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Phenotypic Distribution, Allelic Diversity and Degree of Differentiation at *ABO* and *Rh* Loci in the Population of Haripur District, Khyber Pakhtunkhwa, Pakistan

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Abstract.- Haripur is the main Hindko speaking district in Hazara division, Khyber Pakhtunkhwa. To get a preliminary insight into the genetic structure of Haripur population, we have employed immunogentic markers of *ABO* and *Rh* loci. During the course of five years (2006-2010), phenotypic record of 2,140 donors and 2,099 patients was ascertained from the District Headquarter Hospital Haripur. In the donors, the percentages of blood groups 'A', 'B', 'AB' and 'O' were observed to be 20.75, 34.39, 5.84, and 39.02, respectively, while Rh(-) type was 10.14%. The frequencies of alleles p[A], q[B] and r[O] at the *ABO* locus were established to be 0.1436, 0.2275, and 0.6289, respectively, and Rh[d] allele at *Rh* locus was calculated to be 0.3184. Collectively, the samples of donors and patients were in conformity with Hardy-Weinberg Equilibrium. Gene diversity analyses revealed that heterozygosity at sub-population level (H_S) was not depleted from the overall heterozygosity (H_T) and hence, coefficient of differentiation and absolute gene diversity were not significant (G_{ST} =0.0026, D_{ST} =0.0012; respectively). Curiously however, there was marked difference at the phenotypic proportions, allelic frequencies and heterozygosities at the studied polymorphisms when gender specific data were considered. Differentiation was very high in the sample obtained from female patients compared to males (0.0067 vs. 0.0027), resulting in very high absolute gene diversity and more stratification in the female sample compared to males (0.0030 vs. 0.0014). The specific reasons for this discrepancy remained unknown, but it is quite likely that there were differences in the ascertainment of male and female patients in the hospital. The estimation of Nei's genetic distance demonstrated that the Haripur sample was closer to Swat and Rawalpindi populations than the Swabi, Peshawar and Abbotabad. This pilot study highlights various interesting aspects of Haripur population. Further studies through microsatellite markers are required to fully understand the dynamics of this population.

Key words: *ABO* and *Rh* genes, genetic differentiation, Haripur Hazara, Pakistani population, population genetics

INTRODUCTION

Haripur is one of the five districts of Hazara division in Khyber-Pukhtunkhwa (KPK). It is the fourth populous town after Mansehra, Abbottabad and Batagram districts and comprised about one million individuals according to the 2005 census estimate (PAP, 2011). It has an area of 1,725 km^2 (geographic coordinates: 34°44' to 34°22' NL and 72°35' to 73°15' E). About 88% of district's population resides in rural areas. Hindko and Urdu are the predominant languages and are spoken by >70% of the population.

ABO and Rh blood groups as immunogenetic markers are very favorable traits for the study of genetic structure of human populations since they are polymorphic and their genotypic and allelic frequencies can be readily calculated. Despite their low resolution power and the emergence of more polymorphic markers like microsatellites, these markers still remain popular due to their inexpensive typing and applications in blood transfusion and medico-legal cases. Previously, blood group polymorphisms study had been conducted only for Abbottabad district of Hazara division (Khaliq *et al*., 1984). On the other hand, many studies are available for various districts of KPK and Punjab provinces, *e.g.*, Mardan (Mabood *et al*., 1993), Sawabi (Khurshid *et al*., 1992), Nowshehra (Babar *et al*., 1999), Attock (Ali *et al*., 2005), Rawalpindi/Islamabad (Khan *et al*., 2006; Iqbal *et al*., 2009), and Southern Punjab (Malik and Amin-ud-Din, 2013). No study, however, is available on the population of Haripur for any genetic or anthropological parameters. It is therefore, imperative to explore the blood group polymorphisms in this population.

