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THE UTILITY OF MOLECULAR DIAGNOSTIC HAEMATOLOGY IN THE CONTEXT OF A DEVELOPING COUNTRY

Rusla Marianne Dubreuil Lastrucci

DECLARATION

I, Rusla Marianne Dubreuil Lastrucci declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any other degree or examination at this or any other University.

.....RMDLastrucci.....

...08..... day of September., 2000.

As first author in the six journal articles and 8 of the 12 presentations documented in this thesis, I have been intimately involved with the initial concepts, experimental design, laboratory work and manuscript preparation. My co-authors provided technical assistance, patient samples, manuscript editing and/or played a supportive role, for which I thank them. In the remaining 4 presentations my contribution was a portion of the laboratory work and written components.

"Sweet are the uses of adversity ..." Duke senior

William Shakespeare (1564–1616), English poet and playwright.

As You Like It. (1599); Act 2, Scene 1.

PREFACE

With a mean annual household income of less than US$10 000, South Africa qualifies as a developing country in terms of the World Health Organization definition. However the economic heterogeneity is such that it has access to certain resources not available in many other developing countries. One such resource is a medical infrastructure, which is as demanding of state-of-the-art services, including diagnostic pathology services, as those available in the developed world.

However in comparison to developed countries South Africa's haematological infrastructure is severely under-resourced. Despite a shortfall in trained personnel, South Africa has to deal with a greater national burden of haematological diseases, exacerbated by highly prevalent nutritional deficiencies and infectious diseases with haematological manifestations or complications, including those oncological. It is self evident therefore, that an attempt to address these issues is of fundamental importance in order to invest in the most cost effective and potent health care system.

Motivated by the documented success of molecular diagnostics in addressing the highly prevalent haemoglobinopathies in the Mediterranean littoral countries, the Department of Molecular Medicine and Haematology of the University of the Witwatersrand has embarked on a programme to try to harness some of the power of molecular technology in addressing its own burden of haematological disease.

Haemoglobinopathies are not a major public health hazard in South Africa, as it lies outside of the malaria belt. The diagnosis of malignant disorders of the haemopoietic and

lymphopoietic systems, however, constitute a formidable challenge, given the paucity of pathologists to perform this task. This problem has been exacerbated by the growing HIV/AIDS epidemic in South Africa, where 18 – 22 % of sexually active young adults are already infected.

This thesis aimed at the outset to explore ways in which molecular technology could enable a human resource-poor pathology service to make affordable yet reliable diagnoses of haematological malignancies. In the absence of these human resources many of the existing methods had to be modified to incorporate molecular checkpoints and controls to enable the issuing of a diagnosis with confidence, or to bypass certain facilities taken for granted in the first world. In some instances completely novel approaches had to be employed.

In setting up and developing a molecular diagnostic service for haematological malignancies, it became apparent that the same service could be readily adapted, without any additional expertise or major expense, to other diagnostic domains, effectively crossing interdisciplinary barriers to encompass the molecular diagnosis of other haematological disorders inclusive of those microbiological. The section dealing with molecular diagnosis of inherited haematological disorders is included in this report as confirmation of this concept.

R M Dubreuil Lastrucci, B V Mendelow. Molecular Medicine, the Next Millennium and the Developing World. Presented at the Association for Molecular Pathology Annual Meeting, St Louis, 1999. Abstract published in the proceedings of the above meeting in the Journal of Molecular Diagnostics, November 1999.

Instructions to Reader

This thesis is presented for examination as a collection of publications in terms of the options available for PhD candidates of the Faculty of Health Sciences at the University of the Witwatersrand. It is made up of 6 complete manuscripts (5 of which are in print or in press), 12 congress abstracts (7 international and 5 national), 5 abstracts published in international journals, general background and additional work covering areas not yet published. The publications and abstracts are printed on yellow paper, with the remainder printed on white paper. The section on Boolean logic has not been published due to potential patent possibilities in collaboration with Research Genetics, Alabama.

As the manuscripts had to comply with requirements of the different scientific journals to which they were submitted, editorial, English and reference inconsistencies are evident. The references appear at the end of the publication and are also referenced at the end of thesis by a superscripted cross-reference. The superscripted figure and table numbers however follow the order of the thesis to enable adequate indexing.

The provided computer disc is available to the reader if he/she prefers to read the manuscript electronically; this will enable the use of Microsoft Word referencing and cross-referencing capabilities. In order to be fully functional the web toolbar in Word should be enabled. Should the reader wish to see a table (e.g. Table 2) that is referred to in the text, he/she should click where the grey area and hand is displayed and will

automatically be taken to the relevant table. The same applies to the figures and table of contents. In order to return to the original text, the 'back' button on the web toolbar can be selected. It is possible to toggle between the forward and back buttons. Due to the size of the picture files, they are kept in separate folders. Once on the figure page, the hypertext can be selected to visualise the picture.

The first time a reference is quoted, by placing the pointer on the superscripted reference number, the whole reference will appear in a grey box on the screen. Subsequent to that, a cross-reference appears to the first use of that reference. There are a number of hypertext links to the Internet.

PUBLICATIONS

1. R. du Breuil, J. Patel, B. Mendelow. Quantitation of specific mRNA transcripts using xeno-competitive PCR. PCR Methods and Applications 1993; 3: 57-59.

2. Rosla M Dubreuil Lastrucci, Wendy S Stevens, Barry V Mendelow. Extension of a cold -labelled oligoprobe to analyse polymerase chain reaction products. *Technical Tips Online*. 5/8/98. #01419. http://tto.trends.com.

3. R M Dubreuil Lastrucci, Debbie Dawson, Marion Münster. Development of an internal restriction control in the PCR detection of the prothrombin 20210 A mutation. Clinical and Laboratory Haematology 1999; 21(4): 281-283.

4. R M Dubreuil Lastrucci, Debbie Dawson, Marion Münster. Development of an internal restriction control in the PCR detection of the methylenetetrahydrofolate reductase (MTHFR) C677T mutation. Molecular Diagnosis 1999; 4(2): 159-161.

5. Rusla M Dubreuil Lastrucci, Debbie A Dawson, James H Bowden* and Marion Münster.

*Molecular Pathology, University of Virginia, Charlottesville, VA, USA.

Development of a simple multiplex PCR for the simultaneous detection of the factor V Leiden and the prothrombin 20210 A mutations. Molecular Diagnosis 1999; 4(3): 247-250.

Pending:

6. Rusla Dubreuil Lastrucci, Gwynneth Stevens, Wendy Stevens, Elaine Wick Poplin*, Kel Locklar*, Carol Crowther, Lesley Scott and Barry Mendelow.

*Research Genetics Inc, Huntsville, Alabama, USA.

Use of a cDNA micro-arrays to analyse gene expression patterns without the use of radioactivity. Submitted.

Note: All the above authors are members of the Department of Molecular Medicine and Haematology, School of Pathology, University of the Witwatersrand/SAIMR, unless otherwise stated.

PRESENTATIONS

A International

1. N. Sioutos, A. Bagg, R. Dubreuil Lastrucci*, W.C. Pugh, J. Locker, J. Cossman. Georgetown University of Washington D.C., USA, *University of the Witwatersrand, Johannesburg South Africa. M.D. Anderson Cancer Center, Houston TX, University of Pittsburgh, PA.
Immunoglobulin gene PCR: diagnostic application and interpretation.
International Academy of Pathology, San Francisco, March 1994.

2. RMD Lastrucci, NP Carter*, TL Coetzer, BV Mendelow. * Sanger Centre, Wellcome Trust Genome Campus, UK.
Identification of cancer associated chromosomal translocations using differentially labelled chromosomes and Boolean logic
American Society of Haematology, 39th Annual Meeting and Exposition, December 1997, San Diego CA, USA.

3. W Stevens, G Stevens, G Sherman, R du Breuil, B Mendelow.
The feasibility of molecular diagnostics in the routine laboratory : a third world experience.
ISH-EHA, Amsterdam, Netherlands, July 1998.

4. R M Dubreuil Lastrucci, Debbie Dawson, Marion Münster.

Development of an internal restriction control in the PCR detection of the prothrombin 20210 A mutation.

Association for Molecular Pathology Annual Meeting, Arlington VA, USA, November 1998.

5. R Lastrucci, G Stevens, W Stevens, B Mendelow.

Micro-array technology : the analysis of gene expression patterns using a chemiluminescent detection protocol.

International Society of Haematology, Durban South Africa, September 1999.

(Presented again by request at BIOY2K Combined Millennium Meeting, Grahamstown, South Africa, January 2000.)

6. M Münster, DA Dawson, RMD Lastrucci.

Prothrombin 20210 A mutation is absent in the South African Black population

International Society of Haematology, Durban, South Africa, September 1999.

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The Association for Molecular Pathology Annual Meeting, St Louis, November 1999.

B National

1. R. du Breuil, B. Mendelow. Quantitation of specific mRNA using xeno-competitive PCR (X-PCR).
South African Biochemistry Congress, June, 1992.

2. RMD Lastrucci, DJ Clifford, BV Mendelow. Quality control in molecular diagnostics with particular reference to the polymerase reaction (PCR).
Federation of South African Societies of Pathology Congress, Bloemfontein, July, 1995.

3. RMD Lastrucci, W Stevens, BV Mendelow. Extension of a cold labelled oligoprobe to analyse polymerase chain reaction products. Outreach into Africa.
Federation of South African Societies of Pathology Congress, Cape, July, 1997.

4. D Clifford, W Stevens, R Lastrucci, BV Mendelow. Quality control in the routine molecular haematology laboratory – nightmare or reality?
Outreach into Africa, Federation of South African Societies of Pathology Congress, Cape, and July, 1997.

5. RMD Lastrucci, WS Stevens, C Crowther, D Brittain, G Stevens. Micro-array technology: comparison of gene expression in B-chronic lymphocytic leukaemia and normal peripheral blood.

BIOY2K Combined Millennium Meeting, Grahamstown, South Africa, January 2000.

Abstracts published in international journals:

1. RMD Lastrucci, NP Carter*, TL Coetzer and BV Mendelow

*Sanger Centre, Wellcome Trust Genome Campus, UK

Identification of cancer associated chromosomal translocations using differentially labelled chromosomes and Boolean logic.

Blood, November, 1997, 90 (10) supplement 1, 217b

2. Stevens W, Stevens G, Sherman G, Du Breuil R, Mendelow B

The feasibility of a routine molecular diagnostic laboratory : A third world experience.

British Journal of Haematology, 1998, 102,1, 293

3. Dubreuil Lastrucci, R M. *et al* . Extension of a cold -labelled oligoprobe to analyse polymerase chain reaction products. *Technical Tips Online*.

(http://www.elsevier.com/locate/tto.) #01419

Trends in Cell Biology, 1998, 8, 387

4. Lastrucci Rusla, Dawson Debbie, Münster Marion

Development of an internal restriction control in the PCR detection of the prothrombin 20219 A mutation.

American Journal of Pathology, 1998, 153 (5), 1648, G2.

