



Characterizing Endothelin-1 and ABO Blood Type in Cerebral Malaria

Kathleen Ireland¹, Gregory S. Park¹, Robert Opika-Opoka², Chandy C. John¹

¹Department of Pediatrics, University of Minnesota, Minneapolis, MN; ²Makerere University Faculty of Medicine, Kampala, Uganda



Abstract

Malaria remains one of the most life-threatening problems in the world today. Cerebral Malaria (CM) caused by *Plasmodium falciparum* infection is a severe form of malarial disease that kills more than two million people annually, most of which are children. CM affects the brain and is associated with cognitive impairments, coma, and death. Left untreated, it is fatal within 24-72 hours. Quicker diagnosis is needed to provide adequate and superior treatment. In order to do so, a better understanding of the disease pathogenesis is needed. The goal of my proposed research was to further characterize endothelial cell activation in children with cerebral malaria, as well as the role of blood type as possible clinical markers that may play a role in the disease. The first aim of the project was to measure endothelin-1 levels in samples previously obtained at Mulago Hospital in Kampala, Uganda. Samples were comprised of children ages 4-12 with varying levels of disease severity. Endothelin-1 levels were measured by an immunoassay and analyzed with relation to known clinical outcomes. My second aim was to develop a polymerase chain reaction assay to determine ABO blood type from the Ugandan children's genomic DNA. I have developed a protocol using positive and negative controls that I will apply toward genotyping samples from the patients. Though this project is still in progress, I expect my results will help to characterize the role of endothelin-1 and blood type in cerebral malaria pathogenesis.

Introduction

The molecular mechanisms involved in the pathogenesis of Cerebral Malaria are not yet well known. One proposed mechanism of the pathogenesis is the sequestration of parasitized erythrocytes in brain blood vessels, which activate endothelial cells and result in complications associated with the disease [6]. Two activated endothelial cell responses are the secretion of Endothelin-1 (ET-1) and von Willebrand Factor (VWF). ET-1 is an amino acid peptide best known for its role as a vasoconstrictor and role in controlling inflammatory response. VWF is an adhesive glycoprotein that circulates in the plasma and is associated with playing a major role in blood coagulation. The role of ET-1 in the development of CM has not yet been well studied, though it has been suggested in a mouse model that there may be a significant increase in ET-1 levels associated with CM [3]. A recent study has demonstrated the possibility of VWF levels being higher in children with CM than those with uncomplicated or no disease. It has also been suggested that variations in plasma VWF levels are genetically linked by ABO blood group [5]. Therefore, ABO blood group may be related to CM. In fact, studies in the past have shown a relationship between ABO group and severity of malarial disease. However, ABO group connection to CM has not been fully characterized.

I hypothesized that serum samples with CM will have higher ET-1 levels than those of uncomplicated or no disease. I additionally hypothesized that disease severity will be higher among non-O blood groups based on the implications made by other, similar studies.

Methods

Human serum samples were previously obtained at Mulago Hospital in Kampala, Uganda [2]. The samples were from 264 children, ages 4-12 years old, and written informed consent was obtained from the parents or guardians of study participants. 88 of the study participants had CM (severe disease), 76 had uncomplicated malaria (UM, non-severe disease), and 100 had no disease (HC, healthy community controls).

Samples were tested using a commercial human endothelin-1 immunoassay (*R&D Systems Inc.*, Minneapolis, MN). Endothelin-1 levels were measured in Relative Light Units by a microplate luminometer, and concentrations back calculated from a standard curve. Samples were diluted in a 1:4 ratio in the assay. Values obtained for all groups were not normally distributed. Thus, differences between group endothelin-1 levels were assessed by non-parametric, Wilcoxon ranksum (Mann-Whitney) test using *Stata Release 10* (College Station, Texas).

