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RHD allele distribution in Africans of Mali

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Abstract

Background: Aberrant and non-functional *RHD* alleles are much more frequent in Africans than in Europeans. The *DAU* cluster of *RHD* alleles exemplifies that the alleles frequent in Africans have evaded recognition until recently. A comprehensive survey of *RHD* alleles in any African population was lacking.

Results: We surveyed the molecular structure and frequency of *RHD* alleles in Mali (West Africa) by evaluating 116 haplotypes. Only 69% could be attributed to standard *RHD* (55%) or the *RHD* deletion (14%). The aberrant *RHD* allele *DAU-0* was predicted for 19%, *RHD Ψ* for 7% and *Ccde*^{s} for 4% of all haplotypes. *DAU-3* and the new *RHD* allele *RHD*(L207F), dubbed *DMA*, were found in one haplotype each. A PCR-RFLP for the detection of the hybrid *Rhesus box* diagnostic for the *RHD* deletion in Europeans was false positive in 9 individuals, including all carriers of *RHD Ψ*. Including two silent mutations and the *RHD* deletion, a total of 9 alleles could be differentiated.

Conclusion: Besides standard *RHD* and the *RHD* deletion, *DAU-0*, *RHD Ψ* and *Ccde*^{s} are major alleles in Mali. Our survey proved that the most frequent alleles of West Africans have been recognized allowing to devise reliable genotyping and phenotyping strategies.

Background

The D antigen of the RH blood group (ISBT 004.001; RH1; CD240D; "Rhesus D") is the most important blood group antigen determined by a protein. Anti-D antibodies remain the leading cause for the hemolytic disease of the newborn [1], and antigen D compatible transfusion is standard practice in modern transfusion therapy.

Among Europeans, the presence and absence of the antigen D on the red blood cells (RBC) correlates closely with the presence of the "standard" *RHD* allele and a deletion of the whole *RHD* gene, respectively. Only about 1% of Europeans carry aberrant *RHD* alleles encoding variant antigen D [2–4], which may cause typing problems or are permissive to immunization by a normal antigen D.

For people of African descent a different scenario emerged: D negative Africans often carry *RHD* alleles like *RHD Ψ* [5] and *Ccde*^{s} [6], which harbor large remnants of the *RHD* gene. Furthermore, some partial D alleles

appeared to be quite frequent [7], and anti-D immunizations in D positive individuals were reported to be more frequent than in Europeans [8].

Despite the apparent complexity of *RHD* alleles in Africans, the variety of *RHD* alleles in people of African descent was less well defined than the allele distribution in Europeans. The genetic heterogeneity described so far relied heavily upon characterization of certain alleles like *RHDΨ* [5], *DAR* [6] or *DIIIa*. Thus, it may not be considered surprising that only recently the *DAU* alleles were discovered, which represented a whole cluster of *RHD* alleles on its own [9]. A comprehensive study of *RHD* alleles occurring in Africans was lacking.

We performed a random survey among blood donors from Mali and sequenced their *RHD* alleles to describe the genetic variability of *RHD* alleles extant in a native African population. Aberrant *RHD* alleles were frequent with *DAU-0* being the most prevalent one.

Results

We performed a random survey to determine the molecular structure and frequency of *RHD* alleles present in the inhabitants of Mali, who are representative of a native West African population of Mali.

Nucleotide sequences obtained

In total 1,251 bp coding and 1,230 bp adjacent non-coding nucleotide sequences were determined in 58 blood donors. At these 2,481 nucleotide positions, only 23 nucleotide positions were polymorphic (Table 1 – see Additional file 1). A total of 24 different combinations (patterns 1 to 24) of polymorphic residues were observed. The number of 24 different patterns obtained in an African population was much larger than the heterogeneity expected for a similar sized set of European samples.

