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%, RHDΨ for 7% and Ccdes 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 RHDΨ. 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, RHDΨ and Ccdes 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 RHDΨ [5] and Ccdes [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 DIIIα. 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), 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 Cede 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.

**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 transmembrane 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 Cede 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 Cede 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                   | T379M                  | 42                        | [20]      |
| DAU-0    | 1136C > T              | T379M                  | 18                        | [9]       |
| RHDΨ     | RHDΨ†                  | M218L, F223V, S225F, Y269X | 7                         | [5]       |
| Cede‡    | 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 intron 3/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.
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 RHDΨ, 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, RHDΨce and the RHD deletion. Among the D negative alleles, the RHD deletion was considerably more frequent than RHDΨ and Ccde 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...
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 indistinguishable 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 transmembrane protein segment, making anti-D immunization unlikely.

Previous studies of aberrant RHD in Africans focused on DAR [7], RHDΨ [5] and Ccdes [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 DIIla, 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 Ccdes 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-

### 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%                |       |
| DMAre                             | DMAre                                                | 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                         | 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%  |
| Ccdes                             | Ccdes                                                | 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.
vation, 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 Peuhl (11%). Other ethnic groups are in decreasing frequency: Sarakolé, Sénooufo, 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-Saharan 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 (Genta, 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 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, also by a method adapted to CcDe 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, cttgggctcctcacctcgag, and Dex2b, gttgtgacccacctctcctga.

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 electrophoretograms 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 RHD \( \Psi \)
The presence of the RHD deletion was investigated by a PCR-RFLP established for Europeans [10], that of RHD\( \Psi \) 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\( \Psi \), Ccde 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 Ccde. 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

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