

The implementation of laboratory investigations for diagnosing pyruvate kinase deficiency at the Johannesburg Hospital

Pierre Durand

(Student no: 8604833/H)

A report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfillment of the requirements for the degree of Master of Medicine (Clinical Pathology).

Johannesburg 2007

DECLARATION

I declare that this report is my own work. It is being submitted for the degree of Master of Medicine (Clinical Pathology) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.

.....

Pierre Durand

..... day of 2007

DEDICATION

*I dedicate this report to
postgraduate students and
researchers frustrated and
discouraged by faculty
administration*

ABSTRACT

Pyruvate kinase is an essential enzyme in the anaerobic glycolytic pathway of the erythrocyte. The clinical presentation of this enzyme deficiency is due to the haemolytic process that results from the inability of erythrocytes to generate sufficient ATP. Although pyruvate kinase and glucose-6-phosphate dehydrogenase deficiencies comprise more than 90% of all reported red cell enzyme disorders worldwide, the epidemiology of the disease in South Africa is unknown and there is no assay for pyruvate kinase activity currently being used in South Africa. This report describes the implementation of screening and quantitative assays for pyruvate kinase activity in the Red Cell Membrane Unit at the University of the Witwatersrand Medical School / NHLS. The accuracy, precision and reproducibility of the assay were verified. Furthermore, a patient with pyruvate kinase deficiency was confirmed and found to have 15% of normal enzyme activity at 37°C. The genetic abnormality was identified as a homozygous G1529A point mutation in exon 11 of the pyruvate kinase gene and to the candidate's knowledge is the first mutation described in a South African kindred. The patient's mother was heterozygous for the G1529A mutation and demonstrated an enzyme activity of 58% of normal at 37°C.

ACKNOWLEDGEMENTS

I would like to express my appreciation to the following people

- My supervisor, Prof. T. L. Coetzer for her guidance, expertise and for a creative working environment.
- My family and friends for their encouragement and support.
- My head of department, Prof. W. Stevens for the professional freedom afforded me.
- The PK deficient patient and her mother who permitted the relevant investigations.
- Dr. I. Thomson and Dr. P. Keene for the patient referral.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

- 1.1 The pathology of PK deficiency
- 1.2 Epidemiology
- 1.3 Patterns of inheritance and molecular genetics
- 1.4 Enzyme structure and function
- 1.5 Laboratory testing
- 1.6 PK deficiency testing in South Africa
- 1.7 Aims and objectives

CHAPTER 2: MATERIALS AND METHODS

- 2.1 Blood collection and processing
- 2.2 PK enzyme analysis
 - 2.2.1 Terminology
 - 2.2.2 Qualitative (screening) assay
 - 2.2.3 Quantitative (confirmatory) assay
- 2.3 PK activity of patient samples
- 2.4 Quality control
- 2.5 EA analysis of mutant PK
 - 2.5.1 Qualitative and quantitative assays
 - 2.5.2 Thermal stability of mutant PK
- 2.6 PK mutation analysis
 - 2.6.1 DNA extraction
 - 2.6.2 Primer design
 - 2.6.3 PCR amplification
 - 2.6.4 DNA analysis

2.6.5 Computational analysis

CHAPTER 3: PK ASSAY IMPLEMENTATION

- 3.1 Qualitative (screening) PK assay
 - 3.1.1 Sensitivity of screening PK assay
- 3.2 Quantitative (confirmatory) PK assay
- 3.3 Quality control
 - 3.3.1 Sensitivity of PK qualitative assay
 - 3.3.2 Paediatric size samples
 - 3.3.3 Precision and reproducibility of the quantitative assay
 - 3.3.4 Enzyme stability in whole blood
 - 3.3.5 Enzyme stability in haemolysate
- 3.4 Feasibility of PK assay as a nationwide service
- 3.5 Validation of the PK assay with PK deficient blood
 - 3.5.1 Qualitative PK assay
 - 3.5.2 Quantitative PK assay
 - 3.5.3 Thermal stability of mutant PK

