Pakistan J. Zool., vol. 47(3), pp. 649-656, 2015.

Genetic Heterogeneity and Gene Diversity at *ABO* and *Rh* Blood Group Polymorphisms in Seven Pashtun Populations of Upper Khyber Pakhtunkhwa, Pakistan

Sher Ali and Sajid Malik*
Human Genetics Program, Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, 45320 Islamabad

Abstract.- Pashtun population in the North-West Pakistan is ethnically, culturally and compositionally very distinct from the rest of Pakistanis. Pashtuns are important for the understanding of genetic affinities of Pakistanis with other populations like Afghans, Central Asians and Aryans. To get an overview of their genetic structure, we have assembled the ABO and Rh blood group polymorphisms data of 83,000 subjects from seven Pashtun populations of upper Khyber Pakhtunkhwa, namely Buner, Mardan, Dir-Lower, Swabi, Swat, Nowshera, and Peshawar. We establish that these populations despite their linguistic and ethnic affinities, exhibit ample variation at the blood group markers. The frequencies of alleles *p[A]*, *q[B]* and *r[O]* ranged from 18.7—23.2%, 22.0—26.5%, and 53.9—57.7%, respectively, while allele *Rh[d]* remained 21.5—31.4%. The average heterozygosities ranged from 0.458 to 0.517 in Swabi and Swat populations, respectively. Comparison of genotypic frequencies, homogeneity tests for allelic frequencies, and heterozygosity analyses reveal that a straightforward clustering of these populations was not possible. Furthermore, gene diversity analyses yielded total heterozygosity, H_T =0.4901, and D_{ST} =0.0012, while coefficient of gene differentiation, G_{ST} was calculated to be 0.0024. Nei's measure of genetic distance (DA) was found to be highest between Swabi and Swat samples, and lowest between Dir-Lower and Mardan. These results established substantial differentiation and substructuring among these populations. Further investigations by employing highly polymorphic molecular markers would be indispensable in getting a deeper insight into the genetic diversity of Pashtuns and identifying the underlying evolutionary force causing this differentiation.

Key Words: Genetic heterogeneity, gene diversity, ABO blood groups, Rh blood groups, allelic polymorphisms.

INTRODUCTION

Genetic structure of the Pashtun populations of the North-Western territories of Pakistan deserves special attention due to certain specific features of population development. The Pashtuns are ethnically, culturally and compositionally very distinct from the rest of the Pakistanis. They originated from Afghanistan in 15th and 16th centuries and inhabited the Northern and Western borders of the Indian subcontinent (Afridi, 2003). Today, the Pashtuns in Pakistan occupy the highly inaccessible Hindu Kush rampart between Pakistan-Afghanistan, and between Pakistan-Iran and claim their ancestry with the Yousafzai tribe (Afridi, 2003; Bokawee, 2006). Pashtuns speak Pashto language which belongs to the Iranian branch of the Indo-Iranian subfamily (Khan, 1991). Since migration, Pashtuns have substantially radiated into various sub-tribes which inhabit different geographic locations. However, the nature and dynamics of this diversification has not been much explored (Bernhard, 1967).

The Pashtuns are important for the understanding of the genetic affinity of Pakistanis with the Aryans, Central Asians and Western populations which infiltrated the region in various episodes. Only few studies based on the anthropological and genetic markers have been conducted on these populations, particularly due to their remote mountain terrain and relative seclusion (Chaurdri *et al*., 1952; Ali and Malik, 2014; Rehman *et al*., 2014; Rehman *et al*., 2015). In this context, it seems interesting to examine the genetic structure of representative Pashtun populations. Hence, we have employed classical blood group markers (*i.e*., *ABO* and *Rh*) in order to understand genetic differentiation in seven of Pashtun populations of upper Khyber Pakhtunkhwa (KPK), few of them severely war-affected during the geopolitical turmoil in the last decades. ABO and

* Corresponding author: malik@qau.edu.pk
0030-9923/2015/0003-0649 $ 8.00/0

Rh blood types are basic immuno-genetic systems commonly used in blood transfusions. They have also been employed as polymorphic markers and exhibit considerable variation in different geographic locations reflecting the diversity of human populations.

