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Frequency of HLA antibodies in south India

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Abstract. Sera from 4088 pregnant women (649 antenatal bleeding and 3439 post-partum bleeding) living in Madurai, were collected and screened for anti-HLA. A, B and DR antibodies. 696 of them were screened for anti-HLA DR antibodies. Ten per cent (65/649) of antenatal sera and 13·4% of post-partum sera (463/3439) were positive for HLA A and B antibodies: nonetheless the percentage of monospecific sera were almost the same in both. Screening for HLA DR antibodies were carried out using platelet absorption in test tray technique: seventy three of 696 (10·5%) were positive. The incidence of anti-HLA A, B antibodies correlates to the allelic frequencies in the population. Thus in India, collection and screening post-partum haemorrhage is the simplest and cost effective method of acquiring polyclonal sera for routine laboratory and diagnostic use.

K**eywords.** HLA; anti-HLA antibodies; post-partum; pregnancy.

1. Introduction

The human luecocyte antigen (HLA) system is studied in transplantation matching, population genetics, disputed parentage, disease association and immunology. The system consists of HLA-A, B, C, DR, DQ and DP loci over a region of 3600 kb in p21·3 of C6 (Trowsdale *et al* 1991) and sequence studies have shown that each locus is highly polymorphic (Bodmer *et al* 1992). The products of these alleles (antigens) are expressed in various cells and tissues of the human body and these are identified by specific antisera. There are many sources for obtaining these antisera, *viz.,* multiparous women, transplant recipients, poly transfused patients, immunized donors and monoclonal antibodies. Antibody to HLA was first identified in the serum of a polytransfused patient (Dausset 1958); subsequently matern o-foetal allo immunization was also shown to produce anti-HLA antibodies in pregnant women (Payne and Rolfs 1958; Van Rood *et al* 1958). Pregnant women are the most common source of these antisera, though many monoclonal antibodies have been raised, of late.

Studies on HLA system is very common in Western laboratories: in India, only a handful of laboratories attempted to and continue research in this field. The major limitation for this has been the non-availability of these antisera in the country and one has to depend on importing commercially available sources. In this paper, we present our results describing procurement of sera from multiparous women and screening them for HLA A, B and DR antibodies.

2. **Materials and methods**

2.1 *Screening for antibodies*

Each sixty-well tissue-typing tray (Greiner, Germany) consisted of one positive, one negative and fifty eight sera to be screened, as per tray formats. Thus a total of 71 tray formats to include all the 4088 sera were prepared and used over the period. The trays screened for anti-HLA DR were added with the platlets as described in §2·7, absorbed and tested with B lymphocytes. Each tray was tested with lymphocytes from one volunteer by microlymphocytotoxicity assay. Thirty to fifty such reference cells were used to screen each batch of trays.

2.2 *Samples*

Parous women attending the Government Rajaji Hospital, Madurai and Christian Mission Hospital, Madurai, during 1981–90 were selected at random and included in the study. Their age, number of para, nativity and other relevant details were noted. Cases with high risk pregnancies and caesarian deliveries were excluded from the study, it was not possible to obtain vaginal bleed during caesarian operations and the incidence of HLA antibodies in high-risk pregnancy has been reported to vary (Regan *et al* 1991).

2.3 *Antenatal blood*

Ten to twenty ml of peripheral blood were obtained from out-patient pregnant women during the last trimester of pregnancy. A total of 649 sera were collected between 1981-84.

2.4 *Post-partum blood*

These were collected at the time of parturition (delivery of the baby) in the labour room. Soon after the delivery of the baby and the placenta, the intrauterine bleeding pouring through the vaginal outlet were collected in sterile, shallow basins or scoups kept underneath the bottom. This procedure did not cause inconvenience to the women. The blood was transferred to sterile bottles and stored in a refrigerator, sera separated and frozen at – 20° C. A total number of 3439 sera were thus collected between 1981–1990.

