Exploring association between MBL2 gene polymorphisms and the occurrence of clinical blackwater fever through a case–control study in Congolese children

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Abstract

Background: Blackwater fever (BWF), one of the most severe and life-threatening forms of falciparum malaria, is characterized by acute massive intravascular haemolysis, often leading to acute renal failure. Thus far, the genetics of the underlying susceptibility to develop BWF is not fully elucidated. Deficiency in the MBL protein, an important component of the innate immune system, has previously been suggested to be a susceptibility factor for the development of severe malaria. This study aimed to evaluate the association between MBL2 gene polymorphisms, known to affect the MBL protein level/activity, and the occurrence of BWF among Congolese children.

Methods: This is a case–control study. Cases were patients with BWF, whereas controls, matched for gender and age, had uncomplicated malaria (UM). Dried blood spot was collected for genotyping.

Results: A total of 129 children were screened, including 43 BWF and 86 UM. The common allele in BWF and UM was A, with a frequency of 76.7 and 61.0%, respectively (OR: 2.67 (0.87–8.29) and p = 0.079). The frequency of the C allele was 18.6 and 29.1% in BWF and UM groups, respectively, with p = 0.858. Not a single D allele was encountered. Genotype AA was at higher risk for BWF whereas genotypes A0 (AB and AC) were over-represented in UM group (OR: 0.21 (0.06–0.78)) with p = 0.019. Nine haplotypes were observed in this study: 3 high MBL expression haplotypes and 6 low MBL expression haplotype. One new haplotype HYPC was observed in this study. None of these haplotypes was significantly associated with BWF.

Conclusion: This pilot study is a preliminary research on MBL2 gene and infectious diseases in DRC. The study results show a higher risk for BWF in AA. This suggests that future studies on BWF should further investigate the contribution of a strong immune response to the occurrence of BWF.

Keywords: Mannose-Binding Lectin 2, MBL2, Blackwater fever, Severe malaria, Democratic Republic of Congo

Background

Mannose-Binding Lectin protein (MBL), encoded by MBL2 gene (Mannose-Binding Lectin soluble 2; OMIM: 154545), is an important component of the innate immune system with 4 main functions, including activation of complement, direct promotion of

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opsono-phagocytosis, modulation of the inflammatory response, and promotion of apoptosis [1]. There are also other promoter variants that may affect gene expression [2–5]. The MBL deficiency, also known as ‘dysfunctional MBL’, is one of the most common immune deficiencies in the world [2]. Three non-synonymous single nucleotide substitutions in the exon 1 of MBL2 gene cause dramatic decrease of MBL in heterozygous state or almost complete absence of MBL in homozygous or compound heterozygous state. These include substitutions at codon 52 (CGT → TGT; p.Arg52Cys, rs5030737), codon 54 (GGC ≥ GAC; p.Gly54Asp, rs1800450) and codon 57 (GCA ≥ GAA, p.Gly57Glu, rs1800451) [6–9]. Based on the classic MBL2 polymorphisms codification, substitutions at codons 52, 54 and 57 are referred to as D, B and C derived alleles, respectively, whereas the ancestral allele is known as allele A [10]. Because these three variant alleles cause similar MBL deficiency, the concept of ‘O’ allele is used to describe either of these variants [8].

In addition, 3 substitutions including 2 in the promoter region of MBL2 (~550C/G or rs11003125 and -221G/C or rs7096206) and one in the UTR within the exon 1 (c.4T/C or rs7095891) have been shown to affect the level of MBL protein and influence the outcome of infectious diseases [9, 10]. The derived alleles in the promoter region, the upstream region and the exon 1 have been previously combined into haplotypes [10]. The MBL2 haplotypes HYP_A, LYQA, LYP_A, and HYQA have been associated with high MBL2 expression. Conversely, haplotypes LXPA, LYPB, LYQC, HYPD, LYPD, HYQC, LXPB, and LYQB showed low MBL2 expression [10]. However, a recent haplotype, termed HYPC, was identified in similar sub-Saharan individuals in a study from Zimbabwe [11].