SUBJECTS AND METHODS

Phenotypic data of 83,093 subjects from seven Pashtun populations of KPK were assembled (Tables I, II). For three populations (i.e., Buner, Mardan and Dir-Lower), ABO and Rh phenotyping of 26,614 subjects was carried out on fresh blood samples collected from the healthy volunteer donors and the visitors to the District Headquarter Hospitals at Buner (34°30°41N, 72°29°02E; Dagar), Mardan (34°12°0N, 72°1°60E), and Dir-Lower (35°20°07N, 71°88°28E; Timargara) (Fig. 1). A sample of 2,839 subjects was ascertained from Buner between years 2006-2009; 13,669 subjects from Mardan in 2003-2004; and 10,106 subjects from Dir-Lower in 2007. Data on four adjoining populations (*i.e.*, Nowshera, Peshawar, Swabi and Swat), were extracted from the published literature (Babar *et al*., 1999; Anonymous, 1984; Khurshid *et al*., 1992; Khattak *et al*., 2008).

At *ABO* locus, maximum likelihood estimates of the allele frequencies were obtained. The frequency of the *Rh*(d) allele was calculated from the square root of the frequency of Rh(-) phenotypes. To check the Hardy-Weinberg equilibrium (HWE), goodness-of-fit tests were performed (Mather, 1964; Silva, 2002; Malik and Amin-ud-Din, 2013). Homogeneity was tested for each population for the data acquired in consecutive years and between male and female samples (Neel and Schull, 1954). *Z*-test was employed to check the significance of heterogeneity of blood group proportions among the studied populations. For a meaningful grouping of these populations, homogeneity tests for *ABO* and *Rh* gene frequencies were carried out (Neel and Schull, 1954; Shami and Rasmuson, 1994). Heterozygosity at the individual *ABO* and *Rh* locus and the combined heterozygosities were estimated (Nei, 1987). Finally, the degree of differentiation according to ABO/Rh polymorphic systems was quantified for all seven populations (Nei, 1987). The results were displayed through ternary plot and UPGMA (Unweighted Pair Group Methods with Arithmetic Means) dendrogram (Sneath and Sokal, 1973).

Fig. 1. Map of Pakistan with set-in Khyber Pakhtunkhwa (KPK) province. Seven Pashtun populations studied were: B, Buner; M, Mardan; D, Dir-Lower; Sw, Swabi; St, Swat; N, Nowshera; P, Peshawar.

RESULTS

Blood group proportions and allelic frequencies

In the overall sample, the proportion of 'B' blood group was highest (32.3%), followed by 'O', 'A' and 'AB' types (29.9%, 28.8%, and 9%, respectively). Individually, the preponderance of 'B' blood group was highest in Buner, 'O' in Swabi, 'A' in Mardan, and 'AB' in Nowshera. In seven Pashtun populations, the frequency of blood type 'B' ranged from 28.9—36.7%, 'O' type was 28.8—33.2%, 'A' type was 24.6—32.3%, and 'AB' type was 7.3—11% (Table II). According to the Rh factor, Rh(-) blood group ranged from 04.61% in Swabi to 09.87% in Swat.

The distribution of allelic frequencies at *ABO* and *Rh* loci was also variable in the studied populations. For instance, *A[p]* allele ranged from 18.7—23.2%, *B[q]* between 22—26.5%, and *O[r]* allele between 53.9—57.7% (Table III). At the *Rh*

Table I.- Demographic and linguistic features of seven Pashtun populations of North-West Pakistan.

Population/ District	No. of individuals*	Major Caste / Ethnic group	Percentage distribution by mother tongue					
			Pashto	Urdu	Punjabi	Hindko	Saraiki	Others
Buner	0.74	Yousafzai	96.60	0.19	0.02	0.00	0.02	3.38
Mardan	1.99	Yousafzai	98.44	0.33	0.49	0.00	0.03	0.71
Dir-Lower	1.01	Yousafzai	99.35	0.13	0.04	0.00	0.00	0.48
Swabi	1.40	Yousafzai	96.40	0.20	0.50	0.00	0.10	2.80
Swat	1.77	Yousafzai	92.96	0.18	0.08	0.00	0.12	6.84
Nowshera	1.18	Khattak, Khalils, Yousafzai	91.00	1.30	3.60	0.00	0.30	3.80
Peshawar	2.88	Mix of Yousafzai, non-Yousafzi and non-Pashtun races	85.62	2.76	2.63	5.00	0.18	3.81

*million individuals in 2010

Table II.- ABO and Rh blood groups proportions in seven Pashtun populations of North-West Pakistan.