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SUBJECTS AND METHODS

Blood groups phenotypic data of five consecutive years (August 2006 - June 2010) were obtained from the Blood Bank registry of the District Headquarter (DHQ) hospital, Haripur. The hospital lies in the center of town and receives patients from all locations of the district. The Blood Bank maintains record of blood donors who are volunteers, general visitors or the relatives of patients, while the patient record includes subjects admitted in various wards for in-door treatment. Serological phenotyping was routinely performed by anti-A, anti-B and anti-D antisera. All the ambiguous entries were removed and the disease screening data (*i.e.*, HIV, HCV, HBX, VDRL) were excluded from the analyses.

The patients and donors data were assorted and were further distributed with respect to year and gender. Maximum likelihood estimates of allelic frequencies of *ABO* locus were calculated by the Bernstein method (Strickberger, 2005; Silva, 2002), and the concordance with the Hardy-Weinberg Equilibrium (HWE) was assessed through Chi-test statistics. The allele frequencies at the *Rh* locus were calculated directly from the phenotypic proportions of the recessive phenotype. Variability in the phenotypic proportions and allele frequencies were measured in various sample ascertainment categories. Homogeneity was tested between the samples ascertained for donors and patients, and between consecutive years (Neel and Schull, 1954). Z-test was employed to estimate the heterogeneity of blood group proportions among samples (Garstman, 2008). To understand gene diversity, individual and combined heterozygosities at *ABO* and *Rh* loci were estimated, and degree of differentiation (G_{ST}) and absolute gene diversity (D_{ST}) were calculated (Nei, 1975, 1978). The similarity of Haripur sample with its neighboring populations was sought with the Nei's genetic distance 'D' (Nei and Roychoudhury, 1982).

RESULTS

Phenotypic record of 2,140 donors and 2,099 patients was ascertained (Table I). In the sample from donors, there was minor representation of female subjects (1.45% females compared to 98.55% males), while in the patient's data there were 966 males (46.02%) and 1,133 females (53.98%) (Table I).

Table I.- Year-wise distribution (No. and percentage) of ABO and Rh blood types in donors and patients samples of Haripur population.

Year	No.	Gender		ABO types				Rh types	
		Male	Female	A	B	AB	O	Rh +	Rh -
Donors									
2006	224	220 (98.21)	4 (1.79)	27 (12.05)	94 (41.96)	17 (7.59)	86 (38.39)	196 (87.50)	28 (12.50)
2007	619	601 (97.09)	18 (2.91)	126 (20.36)	219 (35.38)	48 (7.75)	226 (36.51)	555 (89.66)	64 (10.34)
2008	527	523 (99.24)	4 (0.76)	104 (19.73)	183 (34.72)	19 (3.61)	221 (41.94)	476 (90.32)	51 (9.68)
2009	490	487 (99.39)	3 (0.61)	114 (23.27)	148 (30.20)	25 (5.10)	203 (41.43)	440 (89.80)	50 (10.20)
2010	280	278 (99.29)	2 (0.71)	73 (26.07)	92 (32.86)	16 (5.71)	99 (35.36)	256 (91.43)	24 (8.57)
Total	**2,140**	2109 (98.55)	31 (1.45)	444 (20.75)	736 (34.39)	125 (5.84)	835 (39.02)	1923 (89.86)	217 (10.14)
Patients (all)									
2006	222	117 (52.70)	105 (47.30)	25 (11.26)	88 (39.64)	18 (8.11)	91 (40.99)	197 (88.74)	25 (11.26)
2007	600	342 (57.00)	258 (43.00)	116 (19.33)	218 (36.33)	47 (7.83)	219 (36.50)	536 (89.33)	64 (10.67)
2008	521	255 (48.94)	266 (51.06)	110 (21.11)	176 (33.78)	32 (6.14)	203 (38.96)	473 (90.79)	48 (9.21)
2009	482	179 (37.14)	303 (62.86)	113 (23.44)	146 (30.29)	31 (6.43)	192 (39.83)	437 (90.66)	45 (9.34)
2010	274	73 (26.64)	201 (73.36)	72 (26.28)	86 (31.39)	23 (8.39)	93 (33.94)	256 (93.43)	18 (6.57)
Total	**2,099**	966 (46.02)	1133 (53.98)	436 (20.77)	714 (34.02)	151 (7.19)	798 (38.02)	1899 (90.47)	200 (9.53)

Table II.- Year-wise distribution (No. and percentage) of ABO and Rh blood types in male and female patients.