In the Democratic Republic of Congo (DRC), Plasmodium falciparum is the most severe and lethal species of malaria parasite among children below 5 years of age [12–15]. The clinical expression of falciparum malaria consists of a wide spectrum, spanning from asymptomatic to severe forms of infections including malaria [23, 24, 26–33]. On the other hand, MBL deficiency is also thought to be protective against certain complications by preventing excessive activation of the immune response, avoiding thereby deleterious immune-related complications during infections [7, 34, 35]. It has been recently reported that malaria IgG are significantly elevated in BWF [36], which also suggests that unlike other severe forms of malaria, BWF would more likely occur in normal or hyper immune individuals. A straight connection between IgG antibodies and MBL2 alleles have been established in a study on Chlamydia pneumoniae where the mean antibody titre increases with the number of copies of ancestral MBL2 alleles [37]. Although it remains unclear how ancestral MBL2 variants increase antibody titres and whether this matches with known mechanisms of MBL in the immune response, it could be hypothesized that unlike in other severe forms of malaria, people with ancestral MBL2 alleles would be at higher risk to exhibit BWF.

To date, the distribution of MBL2 alleles and their possible association to BWF in the DRC have not been investigated.

Methods

Study aims, design and setting
This study aimed to test the association between MBL2 polymorphisms and Blackwater fever, one of the most severe complications of malaria, and provide the first distribution data for MBL2 haplotypes in Congolese individuals. This is a case–control study conducted over 2 years in 4 medical institutions across Kinshasa, namely University Hospitals of Kinshasa, Kimbaske Hospital, Bondeko Hospital and General Provincial Hospital of Kinshasa. Sampling methods and case definition are published elsewhere [12]. Altogether, 43 cases and 86 controls were enrolled. Ages for cases and controls ranged from 2 to 15 years.

Clinical evaluation
The medical history was obtained from parents, with particular attention to demographic data, including disease history and medications taken before BWF episode. Clinical data were recorded in a customized pre-tested clinical form. Malaria was confirmed by the presence of parasites on blood thick and film.

Laboratory measurements
Twenty mL of fresh urine were collected from each participant. The presence of haemoglobin in urine was first detected by urinary dip strip (Medi test Combi 9, MacheryEur, Paris, France) and then confirmed by spectrometer (Thermo Genesis 10BIO, New York, USA) using protocol of 3,3’ dimethyl benzidine reagent [38]. The
results of urine dip stick were read as either negative (yellow colour) or positive (change in blue colour) 1+, 2+, 3+, which corresponded approximately to haemoglobin concentrations of respectively 0.061 ± 0.0166 mg/L, 0.3986 ± 0.2612 mg/dL and 0.5679 ± 0.27688 mg/L as quantified using the spectrometer.

**DNA extraction and MBL2 genotyping**

Human MBL2 gene was assessed from genomic DNA. Eight drops of blood were collected on FTA card® WB 120067 (GE Healthcare, Amersham, UK) and stored in a fridge until the transfer to the Institute of Tropical Medicine of Nagasaki University in Japan for DNA testing in the Department of Immunogenetics according to a previously described protocol [39].

DNA samples from 43 cases and 86 controls were examined. The promoter region and exon 1 of MBL2 gene were PCR-amplified and Sanger sequenced. Prior to Sanger sequencing, PCR products were verified by gel electrophoresis to confirm the presence of expected band and exclude unexpected inserts. The PCR mixture contained 17.5 μL of ultra-pure water, 2.5 μL PCR 10 × buffer, 4 μL of dNTPs (2 μmol), 0.4 μL (2 units) of Taq polymerase and 0.8 μL of each primer (2.5 μmol). A disc containing between 5 and 20 ng of DNA was punched from the FTA card and added into the PCR reaction tube. In order to identify technical contaminations, a tube a No DNA template was also included in each run. This consisted of a punch from an unsotted FTA card. After an initial denaturation step of 5 min at 95 °C, 35 amplification cycles were applied including rapid denaturation at 95 °C for 1 min, annealing at 65 °C for 1 min and elongation at 72 °C for 1 min. The reaction ended with a final elongation step at 72 °C for 5 min. PCR product was sequenced by dideoxy termination sequencing using Big-Dye® Terminator version 1.1. Sequencing product was analysed on a 3730 DNA ANALYSER, version 3.0, from HITACHI. Haplotypes were double- and triple-checked using visual inspection of sequencing traces.