5. R M Dubreuil Lastrucci, B V Mendelow. Molecular medicine, the next millennium and the developing world. The Association for Molecular Pathology Annual Meeting, St Louis, 1999.

Journal of Molecular Diagnostics, November 1999.

Other Publications and presentations not included this thesis

Invited presentation: CML PCR

Non-radioactive *in situ* Hybridisation Symposium, Boehringer Mannheim, South Africa, 18 August 1994.

Invited presentation: PCR Symposium. Boehringer Mannheim, South Africa, May 1995.

Topics:

Rusla MD Lastrucci and Marianne Pooler. Internal PCR controls for TB PCR.

Rusla MD Lastrucci and Marianne Pooler. Reverse dot blots using non-radioactive methods to identify *Mycobacterium*.

Rusla MD Lastrucci and Marianne Pooler. 'Spoligotyping' to identify *Mycobacterial* strains.

Invited speaker: Molecular diagnostics, technology for a developing country. Department of Haematology, University of the Orange Free State, Bloemfontein. September 1998.

C. Urbani, R Dubreuil Lastrucci and Beverly Kramer. Sexing of heat treated molars from cadavers using DNA PCR. The Journal of Forensic Odonto-Stomatology 1999; 17 (2): 35 – 39.

A. Bagg, F. Adam, R. du Breuil. A specimen bank for haematological malignancies. Federation of the South African Societies of Pathology Congress, October 1989.

R. du Breuil, J. Patel, B. Mendelow. Serum modulation of actin expression in primate macrophages.

South African Biochemistry Congress, January 1991.

RMD Lastrucci. The trials and tribulations of setting up a national DNA database. Perkin Elmer and The African Society for Genetic profiling workshop. 22 May 1998.

Rusla M Dubreuil Lastrucci, Maureen N Twala, BV Mendelow. Ethical and practical issues of setting up a national DNA statistical database (NDSD) and a DNA bank. The African Society for Genetic Profiling, Tau Lodge, May 1998.

G Stevens, R Lastrucci, and W Stevens.
Development of a DNA based assay for clinical monitoring of resistance of human immunodeficiency virus type 1 (HIV-1) to Lamivudine (3TC).
International Society of Haematology, Durban, South Africa, September 1999.

Products:

The novel primers designed as part of this thesis for xeno-competitive PCR, are being sold commercially by Research Genetics, Inc. Alabama, USA:

"β-Actin

These primers amplify a 289 bp region of Exon 3 of human and rat β-actin and serve as excellent controls when performing PCR reactions on genome templates or RT-PCRs on mRNA of these species.

Ref. Du Breuil R.M., J.M. Patel, and B.V. Mendelow. (1993) *PCR Methods and Applications* 3, 57-59."

XAHR 17, catalogue number: M502.10 $20

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(see also http://www.resgen.com/)

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ABBREVIATIONS

AAS	African Academy of Sciences
AIDS	Acquired immune deficiency syndrome
ALL	Acute lymphoblastic leukaemia
AMP	Association for Molecular Pathology
ANLL	Acute non-lymphoblastic leukaemia
APC-SR	Activated protein C sensitivity ratio
ASH	American Society of Hematology
ATRA	All–transretinoic acid
bp	base pairs
CIS	Commonwealth of Independent States
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
D	Diverse region
dATP	deoxy adenine triphosphate
dGTP	deoxy guanine triphosphate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dNTP	deoxy nucleotide triphosphate
DOP	Degenerate oligonucleotide priming

dTTP	deoxy thymine triphosphate
dCTP	deoxy cytosine triphosphate
dUTP	deoxy uracil triphosphate
ECAT	European Concerted Action on Thrombosis
ELISA	Enzyme linked immuno-sorbent assay
EST	Expressed sequence tags
FICTION	Fluorescent immunophenotyping and interphase cytogenetics
FISH	Fluorescent *in situ* hybridisation
FR3	Framework region 3
GDP	Gross domestic product
GERD	Gross domestic expenditure on research and development
GM	Genetically modified
GTG	Giemsa-Trypsin-Giemsa
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
ICRO	International Cell Research Organisation
ICSU	International Council for Science Unions
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain gene
ISH	*In situ* hybridisation/International Society of Haematology
J	Joint region

kb	Kilobase
MCBN	Molecular and Cell Biology Network
MRD	Minimal residual disease
mRNA	Messenger ribonucleic acid
MTHFR	Methylenetetrahydrofolate reductase
MTP	Microtitre plate
NIC	Newly industrialising countries
nt	nucleotide
PC	Personal computer
PCR	Polymerase chain reaction
R	Rand (South African currency)
RCPA	Royal College of Pathologists of Australia
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SAIMR	South African Institute for Medical Research
SKY	Spectral karyotyping
SNF	Supernatant fluid
SOPs	Standard operating procedures
Taq	*Thermus aquaticus* - polymerase
TB	Tuberculosis
TCR	T-cell receptor

TdT	Terminal deoxynucleotidyl transferase
TWAS	Third World Academy of Sciences
TWNSO	Third world Network of Scientific Organisations
U	Units
UKNEQAS	United Kingdom External Quality Assessment Schemes
UK	United Kingdom
UN	United Nations
UNESCO	United Nations Education and Science Commission
US/USA	United States of America
V	Variable region
VNTR	Variable nucleotide tandem repeat
WCS	World Congress on Science

ABSTRACT

Chapter 1- **Introduction**

The major problem confronting health care throughout the world today, but especially in South Africa, is a lack of human resources and funding. In comparison with developed countries, South Africa's diagnostic haematological infrastructure is severely under-resourced. This is reflected in the figures: 67 registered haematologists who are also required for many of the immuno-biological services, as compared with 776 for the United Kingdom. When correcting for the population size this is calculated at 1.59 haematologists per million of the population in South Africa, as compared with 13.3 per million of the population for the United Kingdom. These limited human and financial resources have resulted in a need for innovation and the modification of practices and techniques, to reduce the cost of various investigations in order to accommodate a decline in expertise and financial resources.

The widespread application of molecular techniques to pathology has had an immense impact on the characterisation of molecular defects underlying human disease. They have paved the way for new insights into the understanding of anatomy, physiology and pathology and have thereby delivered new conceptual approaches to the diagnosis and prognosis of patient disease. The further development of these molecular methods to firstly identify and then to routinely monitor disease was pivotal in the establishment of these techniques in the routine diagnostic setting/laboratory. Care should be taken however; not to conceptually categorise 'high tech' methodology for exclusive use by the first world, as the developing world could benefit in many ways from these modern innovations. This thesis strives to highlight the use of molecular diagnostics in the

routine haematological laboratory, and to illustrate the implementation of this incumbent technology in a cost effective, controlled manner to facilitate the diagnosis of haematological disease in the developing world.

Chapter 2 – **Molecular diagnosis of haematological malignancies**

Molecular technology has been applied to the diagnosis of many neoplastic disorders, as these represent somatic mutations in crucial genes and their resultant effects. The ability to detect a molecular marker of clonality is beneficial in the diagnosis and prognosis of neoplastic disease in a patient, as is the ability to detect the differences in gene expression between the malignant cells and their normal counterparts or indeed other types of malignancies. Thus the diagnosis of malignant tumour cells can be carried out at the level of RNA quantitation. The conceptualisation and design of xeno-competitive PCR for simplified RT-PCR based RNA quantitation, using β-actin as a model for proof of concept, is described (section 2.2), as is the development of a non-radioactive detection system to analyse patterns of gene expression using high density micro-arrays (section 2.3). These are two examples of diagnostic molecular technology that is currently out of range for many developing countries, which when modified as described to suit local resources, could become available.

Markers of clonality can be based on clonal expansion of physiologically and/or pathologically rearranged gene sequences. The physiological rearrangement of immunoglobulin and/or T cell receptor genes is the basis for the molecular detection of clonal expansion in lymphoid cell populations, and can be carried out using a simplified PCR assay (section 2.4.5). Pathologically rearranged genes include the well-

characterised t(14;18) and t(9:22) characteristic of follicular lymphomas and CML respectively (sections 2.5 and 2.6.1.1). These models of molecular lesions defining haematological malignancies have been used in this thesis to try to develop principles applicable to the diagnosis of haematological malignancies in the developing world. For the PCR based detection of pathologically rearranged genetic sequences, a cold-labelled oligoprobe was developed, which facilitates the deployment of blot technology in laboratories without access to radioactive facilities (section 2.5). A novel method for the detection of chromosomal translocations, without advanced knowledge of the genes or chromosomes involved, is described. The principles of Boolean logic were used to identify a clonal expansion of gene segments involved in translocations between two chromosomes (section 2.6). While this approach requires more developmental work before a kit based system could be deployed in diagnostic laboratories, proof of concept has been established using chromosomes 14 and 18, in the setting of follicular lymphoma.

Chapter 3 – **Molecular diagnosis of inherited thromboembolic disorders**

Inherited disorders such as the presence of the factor V Leiden, prothrombin G20210A and the methylenetetrahydrofolate reductase (MTHFR) C677T mutations, all place the patient at risk of thromboembolic disease. With the use of the polymerase chain reaction (PCR) and restriction enzyme technology, all the above tests are within financial and practical reach of a routine laboratory, providing that the appropriate controls are in place. In order to implement the prothrombin G21210A and the MTHFR C677T molecular diagnostic tests into the routine work up of patients - in addition to the

conventional controls - it was necessary to incorporate controls for the restriction enzyme digestion of the PCR products generated during these tests. This work is presented in its published form in sections 3.2 and 3.3 of this thesis. Furthermore it was considered prudent and cost effective to multiplex the factor V Leiden G1691A and prothrombin G20210A tests into a single 'run', thus reducing the cost and time required by almost half (section 3.4).

Chapter 4 – **Quality assurance**

No methodology, molecular or otherwise, is beneficial without the stringent quality control measures required to ensure the reliability of the consequent diagnosis. The work presented in this thesis has been intensely cognisant of this fact, and thus attentive in ensuring the maintenance of quality assurance throughout the methodology described. Problems created by the incorrect application and interpretation of diagnostic results illustrate why certain procedures are essential to identify these problems. The techniques need to be optimised for individual laboratories and controls placed to identify false positives, false negatives and other areas where methodological failure could lead to false results. As a final measure to assure quality, standard operating procedures (SOPs) need to be drawn up and adhered to, and the laboratory needs to join up with other laboratories in international sample exchange programmes to monitor their performance and to identify problem areas.

1 INTRODUCTION

1.1 Perspectives – Historical and Modern

1.1.1 General perspectives on discovery, invention and technology

Discovery, followed by invention followed by further discovery, results in the continual development of new inspirations, procedures and devices, and is characteristic of the inventiveness of the human species and its urge, motive and ability to accomplish these objectives [1]. Technology is that special kind of knowledge applied when solving practical problems, a human want or need, or an aspiration. Design of new technology involves creativity and the ability to put together new and existing ideas in novel ways. The scale of these technological innovations and the speed of their implementation today, are quite different from anything experienced in antecedent phases in the evolution of technology [2].

