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ABO Blood Group Phenotype Frequency Estimation Using Molecular Phenotyping in Rhesus and Cynomolgus Macaques

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Abstract

A much larger sample ($N = 2369$) was used to evaluate a previously reported distribution of the A, AB and B blood group phenotypes in rhesus and cynomolgus macaques from six different regional populations. These samples, acquired from 15 different breeding and research facilities in the US, were analyzed using a real-time quantitative polymerase chain reaction (qPCR) assay that targets single nucleotide polymorphisms (SNPs) responsible for the macaque A, B and AB phenotypes. The frequency distributions of blood group phenotypes of the two species differ significantly from each other, and significant regional differentiation within the geographic ranges of each species was also observed. The B blood group phenotype was prevalent in rhesus macaques, especially those from India, while the frequencies of the A, B and AB phenotypes varied significantly among cynomolgus macaques from different geographic regions. The Mauritian cynomolgus macaques, despite having originated in Indonesia, showed significant ($p \ll 0.01$) divergence from the Indonesian animals at the ABO blood group locus. Most Mauritian animals belonged to the B blood group while the Indonesian animals were mostly A. The close similarity in blood group frequency distributions between the Chinese rhesus and Indochinese cynomolgus macaques demonstrates that the introgression between these two species extends beyond the zone of intergradation in Indochina. This study underscores the importance of ABO blood group phenotyping of the domestic supply of macaques and their biospecimens.

Keywords

ABO blood testing; molecular diagnostics; colony genetic management; rhesus macaques; cynomolgus or long tailed macaques

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Introduction

Cynomolgus (*Macaca fascicularis*) and rhesus (*M. mulatta*) macaques serve as experimental non-human primate (NHP) models for immunological, neurobiological and physiological research as well as studies of infectious and metabolic diseases [Fultz, 1993; Rhesus Macaque Genome et al., 2007]. Their genomes are quite similar, with 92% of the Vietnamese cynomolgus macaque genome sequence scaffolds successfully aligning to the Indian rhesus macaque genome [Yan et al., 2011] and 99.21% sequence identity between the Mauritius cynomolgus macaque and Indian rhesus macaque genomes [Ebeling et al., 2011], and both species share the A and B blood group antigens with humans. Researchers have employed macaque A and B blood-group antigen expression to select compatible experimental subjects for transplantation, blood transfusion and stem cell research [Premasuthan et al., 2012; Rhesus Macaque Genome et al., 2007].

NHP A, B and O (H) antigens are not found on the surface of red blood cells (RBCs) but are found in bodily fluids, precluding forward typing agglutination tests [Busch et al., 2006] to determine their presence using anti-sera specific to these antigens. Reverse typing, immunohistochemistry (IHC) and saliva-inhibition tests (SIT) have all been performed to identify the A and B antigens in macaques [Kim et al., 2015; Malaivijitnond et al., 2008; Moor-Jankowski and Socha, 1978; Premasuthan et al., 2012]. Both reverse typing and SIT protocols require fresh macaque blood or saliva samples of sufficient quantity and quality, which are not always available, especially for archived tissue samples. Accurate reverse typing of the A and B agglutinins in macaques by the presence of serum antibodies requires absorbing non-specific (e.g., anti-human) agglutinins from macaque sera with type O human RBCs before reacting the sera with known type A and type B RBCs, a critical step that is sometimes omitted from protocols by researchers that consequently leads to false positive tests for the type O blood group phenotype [Kim et al., 2015]. Differentiation in buccal mucosal epithelia, which are used for IHC analysis, may not always provide the best substrate for IHC, resulting in spurious immunohistochemical test results. In addition, extra purification steps are sometimes necessary in IHC protocols in order to eliminate histo-blood group antigens present in saliva before fixation [Kim et al., 2015]. Although the tests described above can determine A and B antigens when carefully conducted, none of them can directly identify the genetic variants responsible for the A and B alleles.