Year	No.	ABO types				Rh types	
		A	B	AB	O	Rh +	Rh -
Patients (Male)							
2006	117	15 (12.82)	54 (46.15)	11 (9.40)	37 (31.62)	103 (88.03)	14 (11.97)
2007	342	51 (14.91)	143 (41.81)	23 (6.73)	125 (36.55)	296 (86.55)	46 (13.45)
2008	255	56 (21.96)	93 (36.47)	15 (5.88)	91 (35.69)	218 (85.49)	37 (14.51)
2009	179	41 (22.91)	65 (36.31)	10 (5.59)	63 (35.20)	151 (84.36)	28 (15.64)
2010	73	16 (21.92)	34 (46.58)	4 (5.48)	19 (26.03)	64 (87.67)	9 (12.33)
Total	**966**	179 (18.53)	389 (40.27)	63 (6.52)	335 (34.68)	832 (86.13)	134 (13.87)
Patients (Female)							
2006	105	10 (9.52)	34 (32.38)	7 (6.67)	54 (51.43)	94 (89.52)	11 (10.48)
2007	258	65 (25.19)	75 (29.07)	24 (9.30)	94 (36.43)	240 (93.02)	18 (6.98)
2008	266	54 (20.30)	83 (31.20)	17 (6.39)	112 (42.11)	255 (95.86)	11 (4.14)
2009	303	72 (23.76)	81 (26.73)	21 (6.93)	129 (42.57)	286 (94.39)	17 (5.61)
2010	201	56 (27.86)	52 (25.87)	19 (9.45)	74 (36.82)	192 (95.52)	9 (4.48)
Total	**1,133**	257 (22.68)	325 (28.68)	88 (7.77)	463 (40.86)	1067 (94.17)	66 (5.83)

In the total sample of donors and patients, the proportion of blood group 'O' was observed to be highest followed by types 'B', 'A' and 'AB'. Among the donors, the percentages of blood groups 'O', 'B', 'A' and 'AB' were observed to be 39.02, 34.39, 20.75, and 5.84, respectively. The percentages of Rh^+ and Rh^- blood groups were calculated to be 89.86 and 10.14, respectively (Table I). Accordingly, the phenotypic proportions were comparable between donors and patients (Z-stat: $p < 0.05$). However, blood phenotypes were quite variable in the yearly samples (Table I). At the ABO system, highest variability was observed at blood type 'AB' (Coefficient of Variance: CoV=0.294) followed by type 'A' (CoV=0.259), whereas type 'O' was least variable (CoV=0.075).

In the patients sample, the proportions of ABO and Rh blood types in the male and female subjects were markedly different (Z-stat: $p > 0.05$) (Table II). For instance, 'B' blood group was the most representative blood in males (40.27%), whereas 'O' blood group was common (40.86%) in female patients. Additionally, there were 86.13% male subjects with Rh^+ blood type compared to 94.17% in the female patients (Table II). Year-wise distributions of various blood types were highly variable between males and females (Table II). In summary, these analyses suggested more heterogeneity and variability in the samples obtained from the female patients compared to the males.

In the sample of donors, the frequencies of alleles p[A], q[B] and r[O] at the *ABO* locus were established to be 0.1436 (SD± 0.0056; range: 0.1029-0.1754), 0.2275 (SD± 0.0069; range: 0.1964-0.2879), and 0.6289 (SD± 0.0079; range: 0.6024-0.6586), respectively (Table III). At the *Rh* locus, the frequency of Rh[D] allele was calculated to be 0.6816 (SD± 0.0102; range: 0.6464-0.7072) (Table III).