2.5 *Reference panel*

A total of 295 volunteers, employees of our university and nearby colleges, were thoroughly HLA typed panel members. Their HLA phenotypes were determined using any of the three sets of tissue typing sera received from Prof. J Dausset, France, III Asia Oceania Histocompatibility Workshop, 1986 and XI International Histocompatibility Workshop, 1991.

2.6 *Microlymphocytotoxicity test*

Peripheral blood was obtained from reference panel members by cubital venipuncture and lymphocytes isolated by centrifugation in a one step density gradient (Boyum 1968). Microlymphocytotoxicity test described by Terasaki and McClelland (1964) was used for both HLA phenotyping of the reference panel member and for screening the unknown sera. An inverted fluorescence and phase contrast microscope (Leitz, Germany) was used to read the results.

2.7 *Platelet absorption on test tray technique for HLA DR screening*

Platelet absorption on test tray (PATT) described by Thomsen *et al* (1985) was adopted to screen HLA class II antibodies. Sera to be screened (2 μl of each) were dotted in Terasaki trays. Peripheral blood were obtained from 20 random blood donors (5–10 ml each) and platelets isolated by slow centrifugation and pooled. Seven hundred thousand pooled platelets in 1 μl of saline were added to each well of the test tray. The trays were vortexed, incubated at room temperature for 1 h and stored at –20° C until further use.

2.7a *Absorption procedure:* To screen these absorbed sera for DR antibodies, adherent cells (B lymphocytes) of the reference panel members were isolated on a miniature nylon wool (30 mg) adherent column (3 mm × 5 cm) made of plastic straw (8 cm long) (Manickasundari *et al* 1984), labelled with fluoresceine di-acetate (Van Rood and Van Leeuwen 1980) and 3000 cells suspended in 1 μl of TC 199, were added to each well of the test tray. A long incubation *i. e.* 1 h for cells with serum and 2 h for complement was adopted: the complement for HLA DR screening was preabsorbed with pooled peripheral blood white cells and platelets. At the end of the reaction, the trays were flicked to remove the supernatants and 1 μl of ethidium bromide (0·2%) solution to each well was added to label the dead cells. The results were read under blue excitation filter in an inverted fluorescence microscope (Leitz, Germany). Thus 696 sera among the total of 4088 were screened for the detection of HLA DR antibodies.

2.8 *Data processing and analysis*

The reaction of each serum with each cell was correlated to the presence or absence of a HLA antigen. The correlation of a given antigen (HLA allele) to the reactions of a given serum were carried out manually using two by two contingency table and χ^2 and correlation coefficient (r) according to standard methods (Snedcor and Cochran 1968). Further analysis of serum *versus* serum correlations and serograph, as well as serum *versus* antigen correlations were carried out using our computer data base and programmes (Pitchappan and Arulraj 1989). The manual and computer analysis of serum versus antigen compared well. Nonetheless use of computers facilitated the serum *versus* serum and serograph analysis which were cumbersome to perform manually, and also to evaluate serum at various levels of positive scores and to obtain various calculations like r value, % 8 + positive, χ^2, Q scores, list of cells in true positive, false positive, false negative and true negative, if required.

3. Results

3.1 *Incidence of HLA antibodies*

Table 1 presents the number of anti-HLA sera and their specificities identified in the present study. For comparison the percentage phenotypic frequency of various HLA alleles in mixed population of Madurai (Brahmajothi *et al* 1991) are also presented. The incidence followed the expected values of HLA antigens in general.

Table 1. HLA specificities of HLA typing reagents detected in the present study (n = 528) compared to HLA antigen frequency in the population.