At the United Nations (UN) Conference on Environment and Development held in Rio de Janeiro in 1992—widely known as the Earth Summit—politicians and scientists together brought into prominence issues such as climatic change, sustainable development, and the responsible management of global assets and resources. There was a particular regard to environmental pollution, waste disposal, **and a reduction in the gap in technological capacity between developed and developing countries** [3]. In 1988 United Nations Education and Science Commission's (UNESCO) General Conference adopted as one of its four major programmes **"the sciences in the service of development"**, fostering the **"advancement and sharing of knowledge"**, promoting

their "**application in the search for new solutions**" to "**improve the social and cultural environment**" [4].

In this spectacular phase, as the 20th century closes, any characterisation of technology would be incomplete if it failed to acknowledge its inescapable moral obligation to humanity as a whole. There is a responsibility of present generations to safeguard the needs and interests of future generations (UNESCO, Paris, 1998) [4 5]. There is probably nothing inevitable about any technological development. The technology we encounter is the result of decisions that reflect the value judgements (unfortunately in recent history, usually war or financial gain) of those who were in a position to shape the technology. It would seem that form not only follows function, but power as well [2].

Werner Arber, Nobel prize laureate and President of the International Council for Science Unions (ICSU) stated: "There are no barriers between scientists in their discussions" thus they make "good ambassadors for peace". This may well be so, but modern science and the patenting of discoveries could ruin this 'openness' and certainly retard the cycle of discovery into technology into more discoveries [4].

Few people would want to turn back the clock to a century ago. Although sub-Saharan Africa is still poverty stricken and plagued by civil conflict and diseases such as malaria, tuberculosis and AIDS (acquired immune deficiency syndrome), according to UNESCO several developmental indicators reflect the positive impact of science and technology in recent years. The average life expectancy has increased from 39.9 years to

49.9 years between 1960 and 1994. The infant mortality rate dropped by over 40% in the same period, falling from 166 to 97 per thousand live births. The percentage of the population with access to safe water has almost doubled in the past two decades, rising from 24% in the period 1975 to 1980 to 42% in the period 1990 to 1996 [5].

"Today we must realise that the world is one or none", according to Federico Mayor, UNESCO. Although there is progress on the AIDS frontier, there is no money for the millions of AIDS sufferers in Africa and they are not getting treatment. "But we are forgetting that HIV (human immuno-deficiency virus) is a hyper-variable virus. It can generate a mutant that is more aggressive. Today it is only transmitted from blood to blood. But the day there is an aggressive mutant form of HIV, it will be like a boomerang against those who have forgotten that the world is one or none". "We must realise that a given event is not just happening in one place, it is happening in the world, on the Earth" [5 6].

When Japan was modernised after the Meiji Restoration in 1868, the revolutionaries frankly conscripted science and technology experts from already industrialised countries, they however actively sought to avoid the simultaneous importation of foreign values with these technologies. The cry was for "Western techniques but Eastern values". The assimila. on of new technologies was carefully managed to ensure their alliance to an intense patriotism and to the augmentation of the nation's industrial and military capacity [7]. There is probably no concern that modern technology designed to diagnose disease could corrupt 'African values'. On the contrary, first world technology

could certainly have a niche (similar or different) in a developing country. Technologies are not necessarily used by every culture in exactly the same way. For example Gunpowder, invented by the Chinese for fireworks, when brought to Europe stimulated the production of the cannon [8].

1.1.2 Appropriate Technology, directed versus creative research

The idea of a technology being appropriate in the sense of respecting the needs, resources, environment, and lifestyles of the people using it, came under discussion in the 1960s. The economist E. F. Schumacher, who in his book *Small is Beautiful* (1973)[9], wrote of "technology with a human face" and used the term "intermediate technology"[10]. His prescription for intermediate technology required it to make use of modern knowledge and experience; be conducive to decentralisation; be accordant with the laws of ecology; be disciplined in its use of scarce resources; and **serve humans,** instead of making them the servants. However, not all those in the so-called 'developing world' are content to see the cultivation of 'appropriate technology' in their own countries while the industrialised societies are perceived as heading towards a different and 'high-tech' future.

There is a need to be cognisant of both the advantages and disadvantages of a particular technology when either developing, importing or modifying it for use in the developing world. With the speed that technologies are progressing today, there is really no need to settle for anything that does not suit the potential application; modifications can almost always tailor a technology to suit financial and practical constraints.

The jury is still out on the subject of 'directed' versus 'creative' research. The pursuit of knowledge for the sake of knowledge is becoming more difficult in today's goal-driven research, usually for a marketable end product. While shrinking resources is in part the reason for this type of rationalisation, the trend has the long-term potential to stifle creative or innovative work. In many communities, governments provide a significant proportion of the research funds. Increasingly, governments are now demanding a greater influence into the types of work being undertaken. Thus, in subtle ways the State is able to influence the direction that research will take. Although a reasonable request, it has the potential to make research politically driven and can suppress creative individuals who have made significant contributions in the past, but who have difficulty thriving in an increasingly regulated environment. A number of the Nobel Prize winners to date, would not be competitive on current research funding allocations which are based on a strict process of peer review [11]. On the positive side, peer review ensures that there is some form of justice when it comes to distribution of grants, and ensures that money has been spent wisely and that limited resources are directed to where they will be most beneficial [5 11].

The molecular medicine era is one of the most exciting in the modern history of medicine. Many developments have occurred at a time when freethinking or hypothesis-driven research is giving way to goal-driven activities which have attracted funding, because of perceived commercial benefits (sadly). The effects that these shifts in strategies or emphasis will have on society through future developments in molecular medicine will take time to assess [11]. Perhaps it is possible to harness this trend and to

'direct' technologies to where they are desperately needed, with the changes or modifications that they may need to streamline them, make them self contained and cost effective for the developing environment.

Malnutrition, harmful environmental factors, uncontrolled population growth, ageing, degenerative diseases such as cancer and immuno-deficiencies, and the spread of infectious diseases are all areas investigated by molecular and cell biology. "While major advances have been made towards solving these problems in countries with highly developed technology, the rest of the world has not felt an appreciable impact of these solutions. During the past several decades we have witnessed spectacular progress in the application of knowledge derived from basic biological sciences to solving problems in a wide variety of areas of great importance to human welfare. These striking achievements have been to a large extent implemented by the application of new concepts, principles and methods in the area of molecular and cell biology. To enable these (developing) countries to enter the main stream of research and thought it is necessary to make the basic tools and techniques available to them and to establish a suitable environment to the development of solutions to problems employing molecular and cell biological approaches". Prof. Angelo Azzi, UNESCO-MCBN (Molecular and Cell Biology Network) [4].

1.1.3 Biotechnology

In biotechnology, a living organism - either a whole organism, a cell, or a part of a cell, such as an enzyme - acts as an intermediary to transform a starting product into a

desired end product, and has been around for centuries, e.g. when we make bread or wine. According to Albert Sasson, Doctor of Natural Sciences and Special Adviser to the Director-General of UNESCO, biotechnology did not have to wait until 1953 for Watson and Crick to discover the structure of DNA, or for the developments in molecular and cell biology of the 1970s, as Louis Pasteur was a biotechnologist, as was Alexander Fleming [5].

1.1.4 Molecular Technology

Molecular methodology has made it possible to improve our understanding of the living organism and to apply this knowledge to the life and activities of man. Examples are: in agricultural food production, forestry, animal rearing, horticulture, the production of energy, the combating of pollution, public health, vaccines, reproduction, and the diagnosis of disease.

Globally, there is however major concern regarding the long-term safety of engineered or modified organisms, especially for food production [12]. On May 27, 1999, the Nuffield Council –Britain's leading body on bioethics - published a wide-ranging report. The report said that there were no grounds for a ban on genetically modified (GM) products, and found that the genetic modification of plants was insufficiently different from traditional plant breeding to raise moral objections. In addition, the report said that GM crops could prove very beneficial in countries in the developing world, in the fight against malnutrition and poor health. "There is a compelling moral imperative to make genetically modified crops readily available to developing countries

who want them to help comoat world hunger and poverty. However, new measures are needed to minimise risks and to realise benefits that GM crops may offer" [13] [14].

Notwithstanding the raging public debate on genetically modified organisms, many biotechnologies are potentially useful. For example, using micro-propagation one can generate thousands of identical virus-free plants and can supply produce all year round. There are large amounts of 'vitroplants' (test tube plants) produced in the world today, and this technology is used extensively in the flower and arboreal markets. Even poor countries can and have become major producers of vitroplants. More than 30 percent of crop l: ses are due to pests. Biotechnology can reduce these losses by as much as 10 or 20 %, by increasing production through the engineering of resistant seeds [5].

Turning to the field of medicine, diagnosis and therapy account for 68% of the biotechnology industry in the USA, 43.7% in Canada and about 43% in Europe. Examples are:

- recombinant drugs, such as human insulin, growth hormone, interferon and erythropoietin
- recombinant diagnostic kits (HIV, pregnancy)
- recombinant vaccines (Hepatitis B, AIDS)
- production of medicines by transgenic plants or animals (in their milk or urine) [5].

1.1.5 Overview of the history of molecular technology as applied to biomedical science

With respect to medicine, the invention of the microscope was a huge technological leap. Progress since then in the field of cell biology was initially extremely rapid, but began to approach its plateau around the middle of the twentieth century [1 15]. Although DNA was isolated as early as 1869 [16], the discovery that represents the effective beginning of molecular biology was that of the structure of DNA by Francis Crick and James Watson in 1953 [17 18 19]. This was of importance not only because DNA is the molecule which transmits hereditary information from generation to generation (shown by Avery in 1944 [16]), but also because its structure immediately provided an insight into how this was achieved [19 16]. The next question was how this hereditary information influenced the activities of the cell. The central theme of molecular biology was thus discovered: DNA makes RNA makes protein under the laws of the genetic code, and is responsible for inherited traits, genetic disorders and somatic disease [19 16 20]. This heralded the development of molecular technological methods that in turn paved the way for a new dimension in our understanding of anatomy, physiology and pathology. These new insights were essential prerequisites for radically new conceptual approaches to the diagnosis and treatment of patients [21].

Although major advances were made in the 1950s and 1960s, the explosion in molecular biology began in the 1970s with the development of gene-cloning techniques that allowed the isolation of large amounts of a relatively pure DNA fragment [22 23]. This coupled with the development of hybridisation procedures and the ability to radioactively label the DNA molecule, provided a technique called Southern blotting

(after Edwin Southern), that was used to investigate the structure of a gene [24 25]. Similarly, in the related technique of northern blotting, DNA from the gene of interest was hybridised to the RNA prepared from different tissues, allowing the RNA corresponding to that gene to be detected and quantified in a differential manner. These techniques produced much information on gene structure and expression [26].

The elucidation of gene structure led to one of the biggest surprises in molecular biology studies; it was found that in eukaryotes the coding regions of the DNA known as exons, are interrupted by introns [16 20]. These are removed by the process of RNA splicing inside the cell nucleus, transported to the cytoplasm and then translated into proteins by the ribosomes. For example, only 432 nucleotides are required to code for the 144 amino acids of haemoglobin, but there are actually 1356 in the primary transcript of the haemoglobin gene [19]. This provides a potential point where gene expression can be controlled; exons can be spliced together in different ways to produce variety, or can be varied in different tissues or during different stages of differentiation.