The DNA-based assay developed by Premasuthan et al. [2011; 2012] provides a rapid, reliable, convenient and cost-effective molecular diagnostic alternative to the reverse agglutination, SIT and histochemical methods for determining ABO blood group phenotypes. Premasuthan et al.'s [2011 [2012] multiplex real-time quantitative polymerase chain reaction (qPCR) method requires a small amount of DNA, ~1 ng of genomic DNA, and utilizes sequence specific primers (SSP) to target single nucleotide polymorphism (SNP) variants responsible for the A and B antigens in macaques [Premasuthan et al., 2011; Premasuthan et al., 2012]. This method, which can therefore determine A, B, and AB blood group phenotypes, has been shown to be more reliable than serological techniques, which are prone to inconsistent results [Kim et al., 2015]. Premasuthan et al.'s [2011 [2012] methodology, however, assumes that the null allele (O) is absent in macaques. While one study did report the presence of the O allele in wild and semi-wild rhesus and cynomolgus

macaques [Malaivijitnond et al., 2008], the sample size was limited and may not be representative of other populations of these species. Additionally, since the protocol employed in that study omitted the absorption of non-specific agglutinins from sera used for reverse typing, the O phenotypes identified might represent false positives [S. Malaivijitnond, pers. comm. 2016]. The functional mutations responsible for the macaque O phenotype, if it in fact exists, have yet to be identified [Doxiadis et al., 1998; Kermarrec et al., 1999; Yamamoto et al., 1990; Premasuthan et al., 2011; Premasuthan et al., 2012].

Premasuthan et al. [2011; 2012] reported the distribution of the A, B and AB phenotypes in relatively small samples of captive cynomolgus and rhesus macaques based on the SNP analysis. While Premasuthan et al.'s [2011 [2012] conclusions regarding phenotype frequencies were based on more animals than previous reports [Doxiadis et al., 1998; Malaivijitnond et al., 2008; Nakajima and Ogushi, 1965; Terao et al., 1983], an even larger sampling of rhesus and cynomolgus macaques is needed to confirm the accuracy of the phenotype frequency estimates reported in their studies. To characterize the frequency distribution of A, B and AB phenotypes in cynomolgus and rhesus macaques, this study analyzed the phenotypic data from 2369 animals.

Methods

Phenotypes from 1113 cynomolgus and 1256 rhesus macaques with well-documented geographic origins were determined using Premasuthan et al.'s [2011 [2012] qPCR method, and phenotypic frequencies were estimated using the allele counting method, assuming the absence of the O allele (see Table 1). Any samples for which amplification of both the A and B alleles was unsuccessful were considered to be “indeterminate” blood types, and the frequency of these ambiguous phenotypes was calculated for each population. In addition to the cynomolgus (N = 89) and rhesus (N = 78) samples previously analyzed by Premasuthan et al. [2011; 2012], the majority of the samples were obtained from fifteen different macaque breeding and housing facilities in the US and from multiple source countries. Thus, the population samples may exhibit population structure and phenotypes may not conform to Hardy-Weinberg equilibrium expectations. Table 1 describes the regional origins of the samples used in this study, which include India, China, Indonesia, Indochina (Cambodia and Vietnam), Mauritius and the Philippines (Zamboanga).

Each species and regional population was tested for Hardy–Weinberg equilibrium (HWE) at the 0.01 level of probability using a χ^2 test for goodness-of-fit of the observed and expected frequencies of A, B and AB phenotypes. The χ^2 tests were performed with two degrees of freedom (df) unless more than 20% of the expected frequencies were lower than 5 [Yates et al., 1999]. In these cases, the tests were performed with 1 df after the homozygote frequency that was lower than 5 was combined with the AB heterozygote frequency.

The phenotypic distributions of all cynomolgus and rhesus macaques were compared with each other using a contingency χ^2 test for homogeneity with two df at the 0.01 level of probability. The same analysis was conducted to compare phenotypic distributions between pairs of regional populations. When required conditions for the χ^2 test were not met, the same corrective measure as described by Yates et al. [1999] was applied. If one of the four

frequencies for this test was still lower than 5, a Fisher's Exact test was performed [Agresti, 1992]. The probability of randomly selecting a donor/recipient pair exhibiting major incompatibility (IC) was computed for both cynomolgus and rhesus macaques as well as regional populations of each species, where $IC = 2 (f_A f_B) + f_{AB} (f_A + f_B)$ and f_A , f_B and f_{AB} are the frequencies generated for the A, B, and AB phenotypes, respectively.

Results

Of the 1256 rhesus macaques included in this study, 19 were type A, 100 were type AB and 1137 were type B. Among the 1113 cynomolgus macaques, there were 438 type A, 309 type AB and 366 type B animals (Table 1). Thus, rhesus macaques exhibited a lower A allele frequency estimate (6%) compared to cynomolgus macaques (53%). The difference between the blood group phenotypic distributions of cynomolgus and rhesus macaques was highly statistically significant (χ^2 (df = 2, N = 2369) = 881.04; $p \ll 0.01$). At the species level, the blood group phenotypic frequencies in cynomolgus macaques, but not rhesus macaques, significantly departed from those expected under HWE conditions at the 0.01 level of probability (Table 1).