Population	Sample size (n)	Phenotype					
		A	B	AB	O	Rh+	Rh-
Buner *	2,839	24.6	36.7	9.2	29.6	92.7	7.3
Mardan *	13,669	32.3	30.8	8.0	28.8	93.2	6.8
Dir-Lower *	10,106	30.8	28.9	10.3	30.1	92.7	7.3
Swabi	4,360	27.6	30.4	8.8	33.2	95.4	4.6
Swat	22,897	27.9	32.4	10.6	29.1	90.1	9.9
Nowshera	4,510	27.1	32.0	11.0	29.8	92.9	7.1
Peshawar	24,712	27.9	34.3	7.3	30.6	94.6	5.4
Total	83,093	28.8	32.3	9.0	29.9	92.8	7.2

* Data of these populations were ascertained in the current study.

Table III.- Distribution of allelic frequencies, Hardy-Weinberg Equilibrium at *ABO* locus, and heterozygosity estimates for Pashtun populations.

Population	ABO Locus			HWE test; p value	Rh Locus		Heterozygosity		
	p[A]	q[B]	r[O]		Rh+(D)	Rh-(d)	ABO	Rh	Average
Buner	0.187	0.265	0.548	0.11	0.729	0.271	0.594	0.395	0.495
Mardan	0.230	0.220	0.550	inf.*	0.739	0.261	0.596	0.386	0.491
Dir-Lower	0.232	0.220	0.548	0.76	0.729	0.271	0.598	0.395	0.496
Swabi	0.203	0.220	0.577	0.73	0.785	0.215	0.578	0.337	0.458
Swat	0.216	0.245	0.539	0.93	0.686	0.314	0.603	0.431	0.517
Nowshera	0.213	0.245	0.542	0.11	0.733	0.267	0.601	0.391	0.496
Peshawar	0.196	0.238	0.566	inf. *	0.768	0.232	0.585	0.356	0.471
Total	0.212	0.235	0.553	inf.*	0.731	0.269	0.594	0.393	0.494

* highly significant deviation

locus, *d* allele revealed lowest estimates in Swabi (21.5%), and highest in Swat (31.4%). Further, the samples obtained from Peshawar and Mardan were significantly deviating from HWE expectations. The total sample was also not consistent with HWE (Table III).

Genotype frequencies

The comparison of *ABO* and *Rh* genotypic frequencies among the Pashtun populations demonstrated significant differences among them. Highest Z-scores for ABO blood types (depicting maximum heterogeneity against all other

populations) were obtained between Peshawar and Mardan, and between Swabi and Swat. The Swabi and Swat populations were also witnessed to be highly heterogeneous at Rh blood proportions. With respect to the blood phenotypes, the 'AB' type depicted wide fluctuations among seven populations, followed by blood groups 'B', 'A', and 'O' (22%, 52%, and 67% less Z-scores than 'AB', respectively).

Heterozygosity estimates

Heterozygosities were estimated at the individual and combined blood group loci. At the *ABO* locus, heterozygosity was observed to be lowest in Swabi (0.578) and highest in Swat (0.603) (Table III). At *Rh* locus, the lowest estimate was observed again in Swabi (0.337), while the highest estimate was evident in Swat (0.431) (Fig. 2). However, the differences between populations became less conspicuous when combined heterozygosites were considered. The Swabi population showed maximum departure from the averaged heterozygosity for the total Pashtun sample. Taking together, the averaged heterozygosities were 0.495, 0.491, 0.496, 0.458, 0.517, 0.496, and 0.471 for Buner, Mardan, Dir-Lower, Swabi, Swat, Nowshera, and Peshawar populations, respectively.