The development of northern blotting was followed and complimented by *in situ* hybridisation (ISH) allowing the distribution of RNA and DNA (or even protein) within a tissue to be characterised [11]. These studies lead to the conclusion that, in the vast majority of cases, the mRNA encoding a particular protein was only present in tissues and cells which expressed the protein. Therefore the production of different mRNAs and their proteins, was thus central to the functional differences between tissues in normal

individuals and therefore also responsible for the aberrant differences seen in diseased tissue.

Then in 1977, Fred Sanger amongst others read the linear order of the bases in DNA in a process known today as DNA sequencing. By knowing the linear sequence of nucleotides in a gene it was possible to predict the amino acid sequence of the resultant protein using the triplet code. Similarly, by comparing the sequence of a gene implicated in a specific disease to that in normal tissue, it was possible to characterise the alteration in the corresponding protein This may have involved, for example, a base change which lead to a single amino acid change in the protein, or a loss of a DNA segment leading to the loss of a corresponding portion of the protein [19 16 27 28].

Many workers in the field consider molecular medicine to have begun with Linus Pauling, who recognised sickle-cell anaemia as a molecular disease in 1949 by employing chemical and electrophoretic techniques to show that an abnormal haemoglobin was responsible for the sickling phenomenon seen in the patient's red blood cells [28 29]. Neel and Beet, independently of each other, clarified the inheritance of the disease on the basis of the heterozygous-homozygous hypothesis. In 1956 Vernon Ingram, using electrophoresis, chromatography, and trypsin digestion ("fingerprinting") showed that the difference between normal haemoglobin and sickle cell haemoglobin was due to a single amino acid change [16 29]. This demonstrated that a single nucleotide mutation could change a single amino acid in a protein, altering its structure and thus its normal functioning. These ideas led scientists to consider how the functional activity of

a protein was related to its properly folded structure as determined by its amino acid sequence (which in turn is determined by its nucleic acid sequence). In the 1960s, the British biochemist and Nobel Prize winner John Kendrew determined the structure of myoglobin using purified protein and X-ray crystallography [16 30 31]. His colleague and joint prize-winner Max Perutz subsequently went on to determine the more complex structure of haemoglobin. [19 16 32].

The standard approach in anatomical pathology is the microscopic examination of a tissue section. Recent advances have allowed the identification and cellular localisation of certain proteins or nucleic acids by the hybridisation of antibodies and nucleic acid probes to the intact tissue sections respectively (ISH) [11], hinting at the possible mode of action of these molecular entities.

Classical chromosomal analysis involves the morphological study of chromosomes to determine their number and any structural differences that may be present. A karyotype describes an individual's chromosomal constitution, normally 44 autosomes and two sex chromosomes, either an X or a Y, by virtue of their size and characteristic banding pattern. Non-random variations of these features were one of the first clues that these variations were diagnostic and/or prognostic of certain disorders [11]. Several clonal libraries of the human genome have been assembled using large digested (restricted) fragments. Any cloned fragment of DNA can now be localised to a specific chromosomal site by ISH, and viewed by tagging these probes with a fluorescent dye (FISH). By the localisation of these labelled probes to areas of the chromosomes, it is

also possible to establish the presence, absence, duplication and/or relocation of chromosomal material. Many of these aberrations have been shown to be clonal markers of certain cancers. While conventional cytogenetics is performed on the metaphases of living cells, FISH can equally be used on interphase nuclei from fixed specimens [33].

Restriction endonucleases, enzymes able to cut DNA at specific nucleotide sites, were then found in bacteria; used by them as a defence mechanism against marauding bacteriophages and other endoparasites. Smith, Nathans and Arber isolated these enzymes in the late 1960's, early 1970's and they were put to good use in recombinant DNA technology and thus molecular diagnostics (as cited by Trent *et al*, 1987) [11].

In order to fully investigate any DNA fragment, multiple copies need to be made of it. Kary Mullis's invention of the polymerase chain reaction (PCR) in 1983 won him the Nobel Prize in chemistry in 1993 [34 35]. PCR is a remarkably simple method of selectively multiplying specific DNA segments in a short period of time. Previously, DNA could be multiplied in a clone, but not in complete isolation. Scientists could now undertake everything from detecting hereditary disorders, to identifying cancers and infectious agents, to solving impossible murder mysteries, to retracing the very depths of evolution. Lives are already being changed by the PCR machine, now a feature of every biology laboratory in the first world. An American soldier killed in Vietnam, for instance, was identified after more than a generation by matching the DNA in a lock of his baby hair to a single bone found on the battlefield. President Lincoln's suspected

genetic disease, Marfan's syndrome, was finally diagnosed based on his stored bone fragments [34].

The technique of automated single cell analysis, flow or image cytometry bridges a gap between examination of the whole cell and its molecular analysis. Immunophenotypic analysis by means of multiparametric flow cytometry identifies a cell by virtue of its size, surface antigens and DNA content, and classifies it according to its lineage, subset and stage of differentiation [33]. Tissue is often a mixture of several different cell types, only one of which may be of interest or relevant to the diagnosis. By selecting the relevant population, a diagnosis based on its relative abundance and other abnormalities, can be made. Flow cytometry is already well established in cancer diagnostics and immunology, although it does however have its limitations in the diagnosis of certain malignancies [11 33]. Flow cytometers are now considered a routine facility and are a common feature of most large medical facilities including in African developing countries such as South Africa, Botswana, Kenya, Tanzania, Zimbabwe, Zambia, Ethiopia, and Malawi [36] (the latter two of the poorest countries world-wide).

1.1.6 Future Prospects

Molecular biology has achieved much in the 46 years since the structure of DNA was characterised. This progress offers real hope that the complex processes underlying embryonic development and the functioning of the adult organism may one day be sufficiently understood in molecular terms to allow the complete diagnosis and effective treatment of most human diseases.

The widespread application of molecular techniques in the pathology laboratory has had immense impact on the further characterisation of molecular defects underlying disease. With this additional knowledge of anatomy and physiology, the diagnosis, treatment and monitoring of numerous diseases in the patient received an enormous boost and established these techniques in the routine diagnostic setting/laboratory [37].

1.1.7 Ethical issues

Ethics is defined as the system of moral principles by which human actions can be judged as good or bad, right or wrong. Apart form the glaringly obvious issues of privacy and confidentiality, the issue of 'beneficence' (the obligation to do good as well as the avoidance or removal of harm) has far reaching implications. Surely when dealing in terms of access to knowledge, health care, safe drinking water, energy and education, the disparities between the 'haves' and the 'have-nots' is an ethical issue. According to UNESCO's Director-General, Federico Mayer, there is also an "ethics of time" - the postponement of any remedial measure can have irreversible downstream effects [5 6 14].

The promotion of science and technology is a cornerstone of the kind of economic progress that Africa needs if it is to compete in the 21st century. Knowledge is the one aspect of our lives that is still not being globalised, according to UN secretary-general, Kofi Annan [5]. In an age when the acquisition and advancement of knowledge is a more powerful weapon than any missile or mine, surely withholding such a 'weapon' from diseased patients contravenes the concept of beneficence.

1.2 Resources

Molecular diagnostics is generally and erroneously perceived as a first world technology, developed there, perfected there and executed there. It is the purpose of this thesis to present the case for the execution of molecular techniques in the context of a developing country, taking cognisance of the imperatives of quality, cost effectiveness and international standards.

1.2.1 Shortage of Resources

Three-quarters of the world's population, living in 120 developing countries, are not effectively involved in science thus the meaning of 'World Science' needs to be carefully evaluated [5]. Professor J. Vargas President of TWAS (Third World Academy of Sciences) and TWNSO (Third world Network of Scientific Organisations) feels that science and technology can be harnessed by developing countries within their local capacity to solve their current problems. About 20% of the Earth's inhabitants (largely localised in the North) generate considerable wealth and enjoy a high standard of living, sharing over 85% of the world's income and contributing over 90% of the world's current scientific knowledge. The remaining 80% of humanity are unable to master and utilise present day science/technology and are poor, deprived and marginalised. The income share of the poorest 20% is one sixtieth of the richest 20%.

The newest advances in science and technology offer immense possibilities for solving many of the complex problems that are currently impeding social development and economic growth in the less developed nations. Tissue culture, genetic engineering and

biotechnology, for example, can be instrumental in raising agricultural production, reversing land degradation and conserving bio-diversity in the ecologically fragile zones of less developed countries. Many feel that it is important to recognise that the most effective weapon against poverty is science [38]. There is also no need for developing countries to "reinvent the wheel". Professor Vargas feels that developing countries should master modern science and technology and apply them to their own requirements. Radical measures would be needed by the governments in these nations to meet this challenge. These measures include substantially more investment in research and development and full integration of science and technology into national development plans, building national and regional capacities in science and technology, intensifying regional co-operation and establishing strong national and regional alliances between the private sector and research and development institutions [4].

1.2.1.1 Financial

Total global Research and Development expenditure in 1994 was approx. US$ 470 billion. Of this only a small percentage was attributable to developing nations [38]. Experts feel that science and technology investment should be above one percent of GDP (gross domestic product) to have any significant impact on the level of development. Science and technology investment in the most developed countries is closer to two percent of GDP, with Sweden spending the largest proportion (three percent) (Table 1). According to the AAS (African Academy of Sciences) at a meeting in April 1999 - Tunisia, with the exception of a few countries (e.g. South Africa and Egypt) most African countries spend 0.5 percent or much less of GDP on Research and

Development, and there is a call for governments to put aside that one percent for Research and Development [38].

Table 1: GERD* as a percentage of GDP [5]

North America	2.5
Japan and NICs (newly industrialising countries)	2.3
Western Europe	1.8
Oceania	1.5
(World average)	1.4
CIS (Commonwealth of Independent States)	1.0
Central & Eastern Europe	0.8
China	0.8
India & Central Asia	0.6
Sub-Saharan Africa	0.3
South-East Asia	0.3
Latin America	0.3
Arab States	0.2

*GERD: Gross expenditure on research and development - Source: UNESCO World Science Report 1998.

The health budget of the Republic of South Africa was only 3,6% of the Gross Domestic Product (GDP) for 1998/1999, and amounted to approximately R23.22 billion (£2.3 billion) [39]. This is compared to the health budget of the United Kingdom, which

was in excess of £42 billion [40]. Faced with these figures it is questionable whether the developing world can indeed afford what is considered an expensive technology. Molecular diagnostics needs to cost-effectively substitute for existing expensive tests and expensive expertise. Molecular diagnostics is however not as expensive as is often perceived. In fact, were the actual time taken to carry out certain existing tests and the cost of pathologists be scrutinised; in the light of a diagnostic technology which is rapid, extremely accurate and simply executed – it would be hard to not consider molecular diagnostics a cost effective solution in a financially strapped economy. By employing the basic properties of molecular technology and some innovation, it is a simple task to mould diagnostic tests to suit our local environment. That is the specific focus of this thesis.

