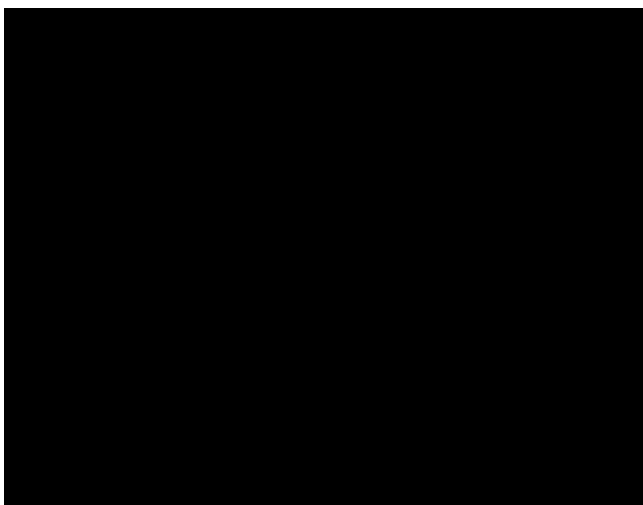


FIGURE 2B. DR2 typing by amplification fragment-length polymorphism. A fragment of 262bp was visible after amplification in individuals positive for DR2.

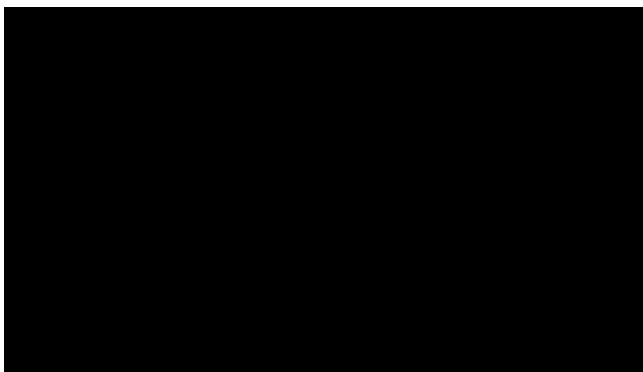


FIGURE 2C. DR4 typing by the sequence-specific primer approach employing mixtures of primers.<sup>13</sup> The fragment of 796bp is a control fragment amplified for ensuring success of the reaction. The fragment at 223 bp level indicates the individuals positive for DR4.

this type) may be indicated here as well. The full implication of this observation is difficult to gauge at this stage as the number of involved cases is small.

As the serologic specificity DRw53 is associated with DR4,<sup>4</sup> HLA-DNA typing using the SSP approach of Olerup and Zetterquist<sup>13</sup> was undertaken and no significant difference was found between the frequencies of controls and RHD patients on the one hand, and between controls and degenerate and congenital degenerate valve cases on the other hand (Table 1).

The previous studies, associating different HLA types with RHD in different populations, employed serotyping. No study on the distribution of HLA types in these patients has so far been done by DNA typing. As HLA-DR4 has earlier been associated with pemphigus vulgaris,<sup>7</sup> rheumatoid arthritis<sup>9</sup> and diabetes mellitus,<sup>8</sup> it appears that this HLA-type is a marker for some other loci whose

involvement has so far not been unveiled. It is also notable that the positive relation referred to rheumatic fever in Turkish children. In contrast to this, a positive association has been reported between DR3 and RHD in the study on Indian population,<sup>4</sup> and a negative association has been reported with previous history of rheumatic fever. These different types of relationships underline the need for further studies on HLA markers and disease associations in order to gain better insights into their nature.

In conclusion, this study by DNA typing has not been able to demonstrate a significant association between HLA-DR4 and RHD obtained earlier<sup>3</sup> by serotyping.

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