Homogeneity tests

To further understand genetic variability among Pashtun populations and to identify the potentially homogeneous group(s), we performed homogeneity tests (Neel and Schull, 1954). Homogeneity was initially tested in pair-wise comparisons, and then three, four and more populations were included in the analyses. It was expected that populations in close geographic proximity might appear more homogeneous compared to the distantly located entities. The analyses showed that simple clustering based on geographic neighborhood or North-South grouping was not possible and highly significant heterogeneities were observed between these populations (Table IV). Heterogeneity with respect to the *ABO* locus was more pronounced than the *Rh* system. Generally, the heterogeneity was increasing with the increasing number of populations included in the analyses. The populations of Peshawar and Swabi were not homogeneous with any population at both *ABO* and *Rh* systems. For comparison, we split seven populations into Northern and Southern groups (Fig. 1). The Northern group (Dir-Lower, Swat, Mardan, Buner) was more heterogeneous at both loci compared to the Southern group (Peshawar, Nowshera, Swabi). Nonetheless, heterogeneity was highly significant when seven populations were considered together (χ^2 =268.1; p<0.0001, and χ^2 =225.4; p<0.0001, for *ABO* and *Rh* loci, respectively).

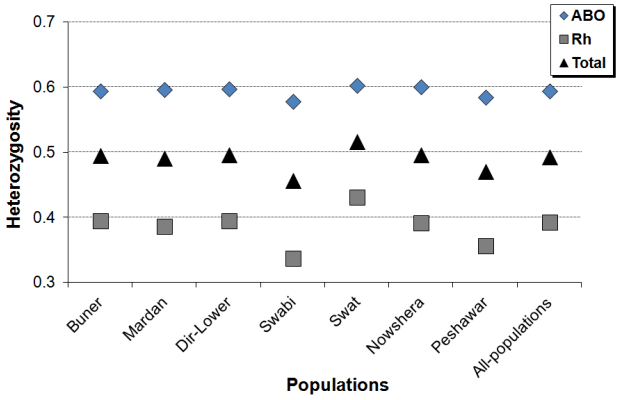

Fig. 2. Comparison of individual and combined heterozygosities at *ABO* and *Rh* loci in studied populations.

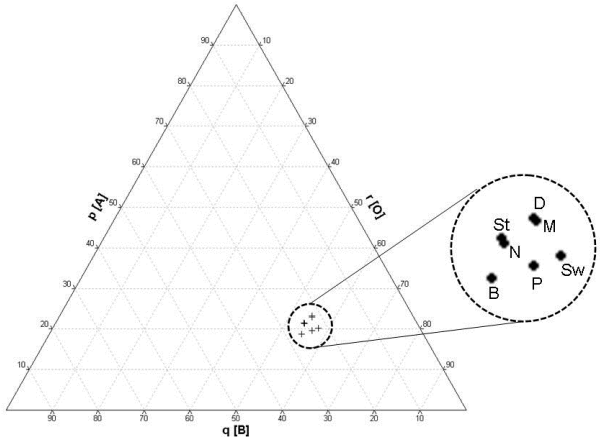

Fig. 3. Ternary plot drawn on the basis of *ABO* allelic frequencies for Pashtun populations (B, Buner; M, Mardan; D, Dir-Lower; Sw, Swabi; St, Swat; N, Nowshera; P, Peshawar.).

Table IV.- Homogeneity tests at ABO and Rh systems in Pashtun populations.

Populations compared	ABO heterogeneity			Rh(D) heterogeneity		
	χ^2	d.f.	p	χ^2	d.f.	p
Buner-Mardan	73.01	2	<0.0001	0.89*	1	0.05
Buner-Dir	73.39	2	<0.0001	0.00*	1	0.05
Mardan-Dir	0.46*	2	> 0.8	2.31*	1	0.05
Swat-Nowshera	0.31*	2	> 0.8	33.47	1	<0.05
Dir-Mardan-Nowshera	29.65	4	<0.0001	2.16*	2	0.05
Buner-Swat-Nowshera	26.37	4	<0.0001	47.54	2	<0.0001
Northern group (Dir-Lower, Swat, Mardan, Buner)	132.33	6	<0.0001	127.85	3	<0.0001
Southern group (Peshawar, Nowshera, Swabi)	29.68	4	<0.0001	25.75	2	<0.0001
All seven populations	268.12	12	<0.0001	225.37	6	<0.0001

* Digits in boldface show homogeneity between populations at *ABO* and *Rh* loci.

Table V.- Gene diversity analysis for *ABO* and *Rh* loci in seven Pashtun populations.