1.2.1.2 Expertise

In South Africa the number of pathologists per million of the population is 14.2, compared to the United Kingdom's 46.5 per million of the population [41 42 43]. As such, alternative means for diagnosis need to be sought in South Africa. Looking specifically at numbers of chemical, microbiological, anatomical and haematological pathologists per million of the population in South African vs. the United Kingdom's (see Table 2), each discipline shows a distinct paucity of the very professionals required to diagnose some of the diseases on the health 'hit parade'. Haematology especially leaves a lot to be desired regarding the diagnosis of haematological disorders such as leukaemia, lymphoma, inherited haematological disorders and even certain infectious diseases; and certainly many patients are going undiagnosed and unattended to, due to a lack of trained staff. These limited resources have resulted in a need for innovation in and modification of practices and

techniques to simplify diagnosis and reduce the cost of various investigations, in order to accommodate a decline in expertise and financial resources.

It is by no means necessary to be a molecular biologist to carry out the applications of molecular medicine in clinical practice. Like computer technology, recombinant DNA technology can be used by non-experts. However, during the developmental years at least, as molecular medicine is finding its rightful place within the science and art of medicine, some scientific knowledge will be useful in order to monitor and examine each new discovery/technology

Additionally, Africa shows a dearth of science graduates. In 1992 a study estimated that Africa had only 20 000 scientists and engineers, 0.36 percent of the world total [5]. Another study showed that this region was responsible for only 0.8 percent of total world scientific publications and its world share of patents was close to zero [5]. In Japan, the USA and Europe, there are two to five scientists and engineers per 1000 population. Parts of Sub-Saharan Africa have less than five scientists or engineers for every 10,000 population - as do India and some countries such as Brazil and Colombia [5].

Scientists are nevertheless not the solution to the lack of medical expertise and funding, however the technology that is produced by them could be. Without the obligatory involvement of scientists and pathologists at every level of the diagnostic process, it would allow these professionals to concentrate on the evaluation of all the data, procuring the correct set of diagnoses, prognoses and treatments to achieve the best fit for many more patients. Society as a whole could benefit economically as well as

medically from such practices. This technology is one day expected to be able to carry out diagnoses in areas far removed from the academic and/or major health facilities.

Table 2: The number of Pathologists from each pathology subspecialty and the number of pathologists per million of the populations of South Africa (SA) and the United Kingdom (UK)

	South Africa		United Kingdom	
Pathologies	**No. of Pathologists**	**No. per Million**	**No. of Pathologists**	**No. per Million**
Anatomical/Histopathology	190	4.51	1151	19.78
Chemical Pathology	73	1.73	218	3.75
Haematology	67	1.59	776	13.33
Medical Microbiology	83	1.97	503	8.64
Other	186	4.41	56	0.96
Total	599	14.22	2704	46.46
Total Population	42130500		58200000	
Pathologists per Million	**14.22**		**46.46**	

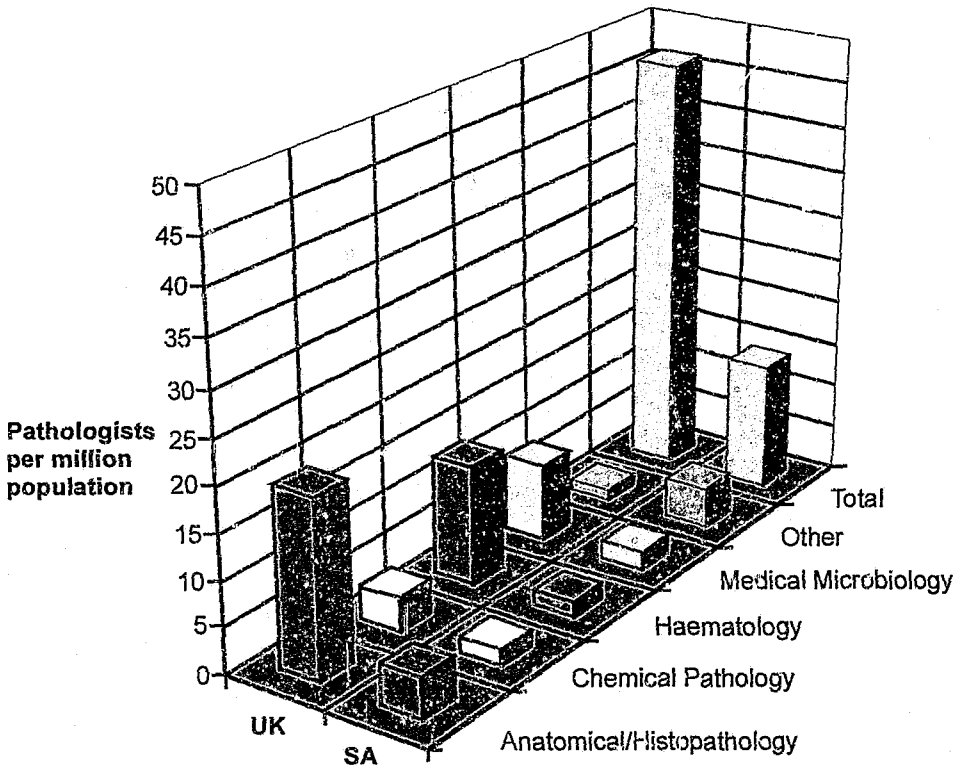

Figure 1: The number of Pathologists from each pathology subspecialty and the number of pathologists per million of the populations of South Africa (SA) and the United Kingdom (UK).

1.2.2 UNESCO

UNESCO at a World Conference on Science (WCS) (Science for the Twenty First Century, Budapest June/July 1999) and as part of one of its next programmes, has committed itself to the "**importance of Cell and Molecular Biology in the future development of science for mankind**", the "**science for knowledge and knowledge for progress**", "**science in society and science for society**" and "**science in response to basic human needs**" [4 44]. UNESCO's technical assistance programme fosters primary and secondary school education, fundamental and adult education, educational administration, scientific information centres, scientific research and science teaching [45]. These global policy makers are striving towards improving the understanding of science (and specifically Cell and Molecular Biology) world-wide, and its application to the developing world's needs. These needs could be described as a need to diagnose more diseases and disorders in a larger percentage of the population, more often and with greater accuracy, at less total cost to the heath care budget.

It is interesting to note that UNESCO's declaration of the human genome and human rights states that "freedom of scientific thought and creativity is an essential freedom that must be protected", "the freedom of research is necessary to the progress of knowledge and part of the freedom of thought" and the advances in biology, genetics and medicine should be "made available to all" [4].

Through MCBN, ICSU and ICRO (International Cell Research Organisation) projects, training activities and generally a huge amount of effort will be directed at strengthening

the science and technology in developing countries and is perceived as a top priority. The aim is to create centres of excellence and to harness creativity in cell and molecular biology (at least partly independent of outside assistance), to alleviate the problems facing the developing world and to enhance the indigenous generation and application of knowledge [4 5].

1.2.3 Cost

A careful costing analysis needs to be performed in a country with limited resources and an inadequate health budget; cost is sometimes the determining factor in the introduction of new diagnostic techniques. We have demonstrated that PCR, for example, can be optimised to rapidly and cost effectively amplify known DNA and RNA sequences. Its enhanced sensitivity, simplicity, speed, small sample requirements, application to archival tissues and low cost, favour it as a diagnostic assay. Often PCR replaces an existing assay at a far cheaper cost and results are obtained sooner. A classical cytogenetic assay which can cost R1481 (£148), and can take 1–3 weeks to obtain a result, can be replaced with an 'in-house' PCR assay that can cost as little as R16.74 per batched test (£1.67) (Table 3), and results can be obtained in as little as one day and less expertise is required. A FISH result, although quicker, costs R705 (£ 71). Various workers have compared genetic testing strategies in chronic myeloid leukaemia (CML), and showed that RT-PCR was by far the most inexpensive testing method, compared with Southern blotting, FISH or routine cytogenetics [33].

Due to its sensitivity, PCR can detect certain disorders at an earlier stage and can potentially save on the cost of expensive and sometimes unnecessary therapy. By obtaining

a more in-depth understanding of the disease's molecular components, therapeutic decisions based on this knowledge could cut costs. To diagnose a patient quickly and definitively has the associated advantage of almost immediate treatment, for the correct disorder, whilst the patient (when he/she is from an out-lying area) is still available at the health care centre.

Table 3: An example of costing an average in house PCR reaction

Item	Cost each (R)	Number used	Cost per un-batched sample (R) #	Cost per un-batched sample ($) #	Cost per sample (R) *	Cost per sample ($) *
1ml pipette tips	0.033	4	0.53	0.09	0.18	0.03
Other tips	0.033	5	0.66	0.11	0.22	0.04
Filter tips	0.623	4	9.97	1.61	3.32	0.54
Microfuge tubes	0.077	5	1.54	0.25	0.51	0.08
PCR tubes	0.690	1	2.76	0.45	0.92	0.15
Plastic pipettes	0.110	2	0.88	0.14	0.29	0.05
Taq	5.000		20.00	3.23	6.67	1.08
Primers	0.250	2	2.00	0.32	0.67	0.11
100 bp ladder	0.724		0.72	0.12	0.97	0.16
Agarose	1.580		19.00	3.06	2.11	0.34
Film	0.666		5.80	0.94	0.89	0.14
Total			**R 63.86**	**$ 10.30**	**R 16.74**	**$ 2.70**

*The cost amortised over 9 batched samples and the cost of 3 controls (positive, negative and blank) per batch is included (× 12 ÷ 9). Labour is not included, but it is estimated that the whole analysis takes approximately 2 hours of actual 'hands-on' time.

The 'worst case scenario' where only one sample was amplified with the appropriate 3 controls.

1.2.4 Molecular diagnosis, a digital diagnosis.

Molecular technology not only offers improved speed but also an unprecedented accuracy and precision to the diagnostic process of medicine. Molecular diagnostics can be applied to most aspects of human disease, and offers an objective, definitive diagnosis based on carefully defined parameters, unfettered by subjective observations or arbitrary cut-off limits. Molecular diagnostics are useful in that they are digital in nature as opposed to the analogue nature of some conventional methods. For example, certain bioassays dictate two populations/ranges that define the patient's disease status. The problem associated with bioassays is spelt out in the scenario of activated protein C resistance (Section 3.1). The detection of a single point mutation via PCR can easily replace a very arbitrary diagnostic bioassay system, where by definition a grey area exists where it is not known whether the patient is in fact positive or negative (Figure 2 (a)). In this case the 'grey area' rapidly disappears with the digital, molecular test where the patient is either positive or negative (Figure 2 (b)), and provided the appropriate controls are in place, the answers are simplified and quality control is assured and maintained [46]. Conceivably, a functional assay is superior when a different mutation to the one screened for, results in the same or a similar phenotype - this would thus not be picked up by a PCR assay directed at a specific mutation. Functional bioassays are dependent on the population that is being screened, so ideally each assay should be re-analysed and the parameters reset for every group studied. Known genetic markers for these functional defects obviously aid the definition of these population specific parameters greatly, if not obviate them altogether. Additionally, mutations that are

specific to the local populations but that give rise to known functional deficiencies, need to be identified and characterised by local molecular research and diagnostic centres.