When regional populations of rhesus and cynomolgus macaques were analyzed separately, none exhibited frequencies that adhered to HWE expectations at the 0.01 level of probability except the Indian rhesus macaques, the Indochinese cynomolgus macaques and the Philippine cynomolgus macaques. The Indonesian cynomolgus macaques were predominantly blood type A (72%) while most of those from the Philippines belonged to type B (73%). More than half of the Mauritius animals were type B (52%) while the proportions of A, AB and B among Indochinese animals were 21%, 35% and 43%, respectively. Though the vast majority of the Indian rhesus macaques (91%) were type B, only half of the Chinese rhesus macaques belonged to this phenotype.

As individual populations, the Indian rhesus macaques and Indonesian cynomolgus macaques appeared to be genetically unique – both these populations were significantly ($p \ll 0.01$) different from all other regional populations included in this study. The Chinese rhesus and Indochinese cynomolgus macaques could not be differentiated, while the Mauritian sample could only be differentiated from Indian rhesus and the Indonesian cynomolgus macaques.

The random probability of major IC was highest for Chinese rhesus macaques (0.45), followed by cynomolgus macaques from Mauritius (0.39), Indonesia and Indochina (0.25), and the Philippines (0.17). With an IC estimate of 0.08, the Indian rhesus macaques reflected the lowest probability of randomly selecting a donor/recipient pair exhibiting major incompatibility, due to their very high frequency of blood group B (Table 1).

Blood groups of indeterminate phenotype occurred at a frequency of 1.4% in all populations. The frequency was higher in cynomolgus macaques (2.3%) than in the rhesus macaques (1.2%), peaked in the Mauritian cynomolgus population (3.9%) and was lowest in the Indochinese and Philippine cynomolgus populations (0%).

Discussion

Both cynomolgus and rhesus macaques can exhibit A, B or AB blood group phenotypes. The A and B blood group phenotyping method developed by Premasuthan et al. [2011; 2012] allows the identification of a rhesus or cynomolgus macaque's phenotype based on the knowledge of the molecular basis of the A and B antigens. This allows a more detailed and reliable determination of the blood group phenotype and therefore a better match for transplantation or transfusion. The present study re-evaluated previous estimates of the A, AB and B blood group phenotypic frequencies using much larger regionally representative sample sizes [Premasuthan et al., 2011; Premasuthan et al., 2012].

Although the degrees of both inbreeding and kinship coefficients were not considered in this study, as this information was not made available by these facilities, relatedness among the analyzed samples may have contributed to the excess in homozygosity observed in this study. While our assumption that the O allele is absent in macaques, if false, would also have inflated the frequency estimates of homozygotes, it is unlikely to be sufficient to have caused this outcome. Alternatively, it is likely that population structure, rather than relatedness or the presence of an O allele, is predominantly responsible for the failure of phenotype frequencies in some populations in our study to conform to Hardy-Weinberg expectations. In humans, the type O phenotype has long been considered a potentially useful universal donor. However, due to the recessive condition of the O allele in heterozygotes and its reported absence, or near absence, in macaques, the likelihood of its presence in potential macaque donors or recipients in transfusion and transplantation is of only minor importance. While the frequency of indeterminate blood groups found in this study is rare [Nei 1987] (i.e., lower than 5% across populations) and may indeed represent the O phenotype, further research is required to determine if this is a result of primer or probe binding site variation, as well as if this phenomenon is observed in other rhesus and cynomolgus macaque populations.

In contrast to Premasuthan et al. [2011; 2012], the frequency distributions of blood group phenotypes of the two species of macaques in the present study were significantly different from each other. Significant levels of regional differentiation in blood group phenotypic frequencies within each species were also observed. The lack of a significant difference (χ^2 (df = 2, N = 101) = 2.23; $p > .328$) between the Chinese rhesus and Indochinese cynomolgus blood group phenotypes supports the hypothesis that the inter-specific gene flow extends beyond the Indochinese hybrid zone and is more widespread than previously thought [Kanthaswamy et al., 2010]. In agreement with Premasuthan et al. [2011; 2012], B blood group phenotypes were far more frequent than A phenotypes in rhesus macaques, especially those from India.