Population	Locus	H_T	H_S	D_{ST}	G_{ST}
Northern group (Dir-Lower, Swat, Mardan, Buner)	ABO	0.598	0.598	0.0007	0.0012
	Rh	0.403	0.402	0.0008	0.0021
	Pooled	0.500	0.500	0.0008	0.0015
Southern group (Peshawar, Nowshera, Swabi)	ABO	0.588	0.588	0.0004	0.0006
	Rh	0.363	0.362	0.0009	0.0026
	Pooled	0.475	0.475	0.0007	0.0014
All seven	ABO	0.594	0.593	0.0007	0.0011
	Rh	0.386	0.385	0.0017	0.0044
	Pooled	0.490	0.489	0.0012	0.0024

Interestingly however, only two homogeneous categories emerged in pair-wise homogeneity tests at the *ABO* locus, *i.e.*, Mardan and Dir-Lower (p<0.001), and Swat and Nowshera (p<0.001). Homogeneity between Mardan and Dir-Lower also corroborated at the *Rh* locus. It is worthwhile to mention that both pairs of homogeneous populations are geographically far apart and are not connected by a land mass directly (Fig. 1). This pairing was also witnessed when a ternary plot was drawn on the basis of *ABO* allele frequencies (Fig. 3).

Gene diversity analysis

To further establish gene differentiation among the Pashtun populations we checked the concept of gene diversity at *ABO* and *Rh* loci. The population groups (*i.e.*, Northern and Southern) appeared considerably subdivided (Table V). The degree of subdivision varied in both regions and was reflected by the differences in the behavior of H_T and H_S. The H_T was higher than H_S at both *ABO* and *Rh* loci indicating a substantial contribution of the inter-population components of genetic differentiation. The total genetic diversity (H_T) of the *ABO* locus was greater than that of the *Rh* locus in both groups. The genetic diversity was relatively higher in the Northern populations than the Southern region (0.598 vs. 0.588) (Table V). At the *Rh* locus also, diversity was higher in the Northern region compared to the Southern (0.403 vs. 0.363). The analysis of H_T averaged over the two loci showed that the genetic diversity was relatively high in the Northern group (0.500) with wide inter-population variation, H_S (0.499). On the other hand, in the Southern populations H_T remained 0.475 with comparatively lower estimates of inter-population variation (0.474). The D_{ST} for the *ABO* (or *Rh*) locus

in the Northern region was higher (~20%) than Southern. Furthermore, the coefficient of gene differentiation, G_{ST}, was increasing with the increasing number of populations.

Nei's measure of genetic distance (DA) was calculated between the Pashtun populations through DISPAN software (Ota, 1993). It was found to be highest between Swabi and Swat samples (0.0035), and lowest between Dir-Lower and Mardan (0.000). Based on the DA distance matrix between populations, dendrograms were constructed as per UGPMA method (Fig. 4). These analyses reaffirmed the affinities between Mardan and Dir-Lower populations which emerged as the closest pair among seven populations. Nowshera also joined this cluster as a third member. Interestingly however, Swabi and Peshawar appeared connected but as out-group to other five populations.

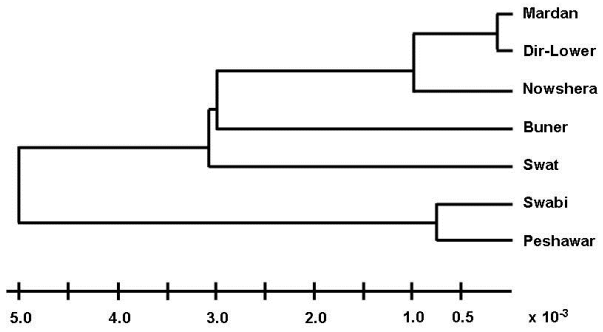

Fig. 4. Dendrogram based upon DA-UPGMA showing the genetic relationship between Pashtun populations.

DISCUSSION

The Pashtun populations of Pakistan, remaining in isolation for centuries are no more restricted to the North-West Pakistan and have substantially expanded in various directions making close endogamous communities. For instance, the Pashtun community constitutes the second largest minority of the Karachi population (>11% of 17 million) at the Southern costal extremes of Pakistan.