RHD *alleles detected*

For each combination of polymorphism observed, a probable genotype was derived. Disregarding polymorphism in the non-coding regions, the set of data shown in Table 1 (see Additional file 1) could be efficiently explained by the occurrence of 8 *RHD* alleles (Table 1) and the *RHD* deletion. The regular *RHD* allele was most frequent and found in 42 donors. Amongst the aberrant *RHD* alleles, the *DAU* cluster was predominant with *DAU-0* observed in 18 donors. Three novel D positive alleles were found: *DMA* encoded a leucine to phenylalanine substitution located in the seventh transmembraneous segment adjacent to the intracellular part of the RhD protein. *RHD*(384T > C) and *DAU-0.1* were variants of regular *RHD* and *DAU-0*, respectively, carrying one silent mutation each. Apart from the *RHD* deletion, two D negative alleles were observed: *RHDΨ* occurred in 7 donors and *Ccde*^{s} in 5 donors.

Polymorphism in non-coding regions

Our sequencing approach could also detect polymorphism in 1,230 bp non-coding DNA segments flanking the *RHD* exons. We recorded these polymorphism (Table 1 – see Additional file 1), because they were useful in proving the heterozygosity for two *RHD* alleles in 13 donors whose *RHD* coding sequence alone was not revealing. These polymorphism could also be helpful in delineating the phylogenetic relation of some extant alleles. Nine different polymorphisms were detected (Table 2), four of which were strictly associated with a specific allele: Three substitutions in intron 2 were invariably observed in *Ccde*^{s} and a thymidine at intron 6 position 29 in *RHDΨ*. The most frequent intronic variation was a cytosine at position -28 in intron 1 found in 23 donors. An A > G substitution in the 3' untranslated region (3' UTR) of exon 10 was found in 7 donors. Other aberrations were confined to a single sample each.

Table 2: *RHD* alleles predicted from coding sequence polymorphism

Allele	Nucleotide aberrations	Amino acid aberrations	Donors carrying the allele	Reference
RHD	None (reference sequence)	None	42	[20]
DAU-0	1136C > T	T379M	18	[9]
RHDΨ	*RHDΨ* †	M218I, F223V, S225F, Y269X	7	[5]
Ccde^{s} ‡	186G > T, 410C > T, 455A > C	L62F, A137V, N152T	5	[6]
DAU-0.1	579G > A, 1136C > T	T379M	2	This work
RHD(384T > C)	384T > C	None	1	This work
DMA/	621G > C	L207F	1	This work
DAU-3	835G > A, 1136C > T	V279M, T379M	1	[9]

* The sum is less than 116 alleles, because homozygous occurrences were not accounted for. † All *RHDΨ* alleles detected carried the 37 bp duplication at the intron3/exon 4 junction and the five single nucleotide substitutions 609G > A, 654G > C,667T > G,674C > T, and 807T > G as previously described by Singleton et al. [5]. ‡ In three donors, the observed aberrations would also be compatible with the presence of the *DIII type 4* allele, because normal *RHD* sequences occurred *in trans*.

Table 3: Polymorphism in non-coding regions and haplotype association

Polymorphism			Number of samples carrying a	
DNA segment	position	Haplotype association	double peak *	single peak *
Intron 1	-28G > C	*DCe*†, *Dce*, *DcE*	14	9
Intron 6	28C > T	*RHDΨce* ‡	4	3
Exon 10, 3' UTR	1347A > G	*Dce*	6	1
Intron 2	51C > T	*Ccde*^{s}§	3	2
Intron 2	135A > G	*Ccde*^{s}§	3	2
Intron 2	-26G > A	*Ccde*^{s}§	3	2
Intron 2	36A > G	*Dce* or *DAU-0ce*	1	0
Intron 5	12G > A	*DcE* or *RHD(384T > C)ce* or *Dce* or *RHD(384T > C)cE*	1	0
Exon 10, 3' UTR	1377G > A	*Dce* or *DAU-0ce*	1	0

* The presence of a double peak in the electropherogram indicates another *RHD* allele *in trans*. A single peak may indicate the presence of an *RHD* deletion *in trans*, the homozygous presence of an allele with the given polymorphism, or – in case of intron 1 position -28 – the presence of *Ccde*^{s} *in trans*. † -28G > C was present in all 13 *DCe* haplotypes, but only in 13 of 43 *Dce* and 1 of 8 *DcE* haplotypes. ‡ Present in all *RHDΨ* donors. § Present in all *Ccde*^{s} donors.