HLA	% PF*	No. of sera	r	HLA	% PF	No. of sera	r
A1	32·4	33		B5	25·0	22	
A2	25·0	54		B51	13·2	24	
A3	14·1	24		B52	14·3	11	
A9	32·9	9		B7	18·8	25	
A24	32·7	23		B8	3·5	14	
A26	8·9	14		B12	11·6	11	
A11	28·2	37		B44	14·3	13	
A19	27·9	12		B13	9·2	15	
A31	10·2	8		B15	10·8	7	
A32	3·1	5		B62	10·2	7	
A33	14·3	2		B16	2·7	—	
A28	12·4	10		B39	4·1	—	
A68	6·1	6		B17	21·0	18	
A69	4·1	2	0·806	B57	8·2	—	
				B58	8·2	2	
DR1	10·5	11		B18	0·7	2	
DR2	32·4	4		B21	5·5	1	
DR3	21·6	2		B49	4·1	12	
DR4	16·7	6		B50	2·1	0	
DR5	25·1	6		B22	7·9	6	
DR11	6·6	9		B56	3·1	12	
DR12	15·4	1		B27	1·0	3	
DR6	25·8	9		B35	24·0	37	
DR7	23·0	8		B37	9·2	9	
DR8	7·7	9		B40	23·5	22	
DR9	10·5	2		B60	1·0	—	
DR10	12·5	13	0·701	B61	19·4	7	0·884

*HLA phenotype frequencies (% PF) are from Brahmajothi *et al* (1991)
r=coefficient of correlation between the antigen frequencies of a given locus and the incidence of antibodies to various antigens in the population, observed in the present-study.

Table 2 presents a comparison of the two collection methodologies and the number of anti-sera directed to various HLA loci. Ten per cent of antenatal collection was positive for HLA A and B antibodies, while it was 13·46% in the post-partum collection; the increase in percentage in post-partum collection was due to higher incidence of mono, duo and poly specific antisera (ref. table 2). While 85% of the positive sera in the antenatal collection were monospecific, only 70% of the positive sera in the post-partum collection were monospecific; nonetheless the

Table 2. Incidence of anti-HLA antibodies in 4088 pregnant women in Madurai.

	Number of sera positive for									
	HLA A and B								HLA DR	
	Antenatal			Post-partum			Total		Total	
	N	%T	%P	N	%T	%P	N	%T	N	%T
Monospecific	55	8·5	85	323	9·4	70	378	9·2	56	8·0
Duospecific	9	1·4	14	95	2·7	21	104	2·5	14	2·0
Polyspecific	1	0·2	2	45	1·3	10	46	1·1	3	0·4
Total + ve	65	10	100	463	13·4	100	528	12·9	73	10·5
Negatives	584	90		2974	86·5		3560	87·1	623	89·5
Total screened	649	100		3439	100		4088	100	696	100

N, Number; %T, percentage among total sera screened; %P, percentage among total sera positive.

yield of monospecific sera was slightly higher in post-partum collection (9·39% as against 8·47%). No attempt was made to analyse the results for HLA C locus since there was a higher percentage of HLA C locus blank in our panel In the screening for HLA DR antibodies 10·48% were positive and these were not analysed based on the methods of collection because of the smaller sample size in antenatal collection.

3.2 *Comparison of collection methodologies*

Comparison of two different collection methodologies employed, *viz.* antenatal and post-partum, with reference to total volume of sera obtainable indicated that while 94·8% of the antenatal sera were of less than 10 ml volume (615/649), 94·8% of the post-partum sera were 10 ml and above (3260/3439); (14·6%, 10–19 ml volume, 21·6%, 20–29 ml, 31·7%, 30–49 ml, 23·3%, 50–99 ml and 3·6%, above 100 ml). The only problem with the collection of post-partum haemorrhage was a higher percentage (around 5%) of contamination by faecal matters, amniotic fluid and foul smell. These sera were discarded.

3.3 *Comparison of primi and multiparous women*

Table 3 presents the results on the correlation between the parity of the women and the incidence of the anti-HLA A, B antibodies. Irrespective of the number of the past pregnancies, the incidence of the anti-HLA A and B antibodies were almost the same. The primiparous women produced the same percentage (10%) of anti-HLA antibody as those of multiparous women. This however is not the international experience.