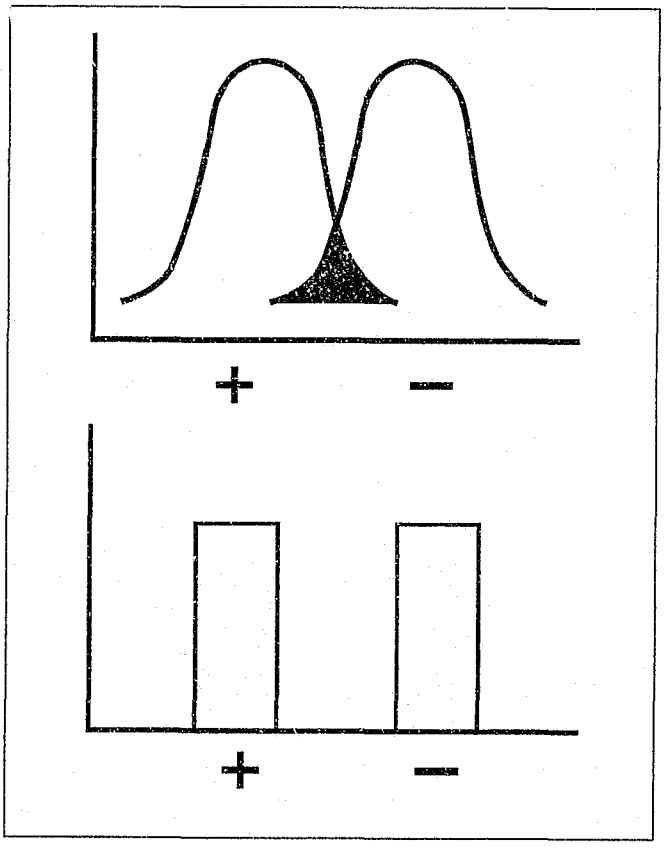

Figure 2: Representation of the analogue (a) and digital (b) nature of diagnosis.

1.2.5 Molecular diagnostics as a solution

The paucity of expertise (e.g. pathologists) may be partly alleviated in the face of unambiguous, definitive, digital diagnoses. Not only has molecular technology answered many questions on the fundamentals of molecular defects underlying human disease, but has also provided us with the tools to diagnose them. Due to speed, simplicity, cost-effectiveness and accuracy of molecular diagnostics, it has already irreversibly established itself in the routine diagnostic laboratory setting [47]. The next logical step is to take it to the point of care; the patient's bedside.

1.2.6 Potential for Innovation

Although molecular technology is well suited to a developing environment, the infrastructure so readily available and taken for granted in the execution of molecular diagnostics in the first world, is sometimes limited or even absent in developing countries. The existing first world molecular techniques sometimes need to be modified and adapted to the availability of expertise and/or equipment in the developing world.

Financial and practical aspects are relevant when considering the ability of the staff dealing with the technique to troubleshoot and to confidently produce unambiguous results. Over and above training, the latter requires that there are numerous, stringent, inbuilt controls in place, and that they are intimately associated with any technique, thus alerting the technician to a problematic result and to facilitate troubleshooting at the bench-level. These could include controls used to detect contamination, inhibition, partial digestion, and spurious amplification. Some of the standard methodology

required to check the validity of a result is often unavailable, or the facility and expertise is not at hand, for example, sequencing or the use of radioactively labelled probes. Innovative manipulations of these existing protocols have enabled their routine use [48 49].

The demand for testing at the molecular level is increasing, and consequently so is the workload of the routine molecular laboratory. Efforts to lighten this workload, economise on time and reduce costs are being made world-wide. The ability to combine more than one test, and still at all times attain and maintain interpretable results and rigorous quality control, would obviously be expedient and prudent in a busy routine laboratory [50].

Cost of molecular diagnostics is not necessarily great, especially if taken relative to the cost of health care professionals and of some of the existing technology. Certain of the expenses can be alleviated by innovative and discerning modifications to existing techniques and protocols. It is thus a considered and possible goal, to make affordable state-of-the-art molecular diagnostics available to all, as well as relevant to the local situation, while at the same time ensuring that good quality control procedures are in place.

1.3 Disease at the molecular level

Molecular pathology, or rather the knowledge of molecular mechanisms, is very adept at breaking down the divisions (all be they artificial) between medical disciplines. Ultimately the molecular profile rather than its anatomical location will define a disease.

Such is the inevitability of this phenomenon that, focussing on the patient's own endogenous genome as the genetic basis of disease, already broad categories appear of 'inherited' versus 'acquired' genetic disorders. In comparison to this, is the definition of other diseases as caused by exogenous 'infectious' agents'. Much more is known about the biology of the blood than any other tissue because of the ease of sampling and the associated relative lack of discomfort. Haematologists have had a major advantage in that blood is so accessible for microscopic, and now, molecular examination.

1.3.1 Neoplastic Disease

Molecular technology has been applied to the diagnosis of many neoplastic disorders. These represent somatic mutations in crucial genes (oncogenes) of only the cells comprising the tumour [21 51]. The ability to detect a molecular marker of clonality is beneficial in the diagnosis of a tumour in the patient. Such markers of clonality are scattered throughout the genome (not to mention the literature) and are particularly well characterised in the leukaemias and lymphomas (see Chapter 2).

Cancer or neoplastic disease results from an acquired defect in the DNA of a cell causing deregulation of its growth processes. The damaged cell transforms from benign to malignant and becomes independent of normal regulatory signals. This transformed cell multiplies into a clone of malignant cells, eventually developing into a tumour or monoclonal population of cells.

The first breakthrough in our understanding of the underlying mechanisms of cancer was in 1911 when Nobel prize laureate in 1966, Peyton Rous, showed that a 'filterable' agent was capable of inducing cancer in chickens (as cited by Raven, 1996). Only in the 1980s was a DNA sequence from a human, bladder cancer cell line cloned and also shown to have transforming capabilities [11]. Oncogenes were soon shown to be the culprits in both these cases. This was followed shortly by the discovery of the so-called anti-oncogenes or tumour suppresser genes [11]. Oncogenes were found to disrupt the cell's normal growth regulation usually due to mutations or duplications, and thus provided the foothold for elucidatory and diagnostic testing.

Cancer or neoplastic disease is increasingly becoming a major health concern in all age and economic groups. In addition to the inherited predispositions to cancer and the occurrence of childhood cancers, more and more of the population are living to an age where they are presenting with age related neoplastic disease. World-wide changes in lifestyle, such as changes in diet, consumption of alcohol and use of tobacco, all have associated risks of cancer development [19]. In addition, the increased availability of health care to most populations predicts an increased incidence of neoplastic disease, due to an increase in its detection and diagnosis [6].

Until recently, the methods of laboratory classification of cancer have been based on microscopic examination of tissue from the diseased organ, and are still considered as a major ally to the clinician. Flow cytometry in its turn has contributed widely to the routine diagnosis of cancer. As molecular technology has started to blaze trails into the

sub-microscopic area of the cell, a whole spectrum of potential disease causing entities has revealed itself, and this knowledge will undoubtedly aid in diagnostic processes. Somatic mutations or other changes in crucial genes of the cells comprising the tumour, could be used to recognise and identify that tumour. These are termed 'markers of clonality'. Examples of these markers are particularly well characterised in the leukaemias and lymphomas, and have been extensively described in the literature [11 19 21 33].

Physiologically, lymphocytes rearrange their genome irreversibly as they differentiate into B and T cells. This phenomenon provides the extensive repertoire of molecules available to fight infection. Should such a cell acquire immortality, its immunoglobulin (Ig) genes or the T-cell receptor (TCR) genes are identically rearranged in the neoplastic cell and all its progeny. This is an invaluable concept in leukaemia and lymphoma diagnostics as this marker of clonality is detectable using PCR-based diagnostic techniques [52].

There are many translocations that have been non-randomly associated with leukaemias, lymphomas and other tumours. Some of them have been generated during the immunoglobulin gene's propensity to rearrange such as the t(14;18) (bcl2/Jh fusion), t(11;14) (bcl1/Jh fusion) and t(8;14) (myc/Jh fusion). Others have been generated by different mechanisms, some of which are still unknown e.g. t(15;17) (PML/RARα fusion) and t(8;21) (ETO/AML1 fusion). Many of these are of course amenable to PCR

detection [33 53]. Two of the most well characterised translocations are those involving chromosomes 14 and 18, 9 and 22. The presence of the t(14;18) chromosomal translocation (bcl2/Jh) arises form an erroneous fusion between the IgH gene and an oncogene (bcl2) in the former's attempt to rearrange during maturation [21 33]. The PCR detection of the t(14;18) translocation is well known for occasionally giving false PCR bands [54 55 56], and it is essential, as with all other PCR methods, to establish whether the visible band is indeed legitimate, This can be done by a variety of methods using hot or cold labelled probes, sequencing or secondary PCR. The presence of the t(9;22) translocation (BCR/abl fusion) is not only a marker of clonality, but is diagnostically definitive of chronic myeloid leukaemia (CML). The ability to identify such a marker, quantitate it and then to monitor its presence allied with disease progression, is a remarkable tool.

It is not only the presence of the gene but also the differential expression of that gene that is important in the ultimate categorisation, treatment, monitoring and prognosis of a disease. The ability to quantitate and compare gene expression with techniques such as competitive PCR, RT-PCR and gene-expression arrays, will obviously be of great use. When quantitating gene expression using PCR, one has to be aware of the potential problems. Although two tubes are set up and treated in exactly the same manner, there can be tube to tube variation, which results in inconclusive results when trying to carry out a quantitative analysis. The method used by most workers in the field to alleviate the problem, is to incorporate an internal control or 'competitor' into each tube in order to create a direct standard on which to base their quantitative results.

The ability to generate an expression profile of the mRNA from any tumour or diseased tissue relative to that of its normal counterpart has tremendous power in both the research and diagnosis of neoplastic disease; not to mention the potential to track mRNA expression differences during therapy and disease progression. On the cutting edge of molecular technology are commercially available 'gene expression micro-arrays', which for the first time allow a systematic approach to surveying RNA variation and expression profiles. Thus the development of an array for specific disease identification that can be used in the routine laboratory could be extremely useful. For example, simply by identifying a repertoire of genes involved in chronic lymphocytic leukaemia (CLL) as opposed to acute lymphoblastic or acute myeloblastic leukaemias (ALL or AML) [57], these diseases could be distinguished from one another by exclusively grouping the implicated genes together on a smaller, cheaper version of the initial micro-array. There is no doubt that micro-arrays and their findings will have a huge impact on disease management and prognosis [58]. The ability to use a wider and wider repertoire of clonal markers would be of great value to the definition and diagnosis of neoplasms.