In the patient's data alone, the allele frequency estimates at *ABO* and *Rh* loci showed considerable variability among the males and females. In the sample from male subjects, the frequency estimates of alleles p[A], q[B], r[O], and Rh[D] were 0.1347, 0.2714, 0.5939, and 0.6276, respectively; while these estimates were 0.1654, 0.2020, 0.6326, and 0.7586, respectively, in the female sample (Table IV) (χ^2 =27.735 and =32.162 for ABO and Rh loci, respectively). Again, there was generally more variability in the allele frequency estimates in the data from female patients compared to males.

Table III.- Allele frequencies and heterozygosities at *ABO* and *Rh* loci in donors and patients samples.

	ABO locus				*Rh* locus		Heterozygosity		
Year	**p[A]**	**q[B]**	**r[O]**	**HWE***	**Rh(+)**	**Rh(-)**	**ABO**	**Rh**	**Pooled**
Donors									
2006	0.1029	0.2879	0.6092	1.640	0.6464	0.3536	0.5366	0.4581	0.4985
2007	0.1519	0.2456	0.6024	0.109	0.6785	0.3215	0.5541	0.4367	0.4958
2008	0.1253	0.2161	0.6586	4.647*	0.6889	0.3111	0.5043	0.4290	0.4671
2009	0.1542	0.1964	0.6495	1.081	0.6806	0.3194	0.5164	0.4352	0.4763
2010	0.1754	0.2179	0.6067	2.111	0.7072	0.2928	0.5546	0.4149	0.4856
Total	**0.1436**	**0.2275**	**0.6289**	2.368	**0.6816**	**0.3184**	**0.5322**	**0.4342**	**0.4833**
Patients (all)									
2006	0.1011	0.2744	0.6245	4.024*	0.6644	0.3356	0.5257	0.4469	0.4874
2007	0.1463	0.2523	0.6013	0.257	0.6734	0.3266	0.5538	0.4402	0.4975
2008	0.1474	0.2253	0.6273	0.296	0.6965	0.3035	0.5345	0.4232	0.4793
2009	0.1627	0.2047	0.6326	0.058	0.6944	0.3056	0.5320	0.4248	0.4789
2010	0.1919	0.2242	0.5839	0.022	0.7437	0.2563	0.5730	0.3819	0.4783
Total	**0.1512**	**0.2331**	**0.6157**	0.096	**0.6913**	**0.3087**	**0.5439**	**0.4269**	**0.4855**

* significant deviations from HWE at *ABO* locus.

In the donors samples, there was one instance of deviation from HWE, *i.e.*, in year 2008 (Table II). Apparently, this deviation was due to the marked over-representation of 'O' blood type (41.94%) and under-representation of 'AB' type (only 3.61%) (Table I). In the patient's samples, there was also one case of significant deviation from HWE, *i.e.*, 2006. Accordingly, blood type 'O' was highly represented (40.99%), whereas blood type 'A' was in minor proportions among the patients (11.26%). However, the total samples of donors and patients were in conformity with HWE (Table III).

The heterozygosity estimate at the *ABO* locus in the total sample of donors was 0.5322 while it fluctuated between 0.5043 and 0.5546 throughout the study period (Table III). Heterozygosity at the *Rh* locus was 0.4342 (range: 0.4149-0.4581). The combined heterozygosity of both loci was 0.4833 (range: 0.4671-0.4985). In the total male patients sample, the heterozygosity estimates at *ABO*, *Rh* and both loci were 0.5558, 0.4677, and 0.5120, respectively, while these estimates in the female patient's sample were 0.5319, 0.3664, and 0.4494, respectively (Table IV). In conclusion, these analyses demonstrated that there was less heterozygosity and more diversification in the female's sample compared to the male's, as for as the ABO and Rh allelic systems were concerned (explained below).