Table 3. Parity and the incidence of anti-HLA A. **B** antibodies.

Pregnancy	Total sera	Specificity					
		Mono		Duo		Total	
		N	(%)	N	(%)	N	(%)
First	2328	212	(9·1)	36	(1·5)	248	(10·6)
Second	1089	75	(6·9)	17	(1·5)	92	(8·4)
Third	726	68	(9·4)	5	(0·7)	73	(10·1)
Fourth	295	18	(6·1)	6	(2·0)	24	(8·1)
Fifth	92	9	(9·8)	2	(2·1)	11	(11·8)
Sixth	16	—		—		—	
Seventh	15	3		—		3	
Eighth	4	—		—		—	
	$n = 4565$	385		66		451	

%, (N/total sera) × 100.

4. Discussion

4.1 *Postpartum collection*

The study has brought out that it is not possible to collect more quantity of blood from a pregnant woman by cubital bleeding (antenatal) in India, especially considering her anaemic status, malnutrition and unwillingness and the lack of facilities for sophisticated procedures like plasmapheresis. The ease with which post-partum haemorrhage can be collected in labour wards with little discomfort to the woman concerned and the higher volume of yield make this procedure preferable. Though 5% of the sera may be wasted by contamination, the rest 95% of the sera were usable and with the number of pregnant women in this country, this methodology is best suited under the given circumstances in our country. Now we have perfected the method of collection of vaginal bleeds; instead of collecting with a scoup, it can be collected by placing a sterile wide mouth bottle directly on to the vaginal outlet: this eliminates contamination by faecal materials and makes handling much easier to the attending nurses and physicians.

4.2 *Are primiparous women a good source*?

It has been suggested that the incidence of antibodies in pregnant women was related to the number of pregnancies and was 4% lower in post-partum sera than antenatal sera (Simonney *et al* 1984). In another study, the percentage of cytotoxic antibodies did not differ based on the number of past pregnancies (Rodey *et al* 1979). There is a general agreement that foetomaternal alloimmunization against HLA antigens is initiated in the first pregnancy itself (Simonney *et al* 1984). It has also been observed that the cytotoxic antibodies appeared by 18th week of pregnancy in primies but by 8th week in second and third para (Vives *et al* 1976). In Mestizo women from Venezuela, the antibodies were detected only by 34th week in primies and 17th week in second and third pregnancy (Simonney *et al* 1984). Thus there are wide variations in the immunizations and the resultant outcome (ref.

below). In the present study we found the same percentage of positive sera in women at different parity, and this is possible since in the present study the sera were collected only during the last trimester of pregnancy and at childbirth. It is possible that all the 'responder' women may produce anti-HLA antibodies in the first pregnancy itself; in subsequent pregnancies time of appearance of antibodies, titre and specificity (mono or duo) may vary.

4.3 *Incidence of antibodies varies in different studies*

Wide variations in the incidence of cytotoxic antibodies in the sera of pregnant women have been reported. The variation ranges from 7·3% to 36% (7·3%, Nymand *et al* 1971; 18·7%, Rodey *et al* 1979; 21·6%, Decary *et al* 1979; 29%, Mestizo women and 9·65% in Warao women, Simonney *et al* 1984; 36%, Terasaki *et al* 1970). Differences in the incidence between two different ethnic groups, *viz.* Venezuelan of Amerindian and mixed ethnic origins have also been reported (Simonney *et al* 1984): the incidence of antibodies in post-partum sera was 2% in Waravo women and 10% in women of mixed ethnic origin. In another study the incidence of antibodies in successfully completed pregnancies have been reported to be 32% compared to 10% in spontaneous abortions (Regan *et al* 1991). Various reasons can be attributed to this 'apparent' discrepancy like *r* value and percentage 8 + positive; ethnic differences may also account for this or methods to detect and panel size. This study has revealed an average incidence of 10% which is in agreement with some of the published observations. A significant observation of the present study was that this incidence was identified irrespective of the parity.

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