Development of the procedure for genotyping blood group was done using purified genomic DNA, donated by four members of the lab with known blood type. Details of modification methods can be seen at the right. Genotyping of samples was conducted using a modified protocol of Olsson, Chester 1995 [2]. PCR was performed with the following primers:

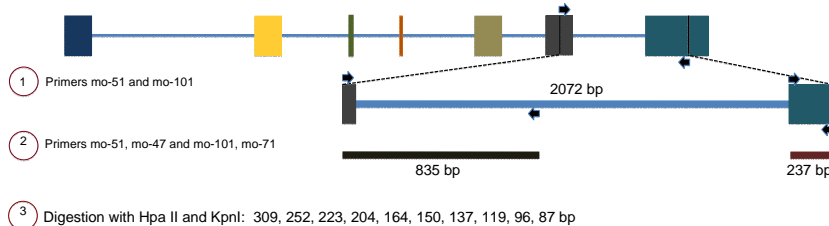
mo-46: 5'-CGGAATCACTCCCACTGCCTGGGCTC-3'
 mo-57: 5'-CGGATCCATGTGGGTGGCACCTGCCA-3'
 mo-101: 5'-CGGATCCCGCTCCGCTGCCTTGCA-3'
 mo-71: 5'-GGGCTAGGCTTCAGTACTC-3'

A 25µl total volume reaction was used with 12.5µl of Denville Hot Start Taq Master mix, 0.5µM of each primer, and 0.1µg/10µl of genomic DNA. BioRad Tetrad2 was used for thermocycling with an initial denaturation at 95°C for 5 minutes and then 10 cycles of denaturation at 95°C for 30 seconds, annealing at 72°C for 30 seconds, and extension for 2 minutes. This was followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 61°C for 30 seconds and extension at 72°C for 2 minutes. 5µl of amplified sample was then digested at 37°C for two hours using 2U each of the restriction enzymes HpaII and KpnI with 1X concentrated NE-Buffer-4 and 1X BSA. Products were then electrophoresed on a three percent agarose gel.

Analysis of blood type was done blinded, based on allele sizes present, and then compared back to known phenotype.

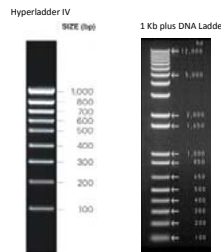
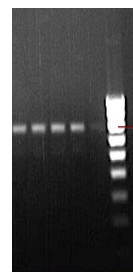
ABO Gene, Exons 1-7

Chromosome 9, location: 9g34.1-g34.2

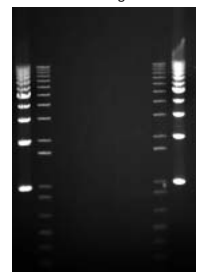


- 1 Primers mo-51 and mo-101
- 2 Primers mo-51, mo-47 and mo-101, mo-71
- 3 Digestion with Hpa II and KpnI: 309, 252, 223, 204, 164, 150, 137, 119, 96, 87 bp

Testing Sample DNA Amplifying HLA-DBR1

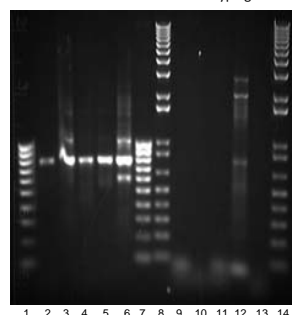


Preliminary ABO Modifications with Annealing Gradient



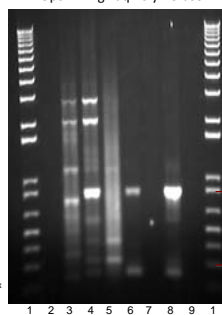
1. 1 Kb Plus Ladder
2. Hyperladder IV
3. Negative Control (H₂O)
4. 59°C
5. 62°C
6. 65°C
7. 68°C

Taq Polymerase Effects on HLA-DBR1 and ABO Genotyping



1. Hyperladder IV
2. Qiagen Taq
3. Takara Hot Start Taq
4. i-Taq DNA Polymerase
5. Denville Scientific Hot Start Master Mix
6. iProof High Fidelity Taq
7. Hyperladder IV
8. 1 Kb Plus DNA Ladder
9. Takara Hot Start Taq
10. i-Taq DNA polymerase
11. Denville Scientific Hot Start Master Mix
12. iProof High Fidelity Taq
13. Negative (H₂O)
14. 1 Kb Plus DNA Ladder