Alleles were designated as suggested by Antonarakis et al. [7] for the 3 variants in the Exon 1. The MBL2*B, MBL2*C and other variants alleles were identified as described by Sumiya et al., Lipscombe et al. and Madsen et al. [3–5, 9].

**Data management and analysis**

Alleles and genotypes frequencies were obtained by direct scoring of electropherogram. Data were recorded using the software Epi Info 7. All analyses were carried out using SPSS 18.0. All records were crosschecked with the original data sheets before the analysis. A non-conditional model was used. This was a binary logistic regression including covariates, anti-malaria drugs, MBL2 gene polymorphism, G6PD and parasitaemia. Multivariate logistic regression analysis was used to evaluate associations between MBL2 haplotypes/genotypes/alleles and the BWF. Odds ratio and confidence intervals were calculated. All tests were two-sided, and the level of significance was set at $p < 0.05$.

**Results**

A total of 129 Congolese children were investigated, including 43 cases and 86 controls. Sixty-eight were girls (52.7%) and 61 boys (47.3%). The mean age was 8.75 ± 3.73 years for all the study population, 8.62 ± 3.84 years and 8.55 ± 3.77 years, respectively, for cases and controls (uncomplicated malaria, UM), only 8 cases (18.6%) were below 5 years, which is the most vulnerable period for severe malaria, versus 20 patients (23.26%) in the control group. The majority of BWF cases (38 cases) occurred during the rainy season (88.4%) and 5 (11.6%) occurred during the dry season. Low parasitaemia was associated to BWF OR: 3.31 (1.41–7.79) with p = 0.005 (Table 1).

Using a non-conditional model, a binary logistic regression, including covariant, anti-malaria drugs, MBL2 gene polymorphism, G6PD and parasitaemia, it was observed that MBL2*AB or AC is protective factor in the development of BWF. OR: 0.09 (0.01–0.63), with p = 0.015. The association with quinine intake and low parasitaemia, observed in this study (Table 2), was already published [12].

The association between alleles and genotypes, and each of the 2 clinical groups was also assessed. The A allele was the most common in BWF group as well as in the UM group with allele frequency of 76.7 and 61.0%, respectively, and the difference was not statistically significant, OR: 2.67 (0.87–8.29 and $p = 0.079$ (Table 3)). Conversely, the C allele frequency was 0.186 and 0.291 in BWF and UM groups, respectively, and the difference was not statistically significant ($p = 0.853$). Not a single D allele was encountered in the present study population (Table 3). Regarding the genotypes; the proportion of homozygote’s AA was higher in the BWF group (72.0%) compared to the UM (50.0%). Conversely, the 00 genotype was proportionately more frequent in the UM (27.9%) than in BWF (18.6%) (Table 3). A0 genotype is significantly over-represented in UM population compared to BWF patients, OR: 0.21 (0.06–0.78) with p = 0.019 (Table 3).

Nine haplotypes were encountered in this study cohort, including 3 high MBL expression haplotypes and 6 low MBL expression haplotypes (Table 4). The high expression MBL2*LYQA haplotype was the most prevalent haplotype in BWF as well as in UM, with 46.3 and 39.5%, respectively. Low MBL expression haplotypes were;
MBL2*HYPB; MBL2*HYPC; MBL2*LYQC (Y16578); MBL2*LYPC, MBL2*LYPB (Y16579); MBL2*LXPA and were not significant. Only MBL2*LYQA haplotype was consistently over-represented in UM group, but not significantly (Table 4). None of the groups deviated from the Hardy–Weinberg expectations [40] as showed in Table 3.