The most surprising finding in this large scale study is that the Mauritian and Indonesian animals display significantly different ABO phenotype distributions (χ^2 (df = 2, N = 1043) = 369.12; $p \ll 0.01$). The inverse frequency profile of the ABO phenotypes between these Mauritian and Indonesian populations highlights the genetic distinctness of the Mauritian population that resulted from a strong founder effect that occurred when individuals from Indonesia [Sussman and Tattersall, 1986; Sussman and Tattersall, 1981] were introduced to

Mauritius in the seventeenth or eighteenth century. Reduced heterogeneity due to effects of small founder representation are also evident across other regions of the genome of Mauritian cynomolgus macaques, including several microsatellite or short tandem repeat (STR) [Bonhomme et al., 2008; Kanthaswamy et al., 2008] loci, the major histocompatibility complex (MHC) [Kawamoto et al., 2008], mitochondrial DNA (mtDNA) [Smith et al., 2007] and Y chromosome haplotypes [Kawamoto et al., 2008; Tosi and Coke, 2007]. This may also explain the elevated frequency of indeterminate blood types in the Mauritian cynomolgus macaque population, which was almost six times greater than that of the Indonesian cynomolgus macaque population.

Given the significant species-level distinction and the regional variation among conspecific individuals, the determination of A and B blood group phenotypic distributions among wild caught samples of macaques of both species is important. For instance, because inter-specific admixture has occurred between rhesus and cynomolgus macaques in Indochina [Kanthaswamy et al., 2010; Stevison and Kohn, 2009], (the principle source of cynomolgus macaques employed for biomedical research in the US), the ramifications of using animals from these regions in transplantation, blood transfusion and stem cell research should be carefully considered. The blood group phenotypic frequency distributions in macaques, particularly the Chinese rhesus and Mauritian cynomolgus macaques, suggest that high probabilities of a dangerous immune response could occur among these animals if they are indiscriminately used in transfusion and transplantation studies. This risk is significantly reduced by the exclusive use of Indian rhesus macaques in such studies. The danger of triggering adverse immunological effects during these studies is further compounded by the absence or rarity of the O blood group phenotype, i.e. the lack of ideal “donors” among these macaques, which makes it extremely critical to screen for incompatible donor/recipient animals. Based on samples obtained from at least 15 breeding and research facilities, this study underscores the relevance of blood group phenotyping in the domestic supply of primates and the bioproducts derived from them and its immediate implication in US biomedical research. It is reasonable to suggest that an ABO blood type be incorporated into the permanent medical record of all research macaques and regarded as essential for all transfusion and transplantation studies involving either macaque species to ensure compatibility of blood group phenotypes of donor and recipient research animals.

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Table 1

Species, geographic ancestry, sample size, number of individuals with each phenotype, and estimates of probability of randomly selecting incompatible donor/recipient pairs (IC) among rhesus and cynomolgus macaques. New data (parenthesized) has been combined with those from Premasuthan et al.'s [2012]. Among the rhesus samples, only those from China were not in equilibrium. Apart from the Indochinese and Philippine samples, all other cynomolgus populations departed from HWE χ^2 ($p = 0.01$).

Species/regional population	Observed phenotypic nos.			Total samples nos.	IC	Indeterminate phenotype frequency	χ^2 test	
	A	AB	B				df	p
<i>Macaca mulatta</i>	19 (3)	100 (78)	1137 (1097)	1256 (1178)	0.101	0.012	1	0.1704
India	5 (2)	89 (77)	1112 (1096)	1206 (1175)	0.076	0.012	1	0.7508
China	14 (1)	11 (1)	25 (1)	50 (3)	0.452	0.020	2	0.006*
<i>M. fascicularis</i>	438 (420)	309 (281)	366 (323)	1113 (1024)	0.459	0.023	2	0.0001*
Indochina (Cambodia and Vietnam)	11 (4)	18 (2)	22 (5)	51 (12)	0.262	0.000	2	0.1800
Indonesian	325 (322)	100 (97)	24 (20)	449 (439)	0.250	0.007	2	0.0001*
Mauritius	100 (94)	188 (182)	306 (298)	594 (574)	0.390	0.039	2	0.0001*
Philippines	2	3	14	19	0.172	0.000	1	0.4682

* : statistically significant at the 0.01 level of probability.