CHAPTER 4: MUTATION ANALYSIS OF A PK DEFICIENT PATIENT

- 4.1 Patient history
 - 4.1.1 Family and social history
 - 4.1.2 Physical findings
- 4.2 Routine haematology investigations
- 4.3 Qualitative and quantitative PK assays
- 4.4 Mutation analysis
 - 4.4.1 DNA extraction and PCR amplification
 - 4.4.2 DNA sequencing
 - 4.4.3 Structural analysis of the mutant PK

CHAPTER 5: DISCUSSION

- 5 . 1 Qualitative PK assay
- 5.2 Quantitative PK assay
- 5.3 PK assay limitations
- 5.4 Quality assurance
 - 5.4.1 Sample handling and processing
 - 5.4.2 Reagents
 - 5.4.3 Accuracy, precision and reproducibility
 - 5.4.4 Controls
- 5 . 4 . 5 Skills, training and equipment
- 5.4.6 Reporting
 - 5.4.6.1 Qualitative assay
 - 5.4.6.2 Quantitative assay
- 5.5 PK deficient patient
 - 5.5.1 History
 - 5.5.2 PK assay results
- 5 . 5 . 3 Mutation analysis and thermal stability of the Arg510Gln mutant
- 5.6 PK deficiency in South Africa
- 5.7 PK deficiency and malaria
- 5.8 Concluding remarks

REFERENCES

APPENDICES

- Appendix 1: Suppliers
- Appendix 2: Reagents
- Appendix 3: SOP: PK screening assay

- Appendix 4: Projected PK qualitative assay costs
Appendix 5: PK assay: physician guidelines

LIST OF FIGURES

- Figure 1: The glycolytic pathway
Figure 2: Schematic representation of the PK-LR gene
Figure 3: Molecular structure of PK-R
Figure 4: Regions of PK-LR gene amplified in this study
Figure 5: A normal result of a screening assay for PK deficiency
Figure 6: PK screening test sensitivity
Figure 7: Rate of change of absorbance at 340nm as a measure of PK activity
Figure 8: Screening assay for PK deficient patient
Figure 9: Quantitative assay of PK deficient patient
Figure 10: PCR amplification of three regions of the PK-LR gene
Figure 11: Sequence data of exon 11 of the PK-LR gene
Figure 12: Modeling of the mutant Arg510Gln PK protein

LIST OF TABLES

Table 1:	Reagents used for screening and quantitative assays
Table 2:	Oligonucleotides for PK-LR gene amplification
Table 3:	Reagents used for PCR reaction
Table 4:	Two data sets of quantitative PK assays
Table 5:	Quantitative PK assay data from 20 Caucasians and 20 Africans
Table 6:	PK stability in whole blood
Table 7:	PK stability in haemolysate
Table 8:	Quantitative PK assay for 10 patients investigated for haemolysis
Table 9:	Quantitative PK assay data of PK deficient patient
Table 10:	Quantitative PK assay data of PK deficient patient's mother
Table 11:	Thermal stability of mutant PK at 53°C

ABBREVIATIONS

ACD – acid citrate dextrose
ADP – adenosine diphosphate
ARV – antiretroviral
ATP – adenosine triphosphate
AZT – azidothymidine
bp – base pair
CV – co-efficient of variation
DNA – deoxyribose nucleic acid
EA – enzyme activity
EDTA – ethylenediaminetetraacetic acid
G6PD – glucose-6-phosphate dehydrogenase
Hb – haemoglobin
HIV – human immunodeficiency virus
LDH – lactate dehydrogenase
NADH – nicotinamide adenine dinucleotide
NADPH - nicotinamide adenine dinucleotide phosphate
NHLS – National Health Laboratory Service
NRTI – nucleoside reverse transcriptase inhibitor
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PEP – phosphoenolpyruvate
PK – pyruvate kinase
PK-LR – pyruvate kinase liver/red cell gene
SD – standard deviation
SOP – standard operating procedure
3TC – 2,3-dideoxy-3-thiacytidine
TE – Tris-EDTA