**Discussion**

The present study investigated whether some alleles, genotypes or haplotypes were significantly over-represented or under-represented in patients with BWF compared to those with UM. A cohort of 129 patients was recruited from 4 hospitals across Kinshasa. Only a few of them were within higher risk group to develop severe malaria, meaning below 5 years of age, as described in many studies. However, the majority of recruited patients was at risk for BWF as this form of malaria is mostly observed in older children and adults [12, 13, 17, 21, 40–45].

**Allele frequency**

MBL2*A allele was the most common allele within the 2 groups compared to each of the derived alleles individually. However, when considered together, null alleles (allele 0) were more frequent among patients with UM compared to those with BWF, with allele frequencies of 0.39 and 0.233, respectively. 0 includes B, C and D alleles (Table 3). MBL2*C was the most frequent in both groups. Bellamy et al. [46] reported also a higher frequency of the MBL2*C in in the population of The Gambia. Compared to the other null alleles, the MBL2*C has been

| Table 1 Socio-demographic features of patients in the study population |
|---------------------------------------------------------------|
| **Case (n = 43)** | **Control (n = 86)** | **Total (n = 129)** | **OR (IC 95%)** | **p** |
| **Distribution for age** | | | | |
| ≤ 5 years | 8 (18.6) | 20 (23.3) | 28 (21.7) | 1 |
| > 5 years | 35 (81.4) | 66 (76.7) | 101 (78.3) | 1.33 (0.53–3.32) | 0.676 |
| **Sex (%)** | | | | |
| Male | 21 (48.8) | 40 (46.7) | 61 (47.3) | 1.10 (0.53–2.28) | 0.803 |
| Female | 22 (51.2) | 46 (53.5) | 68 (52.7) | 1 |
| **Season** | | | | |
| Rainy | 38 (88.4) | 51 (59.3) | 89 (69.0) | 5.22 (1.87–14.56) | <0.001 |
| Dry | 5 (11.6) | 35 (40.7) | 40 (31.0) | 1 |
| **Plasmodium** | | | | |
| *Falciparum* | 37 (86.0) | 73 (84.9) | 110 (85.3) | 1.10 (0.39–3.12) | 0.860 |
| *Falciparum*-malariae | 6 (14.0) | 13 (15.1) | 19 (14.7) | 1 |
| **Parasitaemia (parasites/µl)** | | | | |
| Low (< 1000 tropho/µl) | 33 (76.7) | 43 (51.8) | 76 (61.3) | 3.31 (1.41–7.78) | 0.005 |
| High (≥ 1000 tropho/µl) | 10 (23.2) | 40 (48.2) | 48 (38.7) | 1 |

| Table 2 Determinant factors of Blackwater fever occurrence |
|-----------------------------------------------------------|
| **Crude OR (95% CI)** | **p** | **Adjusted OR (95% CI)** | **p** |
| **Antimalaria drugs** | | | | |
| ACT | 1 | 1 |
| Quinine | 47.31 (10.64–210.3) | <0.001 | 57.33 (11.65–282.08) | <0.001 |
| **Genotypes** | | | | |
| MBL2*A/A | 1 | 1 |
| MBL2*A/B or A/C | 0.21 (0.06–0.78) | 0.019 | 0.09 (0.01–0.63) | 0.015 |
| MBL2*BC or C/C | 0.58 (0.24–1.43) | 0.237 | 0.71 (0.19–2.66) | 0.608 |
| **Status G6PD** | | | | |
| Normal | 1 | 1 |
| Deficient | 0.35 (0.14–0.54) | 0.017 | 0.70 (0.19–2.56) | 0.586 |
| **Parasitaemia** | | | | |
| < 1000 trophozoites/µl | 3.3 (1.40–7.69) | 0.005 | 5.76 (1.79–18.55) | 0.003 |
| > 1000 trophozoites/µl | 1 | 1 | | |
demonstrated to be extremely common in sub-Saharan Africans with a population frequency of 0.30, whereas the MBL2*B was predominant in Europeans, in Asians and in indigenous people of South America with population frequencies of 0.13, 0.20 and 0.50, respectively [3, 5, 46]. None of the alleles observed in the study population presented a significant preferential distribution between the 2 groups.