In the present study, several tires of analyses were carried out in order to explore the dynamics of *ABO* and *Rh* polymorphisms. Our preliminary analyses through these classical blood group markers could be highly valuable in getting a partial understanding of the genetic structure of Pashtuns. Differences in blood group proportions, genotypic frequencies, and allelic frequencies were evident in all the Pashtun populations recruited in the current study. The test of goodness of fit at the *ABO* locus indicated that the populations of Peshawar and Mardan were significantly deviating from Hardy-Weinberg expectations. Non-random sampling, technical errors in genotyping, and inbreeding could result in deviations from HWE. For instance in the samples ascertained from Mardan, this deviation could be due to overrepresented 'A' blood type (32.31%), whereas 'AB' and 'O' types were fewer than expected. For the Peshawar population, this deviation could be due to overrepresented 'B' type and underrepresented 'AB' blood type. There was a decrease in the number of heterozygotes which may show inbreeding (discussed below). Additionally, Peshawar population is relatively cosmopolitan comprising an assemblage of various ethnic groups (Table I), which may contribute to assortative mating systems and loss of heterozygotes. The overall sample was nonetheless not in conformity with HWE, indicating subdivisions in mating groups within populations (Table III).

Homogeneity tests between various samples revealed considerable heterogeneities between Pashtuns. Interestingly however, pair-wise homogeneity was witnessed between Mardan and Dir-Lower, and between Swat and Nowshera. These results are surprising keeping in view of the fact that Mardan and Dir-Lower have wide geographic distance (~150 km), and are physically separated by Malakand Agency. Additionally, Mardan comprises chiefly plain area with a dense population while Dir-Lower has grossly mountainous and undulating topography with clustered inhabitants. On the other hand, Swat and Nowshera are ~200 km apart and are separated by Mardan and Buner (Fig. 1). Furthermore, in order to identify reasonably homogeneous clusters, seven studied populations were split into northern and southern groups. The genetic diversity was higher in Northern populations than Southern. The coefficient of gene differentiation (G_{ST}) was increasing with the increasing number of populations which strongly suggested that local populations were subdivided

and the genetic differentiation of subpopulations was increasing. Hence, it is conceivable that the high degree of differentiation in both groups is accounted for by a low heterogeneity of subpopulations caused by either specific structured marriage relationships or a drift of the given genes.

The Nei's genetic distance (DA) scores demonstrated that Swabi and Peshawar appeared together as out-group. This apparent association between Swabi and Peshawar however, is not due to their similar genetic compositions, but may confirm their heterogeneous status against all other Pashtun population studied. This may also suggest that similar demographic situations were occurring separately in both populations; for instance, the multiethnic assemblage, cosmopolitan effects, inbreeding in the population isolates, and loss of heterozygosity.

This study has several potential limitations. First, only two genetic polymorphic systems have been employed to explore the dynamics of Pashtun populations. Second, despite their popularity *ABO* and *Rh* loci have limited resolution power and they need to be supplemented with additional molecular markers. For instance, these systems do not provide clues to the underlying factors responsible for the differentiation among studied populations. Finally, there are several other Pashtun populations (*e.g.*, Kohistan, Shangla) for which no data on polymorphic systems is available.

In conclusion, the current analyses on seven Pashtun populations of upper KPK demonstrated that there were wide genetic differences among these populations as far as the *ABO* and *Rh* blood markers were concerned. These analyses revealed that a straightforward clustering among these populations was devoid. There was considerable heterogeneity among these Pashtuns which suggested the likely role of the underlying evolutionary forces. This is rather surprising as different Pashtun populations in North-West Pakistan trace their origin to a common stock of Afghan origin. It is quite likely that high levels of assortative unions within tribes and sub-tribes (Wahab and Ahmad, 1996), geopolitical turmoil in the last decades, and migrations and fragmentations, are responsible for this pronounced diversification in Pashtuns. Furthermore, isolation and the unique socio-cultural structure of Pashtuns which favors consanguineous marriages could have also contributed as strong demographic agents (Wahab and Ahmad, 1996). At present it is not known which of these factors is most important in shaping the diversification in Pashtuns, but it appears that at least some parts are due to inbreeding within the populations. Finally, it would be interesting to see how these immune-genetic markers behave in the other Pashtun populations. Additional studies with the help of highly polymorphic molecular markers are warranted to explore the major evolutionary factor(s) that determined the present genetic differentiation of Pashtuns.

ACKNOWLEDGEMENTS

We highly acknowledge the participation of subjects in this study. This project was funded by QAU-URF and HEC-Pakistan.

Conflict of interest

None.

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(Received 13 November 2013, revised 14 September 2014)