Table 4: PCR-RFLP test for hybrid *Rhesus box* in 31 *RHD* homozygous samples

	Result for hybrid *Rhesus box*		
	positive	negative	total
RHDΨ present	4	0	4
RHDΨ not present	5 *	22	27
total	9	22	31

* These samples had the following genotypes: *DAU-0*/*DAU-3*; *cDe*/*DAU-0*; *cDe*/*cDe* [1346C] (two samples); *cDe*/*cDe* [IVS1:-28C].

PCR-RFLP detection of the RHD *deletion*

We checked for the presence of the RHD deletion by a PCR-RFLP that was specific for the hybrid Rhesus box in Europeans [10]. Recently, Matheson and Denomme [11] showed that this assay failed to correctly predict the presence of the RHD deletion in some individuals of African descent. Patterns 2, 4, 5, 7 to 10, 12, 13, 15 to 17, 19 to 21, 23 and 24 (Table 1 – see Additional file 1) were characterized by the simultaneous presence of two different nucleotides at one or more nucleotide positions. It was concluded that the 31 donors assigned to one of these patterns carried two different RHD alleles. These donors were hence not expected to carry the RHD deletion. However, in 9 of these 31 donors, our assay for the hybrid Rhesus box was unexpectedly positive (Table 3). Four of these false-positive results occurred with RHD*Ψ*, which was invariably associated with a positive result for the hybrid Rhesus box in the previously published PCR-RFLP assay. The genotypes of the remaining five false positive samples indicated that two or more additional haplotypes were associated with variant Rhesus boxes.

Calculation of haplotype frequencies

We calculated haplotype frequencies for each haplotype defined by the encoded Rhesus antigens, the result of the PCR-RFLP for the hybrid *Rhesus box* and the presence of polymorphisms in the coding and non-coding regions (Table 4). As expected for an African population [12], *Dce* and minor variations thereof represented the most frequent haplotype. Surprisingly, *DAU-0ce* was the second most frequent haplotype, representing about 20% of all haplotypes and outnumbering *DCe*, *DcE*, *RHDΨce* and the *RHD* deletion. Among the D negative alleles, the *RHD* deletion was considerably more frequent than *RHDΨ* and *Ccde*^{s} combined.

Conclusion

Clinical experience and screens for certain *RHD* alleles hinted to a high incidence of aberrant *RHD* in Africans [5,6,13], but a comprehensive investigation of the *RHD* allele distribution was lacking for any African population. Such systematic knowledge could have considerable impact for typing and transfusion strategy in populations

Table 5: Frequency estimates of *Rhesus* haplotypes

Haplotype based on			
protein sequence	full information	Calculated frequency	Total
Dce	*Dce*	14.2%	
	Dce(hybrid box positive)	3.6%	
	D(1346T > C)ce	6.0%	
	D(IVS1-28G > C)ce	10.6%	
	D(IVS2 + 36A > G)ce	0.9%	
	RHD(384T > C)*ce*	0.9%	36.1%
DCe	*D(IVS1-28G > C)Ce*	11.7%	11.7%
DcE	*DcE*	5.9%	
	D(IVS1-28G > C)cE	1.0%	6.9%
Any standard RhD *			54.7%
DMAce	*DMAce*		0.9%
DAU-0ce	*DAU-0ce*	13.1%	
	DAU-0ce(hybrid box positive)	3.2%	
	DAU-0 (1376C > T)ce	0.9%	
	DAU-0.1ce	1.7%	18.8%
DAU-3ce	*DAU-3*	0.9%	0.9%
Any aberrant RhD *			20.6%
RHD(del)ce	*RHD(del)ce*	14.0%	14.0%
RHDΨce	*RHDΨce*	6.5%	6.5%
Ccde^s	*Ccde*^{s}	4.3%	4.3%
Any non-functional *RHD*			10.8%