Disease prognosis is indeed possible and very feasible with molecular diagnostics and the following are examples of where specific molecular entities have a bearing on disease outcome: The t(15;17) translocation (PML/RARα fusion) in ANLL-M3 (acute non-lymphoblastic leukaemia-M3) predicts an appropriate response to all-transretinoic acid therapy (ATRA), whereas the t(11;17) translocation (PLZF/RARα fusion) positive ANLL-M3 is resistant to ATRA. Bcl 6 mutations are predictive of aggressive behaviour

and failure to regress after therapy in overt lymphoma or myeloma. The t(12;21) translocation (ATV6/ AML1 fusion)and t(2;5) translocation (NPM/ALK fusion) are being associated with favourable prognosis in paediatric and large cell lymphoma respectively. The t(1:19) translocation (E2A/PBX1 fusion) is associated with a poor prognosis in pre-B-ALL, and the t(14;19) (Jh/bcl3 fusion) shows an overall poor survival in CLL < 40 years of age [33 53 59 60]. On even a finer scale, the p190 BCR/abl product is shown to be a far more potent tyrosine kinase than the p210 BCR/abl product. So the list continues to grow to include other factors such as P53, P15 and ras mutations. Suffice to say that molecular stratification can be used to affect decisions regarding therapeutic options and thus save on costly and unnecessarily protracted therapy [33]. Not to be ignored is the evidence suggesting that there are geographic and ethnic differences in the distribution of specific chromosomal aberrations as Bernstein *et al* (1982) showed in various studies [61]. Black patients (southern African) with ANLL differed from Caucasians with respect to their age at presentation, in contrast to the preponderance of ALL in white childhood leukaemias. Additionally it was shown that there was a higher frequency of variant Ph (Philadelphia) chromosomes amongst black CML patients [62]. It is thus essential that studies are carried out in local populations in order to document and establish the prevalence, diagnostic and prognostic data of relevance to them.

Minimal residual disease (MRD) relates to the residual presence of malignant cells, usually post therapy. Although morphologically there may no longer be evidence of the clone, there is a point beyond this that there may still be residual evidence of disease.

This evidence stems from data supplied by exquisitely sensitive techniques such as PCR that can pick up one tumour cell in a background of 10^5 to 10^6 normal cells [33]. Of course, in order to detect the tumour cell, a clonal marker is needed, indeed one that has been associated specifically with that patient's disease. Translocations remain superior for this purpose, however gene rearrangements have also been used in spite of the tendency of the Ig and TCR genes to continue to change in the tumour itself. The debate continues in the literature as to the relevance of both a positive and a negative PCR result in the MRD setting.

1.3.2 Inherited Disorders

Classical genetics has limitations when trying to probe the complexity of the human genome. The application of recombinant DNA technology to human genetics has advanced our knowledge of inherited disease immensely. Inherited disorders represent that which is present in the genome of **all** the cells from birth, and covers a multitude of disciplines and concepts. Molecular methods have provided us with a handle for the detection of inherited molecular defects underlying many human disorders, including haematological, and the risk of and protection from disease [19 21].

The first human genetic disorders to be diagnosed by identifying the mutant genes were β thalassaemia and sickle-cell anaemia using liquid hybridisation and DNA polymorphism approaches described by Kan *et al* in 1978 (as cited by Trent, 1997) [11]. Today the individual mutations are easily identified by PCR and restriction enzyme technology, and the molecular diagnostics of thalassemias and other haemoglobinopathies has received much attention [16].

Venous thromboembolism, which includes deep-vein thrombosis and pulmonary embolism, is a common cause of death and one that can be avoided [63]. Treatment of affected patients reduces the incidence of fatal pulmonary embolism, but therapy should be based on the balance between the severity of the disease and the potential side effects. Decisions about the duration of therapy should be made by assessing the risks of therapy against the risk of recurrence. Important to determine is the presence of underlying environmental and genetic risk factors that could influence the management and duration of anticoagulant therapy [63]. The past few years have witnessed significant advances in our knowledge of inherited thrombotic disorders. Molecular diagnostics combined with existing laboratory techniques allow the accurate classification of at least half of Caucasian patients presenting with thrombosis as having an inherited thrombotic disorder [64].

The most significant advance has been the routine identification of a common inherited thrombotic disorder manifesting as an activated protein C resistance by factor V due to a mutation (G1691A) in exon 10 of the factor V gene (factor V Leiden) [65]. This disorder was initially described by Dahlbäck in 1993 [66], and may account for up to 52-64% of inherited thromboses in Caucasians [67]. A second slightly less common mutation in the prothrombin gene resulting in hypercoagulability has been described. Prothrombin G20210A results in elevated plasma prothrombin levels and an increased risk of venous thrombosis [68]. Hyperhomocysteinaemia has been identified as a risk factor for coronary artery disease and venous thromboembolic disease. MTHFR (methylenetetrahydrofolate

reductase) is involved in the remethylation of homocysteine to methionine. A common mutation in the MTHFR gene (C677T), has also been described and renders the MTHFR protein thermolabile. In the homozygous state this mutation is thought to interact with environmental factors, such as a deficiency of folic acid to predispose to hyperhomocysteinaemia [69].

1.3.3 Infectious Diseases

For infectious diseases the application of molecular medicine is both straightforward and dramatic. Any clinical sample can be analysed for the presence or absence of foreign DNA. Diseases such as HIV and TB (tuberculosis) are certainly a health care priority and are requiring urgent attention diagnostically as well as therapeutically. Apart from the obvious detection of the presence of these infectious agents, there is a need to quantitate and identify resistant organisms [11 21]. Molecular technology is well placed to serve these needs quickly, cost-effectively and reliably. Largely micro-biological in nature they are dealt with by other pathological disciplines. However it is interesting to note that the infrastructure required to perform molecular diagnosis, once in place, would be able to carry out the required tests within any of the pathology laboratories, again breaking down the interdisciplinary divisions.

1.4 Quality Assurance

As the newest area of laboratory medicine, molecular pathology is still in its infancy with respect to its procedures, and its relevant quality assurance standards are still being evolved and discussed [46 47]. The awareness that the use of non-standardised assays at numerous sites results in variable sensitivity, specificity and reproducibility of these

assays is of concern. There is a thus a need for controls, and safeguards to be present to ensure that molecular diagnostics is indeed definitive and objective.

Quality control and quality assurance is no less of a concern at the tip of Africa than elsewhere in the world. It is essential to establish external quality assurance programmes with national and international laboratories to improve the standardisation of molecular methods.

1.5 The cutting edge, future technology

Launched in the 1980s, the international genome initiative has set out to clone and sequence the human genome. By 2002 – 2003 > 80 000 genes will have been identified, localised and sequenced in the human genome. No doubt clinical medicine will be the principal beneficiary of this work [5 19].

Although PCR is the prototype of molecular diagnostic techniques, it is becoming increasingly obvious that it is only the antecedent of a plethora of innovative technology that is hailing the new millennium. Molecular technology is striving to become more streamlined and compact, with an ability to evaluate more than one disease entity in 'one sitting' so to speak. Such advances in technology are often associated with a reduction in cost and an increase in simplicity, which would benefit a developing world situation.

Methods that are fast becoming the cutting edge of molecular technology include multiplex testing, ELISA (enzyme immuno-sorbent assays) plate formats, fiberFISH, FICTION (simultaneous fluorescent immunophenotyping and interphase cytogenetics), SKY (spectral karyotyping), micro-array technology and DNA-chips, to mention but a few [33 70 80 81]. A matrix of multiple nucleic acid fragments on a semiconductor substrate that can electronically signal the presence of specific DNA fragments is now a reality. Clinical Micro Sensors Inc. has designed a handheld micro sensor that can signal the presence of HIV and HCV (hepatitis C virus) in a few minutes on a liquid crystal display by the simple application of a patient sample to the device. Such is the status of point-of-care molecular diagnostics [71].

1.6 Information Technology

Perhaps the only field that has shown a greater growth than molecular and bio-technology is information technology. It is a given fact that the world is soon to be (if not already) a 'global village'. Access to information and the sharing of knowledge through electronic publications is a vital link. The ability of molecular diagnostics in a developing country (as well as in other medical disciplines), to stay abreast of its first world cousin and for it to succeed in emulating the existing methods of diagnosis relies on this link. Access to this information is just as far as the nearest PC (personal computer) and its Internet link. Now, cellular phones and cheap computers are starting to bring the Internet to the rural areas of developing countries. With satellite links now being put in place, and a variety of software becoming available, 'the sky' is really 'the limit'.

2 MOLECULAR DIAGNOSIS OF HAEMATOLOGICAL MALIGNANCIES

2.1 Synopsis

For the purposes of this thesis the deployment of molecular technology for the diagnosis of haematological malignancies has been at two conceptual levels. These address the issues of (a) quantitation of gene expression and (b) markers of clonality.

(a) A fundamental question with reference to the diagnosis of malignancy concerns the issue of lineage. While this is traditionally based on morphological or biochemical similarities of tumour cells to their normal counterparts, it is becoming very clear that a far more objective analysis is provided by the determination of lineage-specifying gene expression by tumour cells. This can be analysed at a protein level by immunophenotypic methods, or at the level of RNA quantitation. Original contributions to the investigation of gene expression emanating from this thesis include i) the conceptualisation and design of xeno-competitive sequences for simplified RT-PCR based RNA quantitation (paper 2.2), using β-actin as a model for proof of concept, and ii) the development of a non-radioactive detection system to analyse patterns of gene expression using high density micro-arrays (paper 2.3). These are two examples of diagnostic high technology whose standard configuration is currently out of range for many developing countries, but when modified as described could eventually become available more widely.

(b) With regard to markers of clonality, these are in turn divisible into those based on clonal expansion of *i) physiologically and ii) pathologically rearranged gene* sequences [52].

The physiological rearrangement of immunoglobulin and/or T cell receptor genes is the basis for the molecular detection of clonal expansion in lymphoid cell populations. Work performed as part of this thesis contributed towards (abstract 2.4.5) a simplified PCR assay requiring only a single VH/JH primer pair. Examples of pathological rearranged genes within the context of haematological malignancies, include the well-characterised t(9:22) and t(14;18) characteristic of CML and follicular lymphomas respectively. These models of molecular lesions defining haematological malignancies have been used extensively in this thesis to try to develop an inventory of principles applicable to a broad range of malignant disorders of the blood and lymph nodes, essential for a diagnostic laboratory in the developing world (paper 2.5). For the PCR based detection of pathologically rearranged genetic sequences, a simplified cold-labelled oligoprobe was developed, which facilitates the deployment of blot technology in laboratories without access to radioactive facilities. This is described in paper 2.5 which was published in Elsevier's Trends - technical tips online (TTO). The online journal format is itself ideally suited to the developing countries which may lack comprehensive library facilities.