It has been hypothesized that *B, *C and *D alleles are positively selected in order to reduce susceptibility or mortality due to certain infectious diseases [5, 24, 34]. This study did not identify the MBL2*D allele within the 2 groups. This allele has been detected with frequencies up to 0.05 in the northeast of Africa, in Europe and India [3, 10]. Hence, the absence of the MBL2*D may simply indicate a low admixture with European and Indian in the Congolese population examined in this study [47–49].

### Table 3 Alleles and genotypes Frequencies for the 3 polymorphisms in the Exon 1

| Alleles | Blackwater fever | Uncomplicated malaria | Total | Crude OR 95% CI | p values |
|---------|------------------|-----------------------|-------|----------------|----------|
| A       | n (freq)         | n (freq)              | n (freq) |              |          |
| B       | 1                | 7                     | 8      |              |          |
| C       | 3                | 12                    | 15     |              |          |
| D       | 0                | 0                     | 0      |              |          |
| Total allele freq | 86 (1.00) | 172 (1.00)            | 258 (1.00) |              |          |

| Genotypes | n (freq) | n (freq) | n (freq) |          |          |
|-----------|----------|----------|----------|----------|----------|
| AA        | 31 (0.721) | 43 (0.500) | 74 (0.574) | 1        |
| A0        | 4 (0.093) | 19 (0.221) | 23 (0.178) | 0.21 (0.06–0.78) | 0.019 |

| Total genotype freq | 43 (1.00) | 86 (1.00) | 129 (1.00) |          |

| Alleles | n (freq) | n (freq) | n (freq) |              |          |
|---------|----------|----------|----------|----------|----------|
| A       | 66 (0.767) | 105 (0.610) | 171 (0.663) | 2.67 (0.86–8.29) | 0.079 |
| B       | 4 (0.046) | 17 (0.098) | 21 (0.081) | 1        |
| C       | 16 (0.168) | 50 (0.291) | 66 (0.256) | 1.35 (0.41–5.30) | 0.858 |
| D       | 0 (0.00) | 0 (0.00) | 0 (0.00) | –        |

| Total allele freq | 86 (1.00) | 172 (1.00) | 258 (1.00) | –        |

| Genotypes | n (freq) | n (freq) | n (freq) |          |          |
|-----------|----------|----------|----------|----------|----------|
| AA        | 31 (0.721) | 43 (0.500) | 74 (0.574) | 1        |
| A0        | 4 (0.093) | 19 (0.221) | 23 (0.178) | 0.21 (0.06–0.78) | 0.019 |

| Total genotype freq | 43 (1.00) | 86 (1.00) | 129 (1.00) |          |

### Table 4 MBL2 haplotypes (promoter region and exon1) and risk assessment

| Haplotypes | BWF, n (%) | UM, n (%) | Total n (%) | p |
|------------|------------|-----------|-------------|---|
| High MBL expression | | | | |
| MBL2*LYQA (Y16576) | 20 (46.1) | 24 (39.5) | 54 (41.9) | -NS |
| MBL2*HYPA (Y16581) | 6 (14) | 11 (12.8) | 17 (13.2) | NS |
| MBL2*LYPA (Y16577) | 3 (7.0) | 2 (2.3) | 5 (3.9) | NS |
| Low MBL expression | | | | |
| MBL2*HYPB | 0 (0.0) | 1 (1.2) | 1 (0.8) | NS |
| MBL2*HYPC | 1 (2.3) | 1 (1.2) | 2 (1.5) | NS |
| MBL2*LYQ (Y16578) | 5 (11.6) | 20 (23.3) | 25 (19.4) | NS |
| MBL2*LYPC | 0 (0.0) | 1 (1.2) | 1 (0.8) | NS |
| MBL2*LYPB (Y16579) | 1 (2.3) | 3 (3.5) | 4 (3.1) | NS |
| MBL2*LXPA | 7 (16.3) | 13 (15.1) | 20 (15.5) | NS |
| Total | 43 (100) | 86 (100) | 129 (100) |          |