In order to further understand gene diversification at the *ABO* and *Rh* loci, the heterozygosities at various sample types were compared with the total heterozygosities (Table V). It was observed that heterozygosity at sub-sample level (H_S) was not very different (*i.e.*, low) from the overall heterozygosity (H_T), at the *ABO, Rh* and combined loci (Table V). Hence, there appeared no loss of heterozygotes in the donors or patients samples at the studied loci. Nevertheless, genetic differentiation in the total donor and patient samples was not significant. In the donors samples, coefficient of differentiation (G_{ST}) and absolute gene diversity (D_{ST}) were observed to be very small at the studied blood group polymorphisms (0.0026 and 0.0012, respectively).

However, when the gender specific data of patients were considered, there was marked difference between the gene diversities in male and female samples. Differentiation was very high in the female patients compared to the males (G_{ST} =0.0067

Table IV.- Allele frequencies and heterozygosities at *ABO* and *Rh* loci in male and female patients samples.

	ABO locus				*Rh* locus		Heterozygosity		
Year	**p[A]**	**q[B]**	**r[O]**	**HWE***	**Rh(+)**	**Rh(-)**	**ABO**	**Rh**	**Pooled**
Patients (Male)									
2006	0.1173	0.3310	0.5517	0.684	0.6541	0.3459	0.5748	0.4545	0.5168
2007	0.1147	0.2824	0.6030	0.052	0.6333	0.3667	0.5443	0.4652	0.5055
2008	0.1514	0.2421	0.6065	1.135	0.6191	0.3809	0.5517	0.4726	0.5131
2009	0.1555	0.2395	0.6050	1.297	0.6045	0.3955	0.5540	0.4795	0.5182
2010	0.1505	0.3128	0.5367	2.056	0.6489	0.3511	0.5955	0.4588	0.5308
Total	**0.1347**	**0.2714**	**0.5939**	**1.307**	**0.6276**	**0.3724**	**0.5558**	**0.4677**	**0.5120**
Patients (Female)									
2006	0.0836	0.2170	0.6994	3.719	0.6763	0.3237	0.4590	0.4399	0.4516
2007	0.1898	0.2140	0.5963	0.696	0.7359	0.2641	0.5638	0.3895	0.4775
2008	0.1436	0.2097	0.6467	0.088	0.7966	0.2034	0.5182	0.3246	0.4222
2009	0.1670	0.1850	0.6480	0.406	0.7631	0.2369	0.5189	0.3621	0.4412
2010	0.2071	0.1947	0.5981	0.750	0.7884	0.2116	0.5628	0.3345	0.4498
Total	**0.1654**	**0.2020**	**0.6326**	**3.005**	**0.7586**	**0.2414**	**0.5319**	**0.3664**	**0.4494**

* All the samples were in conformity with HWE conditions.

vs. 0.0027). Similarly, absolute gene diversity estimate in female patients was two folds compared to males (D_{ST} =0.0030 vs. 0.0014). Furthermore, genetic differentiation and diversification were more pronounced at the *Rh* locus compared to the *ABO* locus (Table V).

In order to assess the affinities of Haripur sample with the neighboring populations, Nei's measure of genetic distance D was calculated (Nei and Roychoudhury, 1982). Haripur population was closer to Rawalpindi, Swat and Buner populations (D=0.0060, 0.0062 and 0.0069, respectively), as compared to Mardan, Abbotabad, Peshawar and Swabi (D=0.0098, 0.0099, 0.0104 and 0.0129, respectively).

Table V.- Gene diversity indices in the Haripur population samples of donors and patients.