* Standard RhD indicates the presence of an RhD protein with a "standard" RhD protein sequence, aberrant RhD the presence of an RhD protein differing in at least one amino acid from the "standard" protein sequence.

with African admixture; possible difficulties in transfusion therapy and in genotyping could be anticipated and appropriately improved strategies devised. We performed a comprehensive survey to determine the *RHD* allele distribution in the native West African population of Mali. In striking difference to any European or Asian population examined to date but in concordance with a recent *RHD* phylogeny model, alleles belonging to the three "African" D clusters were found frequently. In particular, the most frequent aberrant *RHD* alleles belonged to the *DAU* cluster [9], which was identified only last year.

In contrast to any previous population study on *RHD*, we determined the full length *RHD* coding sequence for all probands without prior consideration of the Rhesus phenotype. This approach allowed an unbiased determination of the *RHD* allele distribution with a proper representation of all *RHD* alleles, even if they were phenotypically undistinguishable from normal RhD. For instance, two alleles, *RHD(384T > C)* and *DAU-0.1*, were found that differed from known alleles by one silent mutation each. Another new *RHD* allele, *DMA*, carried a single conservative amino acid substitution in a transmembraneous protein segment, making anti-D immunization unlikely.

Previous studies of aberrant *RHD* in Africans focused on *DAR* [7], *RHDΨ* [5] and *Ccde*^{s} [14]. Hemker et al. showed *DAR* to occur in 4.9% of South African blacks [7]. Other alleles of the weak D type 4 cluster sharing the T201R and F223V substitutions, like *weak D type 4.0* and *DIIIa*, occurred in that population with a cumulative frequency of 12% [7]. Hence, it was unexpected that none of these alleles were found in our study, indicating a considerable variability of allele distribution among African populations. It was also surprising to find *DAU-0* as the most prevalent aberrant *RHD* allele in Mali, an allele which was previously perceived as rare and occurring in Europeans [9]. In contrast, the frequencies of *RHDΨ* and *Ccde*^{s} in Mali were similar to previous reports [5,13,14] for other African populations.

All clinically relevant *RHD* alleles found in this study had been reported before, despite the current study being the first comprehensive survey in Africans. Although the allele distribution differed considerably from the European sit-

uation, all predicted phenotypes would have been typed reliably with current typing strategies applied in Europe, such as using IgM monoclonal anti-D that do not bind DVI [2]. Our study was limited by the relatively small number of individuals tested. However, our results would not preclude the application of this D typing strategy that was originally devised for patients of European descent.

Our study corroborated the notion that the *RHD* deletion is the most frequent D negative allele not only in Eurasians, but also in Africans [10]. In Europeans, the *RHD* deletion may reliably be determined by testing for the hybrid *Rhesus box* with a published PCR-RFLP [10]. However, Matheson and Denomme [11] detected *Rhesus box* copy numbers that were inconsistent with the phenotype in 8 of 328 samples. All inconsistent results occurred in individuals of African descent. Perco et al. [15] obtained discrepant results depending on the specific method used for the detection of the hybrid *Rhesus box* in one of 83 samples investigated; this sample was of Caucasian origin. Furthermore, they noticed a lack of the control band deriving from the downstream *Rhesus box* in two weak D samples. We confirmed that the PCR-RFLP for the detection of the hybrid *Rhesus box* was unreliable for predicting the *RHD* deletion in Africans from Mali: Nine samples that carried two different *RHD* genes were still typed as positive for the hybrid *Rhesus box*. Hence, this PCR described previously and developed for Europeans [10] should not be used in African populations for the prediction of the presence of an *RHD* deletion. Likewise, this method may not be reliable for the determination of *RHD* zygosity in fathers of African ancestry. The current status of PCR-RFLP for the *Rhesus box* in Africans mirrors the status of *RHD* PCR before *RHDΨ* was recognized [16].