**MBL2 genotypes and BWF**

**MBL might protect against severe disease forms but not against BWF**

Multiple genetic epidemiological studies reported that the presence MBL2 derived alleles and genotypes are associated with an increased risk to infections [4, 28, 29, 32, 50] and might be considered as a prognostic marker in various infectious conditions [29, 32, 51, 52]. Functional studies showed that heterozygotes for a MBL2 variant produces low concentration of MBL protein and this may hamper the phagocytosis of bacteria or parasites, thereby allowing the replication of the pathogen [24, 28, 48, 53, 54]. Based on this group of studies, one would expect individuals with ancestral MBL2 AA alleles to be protected against BWF, a severe phenotype. Unlike in other severe forms of malaria, such association was not observed in this study.

**Homozygotes for ancestral MBL2 alleles are at higher risk for BWF**

The other wildly supported theory is that low levels of functional MBL may decrease excessive activation of the
Haplotypes
The present study revealed 9 haplotypes, including 3 high MBL expression haplotypes (Table 4) and 6 low expression haplotypes. The LYQA haplotype was the most prevalent haplotype both in BWF and UM group with, respectively, 45.5 and 39.5%, followed by MBL2*LYQC in UM population with 23.3%. In Gabon, Boldt et al. defined 14 new haplotypes and reported that MBL2*LYQC, MBL2*LYQA and MBL2*LYPA were the most prevalent haplotypes in the children population [55]. A new haplotype HYPC only described in Zimbabwe individuals was observed in 2 patients: one BWF and one UM. A study in India reported that the MBL2*LYPA haplotypes confers protection, whereas MBL2*LYPA increases the malaria risk. These findings in Indian populations demonstrate that MBL2 functional variants are strongly associated with malaria and infection severity [10]. However, no significant association was find between BWF and a particular haplotype.

Parasitaemia and BWF
Lower parasitaemia was observed in BWF patients. Considering that quinine intake offers a strong clearance of parasite, low parasitaemia observed in BWF may be secondary to the quinine intake that triggers BWF occurrence. In that prospect, the time between quinine intake and the occurrence of BWF may influence parasitaemia. However, this timing remains unclear since reported time-lapses range from 12 h to multiple days after treatment [56–58]. Another reason for low parasitaemia in BWF could be the activity of the immune system in AA individuals. The active immune response would provide a good clearance of parasite and accessoryry cause BWF. Further studies may be needed to investigate this hypothesis.

Limitations of the study
The major limitation of this study is the small sample size. Although BWF is rare in the study setting, the small sample size may have influenced the statistical calculations. Another limitation was that the investigation of G6PD polymorphisms, and the complement activation and MBL protein were not measured. In addition, no data exist in the community about the frequency of MBL2 polymorphism in the country. Despite these limitations, these data provide insights into the relationship between MBL protein level/activity and BWF, and could form a basis for further studies in a large Congolese population.

Conclusion
This pilot study is a preliminary research on MBL2 gene and infectious diseases in DRC. The result shows a higher risk for BWF in AA. This suggests that future studies on BWF should further investigate the contribution of a strong immune response to the occurrence of BWF.

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Data distribution
Anonymized genomic data can be obtained upon request to the corresponding author.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author.

Ethics approval and consent to participate
All information about this study was provided to parents in local languages. Written informed consent from parents for each patient in this study has been obtained. The Ethics Committee of Public Health School of University of Kinshasa approved the protocol under the number ESP/CE/027B/2011.

Consent for publication
Not applicable.
Competing interests
The authors declare that they have no competing interests.

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