Optimizing Taq Polymerase

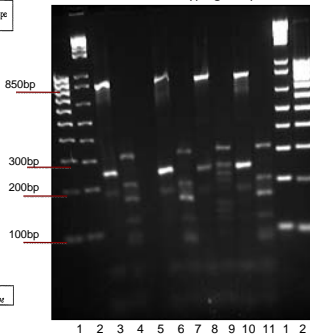


1. 1 Kb Plus DNA Ladder
2. Negative Control (H₂O)
3. iProof High Fidelity Taq
4. iProof, 2µl of DNA
5. iProof, 2µl of DNA
6. Qiagen Taq
7. i-Taq DNA polymerase
8. Denville Scientific Hot Start Master Mix
9. Takara Hot Start Taq

	A ₁	A ₂	A ₁ B	A ₂ B	B	O	Phenotype
87							
96							
119							
137							
150							
164							
204							
223							
252							
309							
	A ₁ A ₁	A ₁ A ₂	A ₁ O	A ₂ O	A ₁ B	A ₂ B	BB
	BO	BO	BO	O	O	O	O
	O	O	O	O	O	O	O
	Genotype						

[4] Olsson and Chester 1995, pp. 245

ABO Blood Genotyping Samples

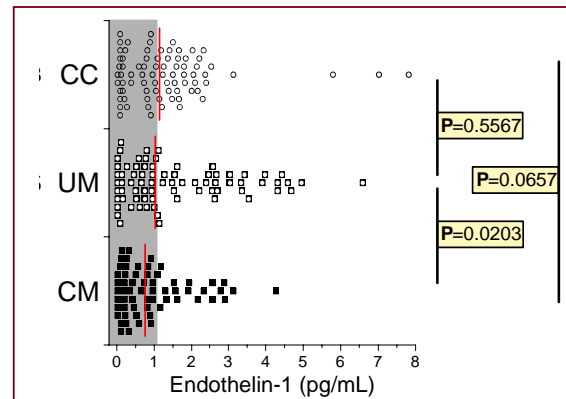


Genotyping Results

Sample	Determined Blood Type
GP	A ⁺ O ⁺
TO	O ⁺ O ⁺
AB	A ⁺ B ⁺
MM	O ⁺ O ⁺

1. Hyperladder IV
2. 1 Kb Plus DNA Ladder
3. Undigested TO
4. Digested TO
5. Negative
6. Undigested TO
7. Digested TO
8. Undigested AB
9. Digested AB
10. Undigested MM
11. Digested MM

Endothelin-1 Levels



Summary

•There is a trend toward decreasing endothelin-1 levels as disease severity increases. However, while endothelin-1 is significantly higher in those children with UM than CM, levels in healthy children are not significantly higher than those with UM or CM.

•The brand of Taq polymerase affected the ABO genotyping protocol.

•The currently developed ABO genotyping protocol can successfully genotype blood group using purified genomic DNA.

Conclusion

The near normal concentrations measured suggest that endothelin-1 levels may vary in malaria-infected individuals, but that levels are not related to severity of disease.

Future Studies

•Determine endothelin-1 levels at different time frames of the disease.

•Reconfirm results by performing the experiment again, or by

•Reconfirming results using a second uncomplicated malaria population.

•Use developed ABO protocol to genotype the Ugandan samples. This would also be a test of the protocols ability, as the samples are isolated from filter papers and have varying, low concentrations.

References

- [1] Cserti and Dzík. 2007. Blood. 110:2250
- [2] John et al. 2006. J. Infect. Dis. 194
- [3] Machado et al. 2005. Exp Biol Med. 231:1176
- [4] Olsson and Chester. 1995. Vox Sang. 69:242
- [5] Orstavik et al. 1985. Am J Hum Genet. 37:89
- [6] van der Heyde et al. 2007. Trends Parasitol. 22:503

Acknowledgements

This project was supported by the Undergraduate Research Opportunities program by grant funding.