For *RHD* PCR, the characterization of *RHDΨ* and its specific detection [5] allowed the development of *RHD* PCR methods reliable also in individuals of African ancestry. Similarly, the accrued data may guide improvements for the detection of the *RHD* deletion by PCR. All four samples that carried a different *RHD* positive allele *in trans* to *RHDΨ* had a false-positive result suggesting that *RHDΨ* carries a hybrid *Rhesus box* or is associated with an aberrant *Rhesus box* that confounds detection of the *RHD* deletion. However, the situation may be even more complicated than for the detection of *RHD*, because our data indicated that at least two additional *RHD* haplotypes were associated with a false-positive hybrid *Rhesus box* PCR-RFLP test result. It should be noted that a positive result for the hybrid *Rhesus box* in our PCR-RFLP assay must not indicate the presence of a hybrid *Rhesus box*. Different assay systems have been found to yield discrepant results [15]. In some samples, aberrant downstream *Rhesus boxes* carried polymorphisms previously considered typical for the hybrid *Rhesus box* [11]. A single nucleotide substitution involving the binding sites of the primers or the restriction site is sufficient to cause such erroneous typing results. Although a full account of the *Rhesus box* variability is still lacking, further investigations in this observed *Rhesus box* variability will allow to improve the specificity and reliability of current genotyping techniques for the *RHD* deletion in Africans.

Methods

Blood samples

EDTA blood samples were drawn from 58 blood donors at the Centre National de Transfusion Sanguine (CNTS) de Bamako, Mali. The samples were collected at random during a one week period in April 2002.

The average draw per week was 300 donors with a total collection of 12,000 donations in 2001 and 16,000 in 2002 at CNTS. Presently, the CNTS does not have any satellite centers, thus, the majority of donors come from the city of Bamako. There are 26 different ethnic groups recognized within the Malian population. In the Bamako donor population, the Bambara ethnicity is most prevalent with about 27% followed by Malinké (15%) and Peulh (11%). Other ethnic groups are in decreasing frequency: Sarakolé, Sénoufo, Sonrhaï, Kassounké, Dogon, Bbo, Bozo, Minianka, Somono and Touareg. The area covered by CNTS was about 220 square kilometers. Bamako is located in the Kouliboro region and the capital city of Mali in sub-Saharian West Africa. The population of Bamako is 1.1 million and the total Mali population is about 10 million inhabitants.

RHD *nucleotide sequencing*

DNA was extracted in Philadelphia using a commercial kit (Gentra, Minneapolis, MN) and forwarded to Ulm, where the ten *RHD* exons were sequenced by an *RHD* allele specific method [3,17,18] and the Rhesus CcDEe phenotype was determined using commercial monoclonal Rhesus typing antisera in tube technique. In addition to the *RHD* coding sequence, the following non-coding DNA segments flanking the *RHD* exons were evaluated: 5' 100 bp and 3' 20 bp for exon 1; 29 and 158 for exon 2; 30 and 6 for exon 3; 25 and 20 for exon 4; 6 and 70 for exon 5; 21 and 40 for exon 6; 40 and 50 for exon 7; 20 and 200 for exon 8; 15 and 140 for exon 9 as well as 40 and 200 for exon 10.

The standard *RHD* sequencing method described in the previous paragraph is known to fail for exon 1 and 2 in *Ccde*^{s} samples [17]. Therefore, we sequenced exons 1 and 2 of samples carrying the A137V and N152T substitutions in exon 3, which are typical for *Ccde*^{s}, also by a method adapted to *Ccde*^{s} as described previously [17]. In one such sample, exon 2 was sequenced using the D-specific sequencing primers Dex2a and Dex2b. Primer sequences

were Dex2a, cttgggcttcctcacctcgag; and Dex2b, gtgtgatgaccaccttccctga.