Locus	**H_T**	**H_S**	**G_{ST}**	**D_{ST}**
Donors				
ABO	0.5343	0.5324	0.0034	0.0018
Rh	0.4348	0.4342	0.0015	0.0006
Both	**0.4845**	**0.4833**	**0.0026**	**0.0012**
Patients (all)				
ABO	0.5447	0.5432	0.0028	0.0015
Rh	0.4248	0.4235	0.0030	0.0013
Both	**0.4847**	**0.4833**	**0.0029**	**0.0014**
Patients (Male)				
ABO	0.5632	0.5610	0.0039	0.0022
Rh	0.4655	0.4650	0.0012	0.0006
Both	**0.5144**	**0.5130**	**0.0027**	**0.0014**
Patients (Female)				
ABO	0.5275	0.5246	0.0055	0.0029
Rh	0.3718	0.3687	0.0085	0.0032
Both	**0.4497**	**0.4466**	**0.0067**	**0.0030**

DISCUSSION

The sample of Haripur population comprised 2,140 donors and 2,099 patients. Expectedly, the phenotypic distributions and the allelic frequencies were similar in both sample types. However, within the samples there were marked differences in gender specific distributions. For example, donors were predominantly males (98.55%). This could be

explained by the fact that in our society, male subjects are chiefly responsible for seeking medical help for their family members. Hence, the likelihood of blood donation by the male subject attending the emergency situation at the hospital, is very high.

Homogeneity was tested between the samples ascertained in consecutive years for donors and patients (*i.e.*, total, male and female). Among the donors, samples were generally homogeneous. However, heterogeneity was observed at blood type 'A' between the samples ascertained in years 2006 and 2007 (Z-score =2.76). Heterogeneity was also observed between the samples obtained in years 2007 and 2008 at 'AB' blood type (Z-score =2.98). In the patient's data, there was heterogeneity at 'A' blood system between the samples obtained in years 2006 and 2007 (Z-score =2.73). Among the samples from male patients, heterogeneity at 'A' blood system was evident between years 2007 and 2008 (Z-score =2.22). Among the female patients, heterogeneity was observed at 'A' and 'O' blood group systems between years 2006 and 2007 (Z-scores =3.34 and 2.64, respectively). Marked heterogeneity was also observed in all pair-wise comparisons between male and female patients samples. At the ABO system, most of the heterogeneity was due to 'B' blood type. Additionally, heterogeneity was more explicit at the Rh system compared to the ABO.

In the data from patients, there were comparable number of males and females (966 vs. 1133). Curiously however, the phenotypic and genotypic proportions of ABO and Rh loci were markedly different in both genders. For instance, 'B' blood group was the common type in male patients while 'O' type was more prevalent in females. The differences were also obvious at the Rh system (Table II). Accordingly, at both loci there were disparities in the allele frequencies between both genders (Table IV). At the *ABO* locus, allele q[B] was the most variable type in the male sample whereas allele r[O] was observed to be more fluctuating in females. The differences in the male and female patients samples were also witnessed in the subsequent analyses at various levels. For instance, in the female sample there was loss of heterozygosity at the *ABO* and *Rh* loci which resulted in very high gene differentiation and absolute gene diversity. In summary, there was discrepancy between male and female samples which could be due to various reasons including non-random sampling. It might well be that the district hospital receives in-door patients preferentially from certain localities. For instance, there could be gender differentials in the hospitalization/medical recruitment. Further studies are required to resolve this apparent lack of concordance between the male and female samples ascertained from male and female patients.

In conclusion, this pilot study in Haripur district revealed various interesting aspects. First, it established the phenotypic and allelic composition of *ABO* and *Rh* loci in the donors and patients samples, and showed that diversification was weak. Secondly, within the patients, marked differences were evident in the male and female patients samples. These differences were explicit at the phenotypic proportions, allelic frequencies and locus heterozygosities. Additionally, discrepancies were also witnessed at the gene diversity and population differentiation estimates. Generally, the male sample appeared more random and less stratified, while the female sample was less diversified. It is worth mentioning that the conclusion drawn from the present data need to be complemented with additional molecular markers. Further studies with the help of highly polymorphic genetic markers are required to further understand the genetic structure of Haripur and neighboring populations.

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