An *RHD* specific amplification of exon 9 was achieved in 29 samples only. In the remaining 29 samples the *RHCE* exon 9 was co-amplified and we determined the relative amplification of the *RHD* and *RHCE* alleles from the heights of *RHD* and *RHCE* specific peaks in the electropheretograms from the nucleotide sequencing machine (ABI Prism 3100 Genetic Analyzer, Applera, Darmstadt, Germany). An *RHD*/*RHCE* ratio of 2:1 was found in 16 samples, a ratio of 1:1 in 9 samples and a ratio of 1:2 in 1 sample. In 17 samples, some co-amplification of *RHCE* occurred for exon 7.

Because *RHD* exon 8 and exon 9 represented a single amplicon, the presence of the same ratio of amplification for a normal *RHCE* allele was assumed in both exons. As *RHCE* carries a C at position 1136 in exon 8 two types of corrections had to be applied: (i) Two samples had a *RHD RHCE* ratio of 1:1 in exon 9 and a T/C ratio of 1:1 in exon 8. Both samples were counted as T only in exon 8. (ii) Six samples had a *RHD RHCE* ratio of 2:1 in exon 9 and a T/C ratio of 1:2 in exon 8. These samples were counted as T and C in exon 8.

Detection of the RHD *deletion and of* RHDΨ

The presence of the *RHD* deletion was investigated by a PCR-RFLP established for Europeans [10], that of *RHD*Ψ by PCR-SSP [17] as described previously.

Estimation of RHD-RHCE *haplotype frequencies*

A haplotype was considered to be defined by (i) the presence of a specific *RHD* allele, (ii) a positive result for the hybrid *Rhesus box* and (iii) the CcEe phenotype. The most probable genotype was determined using the following rules: (i) The sample was considered homozygous for *RHD*, if two different *RHD* alleles were detected or the PCR-RFLP for the hybrid *Rhesus box* was negative. (ii) The multiple substitutions found in *RHD*Ψ, *Cde*^{s} and *DAU-3* were assumed to occur *in cis* and be representative of the known alleles. (iii) If an aberration was observed in several samples and was associated with the presence of a specific haplotype, it was assumed that this aberration occurred *in cis* with the haplotype. (iv) If an aberration occurred only once, it was assumed that it occurred *in cis* with the more prevalent haplotype present in the sample. (v) If different combinations of haplotypes could explain a given sample, the combination requiring the least number of different haplotypes was chosen as the most plausible explanation.

After the initial assignment as described in the previous paragraph, haplotype frequencies were corroborated iteratively by the counting method [19] that yields a maximum likelihood estimate.

Detection of hybrid RHD-CE-D *alleles*

RHD-CE-D hybrid alleles and *RHD* alleles lacking the amplification of certain exons were readily detected if they occurred *in trans* to the *RHD* deletion. The only examples found were two samples with the *RHD-CE(4-7)-D* hybrid allele known as *Cde*^{s}. In these samples, the hybrid allele could be confirmed by *RHD* exon specific PCR-SSP [17]. We found no hint to another *RHD-CE-D* allele in our set of samples. However, we cannot formally exclude the possibility of a rare hybrid allele occurring exclusively masked by a normal *RHD in trans*.

Nomenclature

The name DMA was derived from D detected in Mali.

Authors's contributions

FFW carried out the molecular genetic studies, analyzed and interpreted the data and drafted the manuscript. JMM had the initial idea, collected the samples and isolated the DNA. AT and BK recruited the blood donors and collected the samples. WAF designed the study, interpreted the data and co-wrote the manuscript. All authors read and approved the final manuscript.

Abbreviations

PCR-SSP = polymerase chain reaction with sequence-specific priming;

Additional material

Additional File 1

Patterns of polymorphic nucleotide positions among 2,481 bp of the RHD *gene in 58 blood donors from Mali The additional file documents the polymorphic nucleotides and theirs positions observed in the 58 individuals analysed in this study.*

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[http://www.biomedcentral.com/content/supplementary/1471-2156-4-14-S1.rtf]

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