A skilled, labour intensive investigation which has been used widely in the definitive diagnosis of haematological malignancies is that of cytogenetics, which more recently has been significantly complemented by the advent of FISH techniques. These

approaches have the great advantage of permitting the detection of chromosomal abnormalities, especially but not exclusively translocations, without advanced knowledge of the genes or chromosomes involved. In the final part of this section of the thesis, a method designed to permit the detection of such translocations, again without the prior knowledge of the involved genes or chromosomes, is described. The principles of Boolean logic dictate that any occurrence of simultaneous labelling with probes for chromosome A *and* B must be indicative of a clonal expansion of gene segments involved in translocations between these two chromosomes. While this approach requires more developmental work before a kit-based system could be deployed in diagnostic laboratories, proof of concept has been established using chromosomes 14 and 18 in the setting of follicular lymphoma (section 2.6). Due to potential patent possibilities in collaboration with Research Genetics, Alabama, this work had not been published.

2.2 Quantitative RT-PCR

- Journal article: Quantitation of specific mRNA tra ripts using xeno-competitive PCR.

R.M.du Breuil, J.M. Patel, and B.V. Mendelow.

PCR Methods and Applications 1993, 3 : 57 - 59.

- Abstract: Quantitation of specific mRNA using xeno-competitive PCR (X-PCR).

R. du Breuil, B. Mendelow

South African Biochemistry Congress, June, 1992.

(Appendix 1.1)

- Abstract: Serum modulation of actin expression in primate macrophages.

R.M. Du Breuil, J.M. Patel and B.V. Mendelow.

South African Biochemistry Congress, 1991.

(Appendix 1.2)

(Work carried out outside of the period of registration for this degree).

2.2.1 Journal article

Quantitation of β-Actin-specific mRNA Transcripts Using Xeno-competitive PCR

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PCR is being used increasingly, not just to establish the presence of specific nucleotide sequences, but also to ascertain their quantity. The technique of competitive PCR involves the coamplification of a quantified competitive sequence with the target mRNA. Relative abundance after amplification of the target and competitor is then analyzed using a pre-defined and exploitable difference between the target sequence and that of the competitor (1,2) [72,73] Differences that have been reported previously involve restriction site variations induced by site-directed mutagenesis and incorporation of an intron within the competitor. (3) [74]

In this paper we report a simplified competitive PCR system based on inter-species sequence differences and similarities. The technique has been applied to study quantitative variations in β-actin mRNA within serum-deprived human hepatocellular

carcinoma cells (Hep 3B) in response to serum addition. By comparing the β-actin gene nucleotide sequence of the rat with that of the human (4,5) [75 76] and obtaining a range of consensus sequences, an appropriate set of nucleotide sequences that best fit the criteria for primers and were identical in both species was selected (Fig. 1) (Figure 3). By exploiting existing nucleotide differences between the two species, unique restriction sites present in only one of the sequences could be identified (Fig.1) (Figure 3). Two β-actin primers were designed that produced a 289-bp product after reverse transcriptase-PCR (RT-PCR), which upon complete restriction with *Pvu*II yielded two fragments of 132 and 157 bp in the rat only (Fig.1) (Figure 3).

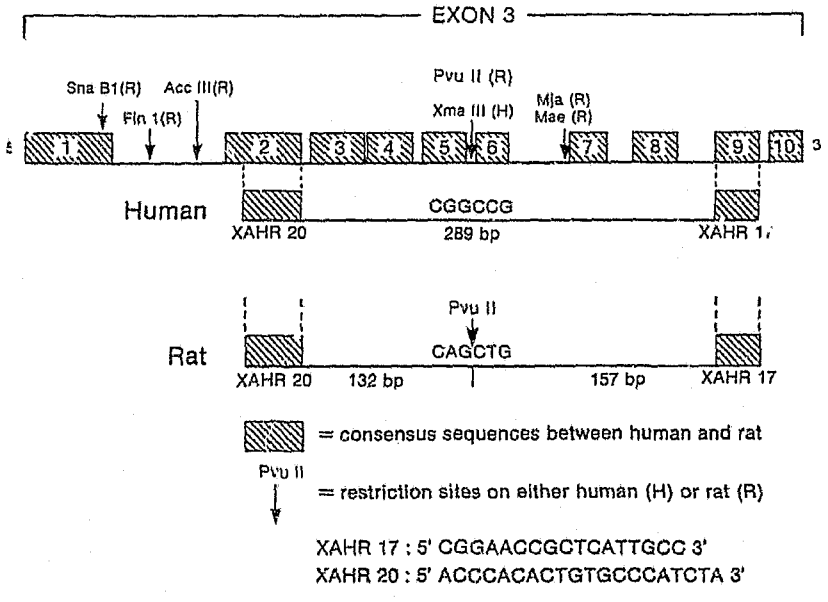

Figure 3: Exon 3 of the human and rat β-actin gene showing consensus regions and restriction sites. Consensus sequences 2 and 9 were chosen for primers. A *Pvu*II restriction site is present in the rat sequence.

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RNA was extracted by the acid-guanidium thiocyanate method. (6) [77] Human or rat RNA was submitted to a RT reaction using random hexamers or the downstream primer XAHR17. Upon the addition of the remaining primer, the cDNA was subjected to standard PCR.

Commercial reagents (GeneAmp RNA PCR kit, Perkin-Elmer) and the manufacturer's suggested reaction conditions were employed for the reverse transcription of the mRNA into cDNA. A master mix consisting of 5 mM $MgCl_2$, 1 x PCR buffer II, 0.35 μM dNTPs, 1 U/μl of RNase inhibitor, 2.5 U/μl of RT, and 0.75 μM of the downstream primer (XAHR17) was used. Prior to PCR, ~0.5 μCi per sample of [α-^{32}P] dATP or [α-^{32}P] dCTP was added to enable sample analysis. (1,2,7) [72 73 78] PCR was carried out in 50- to 100-μl volume of a master mix containing 2 mM $MgCl_2$, lx PCR buffer II, 0.15 μM upstream primer (XAHR20), and 0.025 U/μl of AmpliTaq DNA polymerase at a cycle program for 15-30 cycles of 92°C for 1 min, 54-56°C for 1 min, and 72°C for 2 min, followed by soaking at 15°C. Blank controls were set up with no RNA, rat RNA only, and human RNA only.

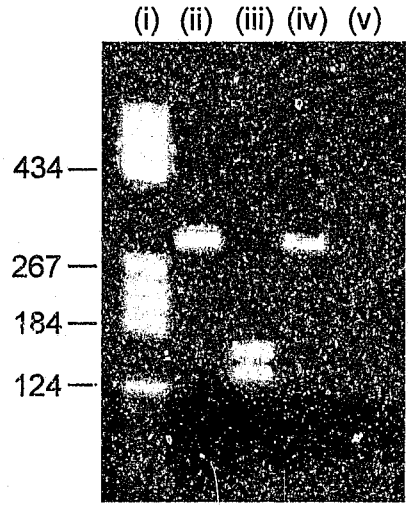

Figure 4: A 2% agarose gel showing PCR products after restriction with *Pvu*II. Amplified β-actin fragments of human (lanes ii and iv) and rat (lanes iii and v) showing no restriction and restriction, respectively. It is preferable that restriction is carried to completion, as seen in lane v, as it would facilitate analysis of the results.

The 289-bp PCR product was then restricted with *Pvu*II to give, as predicted, restriction of the parent fragment into the two smaller fragments in the case of the rat sample (Fig 2. lanes iii and v) [Figure 4] and no restriction occurred in the case of the human sample (Fig 2. lanes ii and v) [Figure 4]. *Pvu*II restriction was carried out directly on an aliquot of amplified DNA with 30 units of enzyme at 37°C for ~12 hr.

The resulting reaction mixtures were then run on a 2% agarose gel with ethidium bromide, and the specific bands were excised and the radioactive counts in each determined. (1) [73] Background counts representing the unrestricted rat fragment were subtracted, and the ratio of rat/human determined and plotted.

To demonstrate "linearity of quantitation", a xeno-competitive PCR standard curve was carried out by modification of the approaches described by Gilliland et al. (1,2) [72 73] In brief, varying volumes (0.25-1μl) of a standardized stock solution of rat competitor were dispensed into master mixes for reverse transcription and aliquoted into varying but known volumes of human mRNA stock, prepared from a human leukemic cell line (HL-60). The amplification step was subsequently carried out by aliquoting a PCR master mix into the human/rat cDNA mixture. The ratios of human/rat counts plotted against the increase of human RNA are depicted in Figure 3 [Figure 5]. Each experiment represented samples run within a single batch, with identical aliquots of rat competitor RNA. For each of the batches analyzed, a linear response of ratios, was obtained for the varying amounts of human mRNA. However, it was not possible to compare ratios among batches. The results suggest that samples within a single batch may be compared

with one another with respect to their relative quantities but that the values obtained are not comparable with those obtained from different batches.

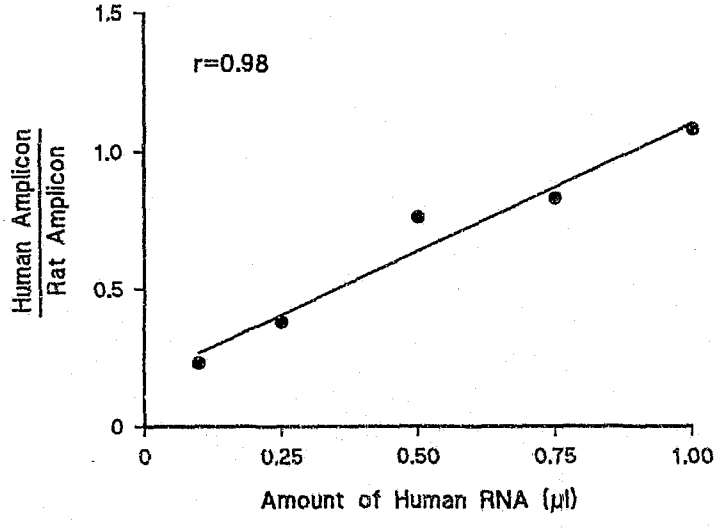

Figure 5: Graphic representation of varying volumes of human stock mRNA (µl) reverse transcribed and amplified with 0.25 µl of competitor rat mRNA. Radioactive counts representing human and rat bands are depicted as human/rat ratios. The ratios which were comparable within but not among, batches, showed a linear relationship to actual quantities of human RNA. Different batches contained varied volumes of rat competitive stock (r for 0.5 µl of rat stock = 0.95: r for 1 µl of rat stock = 0.99).

To test further the within-batch precision of the technique, tube-to-tube variation was deliberately induced by varying the number of PCR cycles for otherwise identical tubes. Four tubes were set up for RT-PCR, each with 0.25 µl of human and 0.25µl of rat RNA stock solutions, and subjected to 15, 20, 25, and 30 cycles, respectively. A duplicate experiment was carried out using one-tenth the volume of human RNA. At 15 cycles, no PCR product was visible, and it was not possible to obtain accurate results. For 20, 25, and 30 cycles it was found that the coefficient of variation was <30% with high amounts of human RNA (0.25 µl) and <20% with lower amounts of human RNA (0.025 µl). In experiments designed to apply the system to a real-life example, the postulate that β-actin mRNA is constitutive and unregulated was tested and found to be flawed. The Hep 3B cell line was cultured under serum-deprived conditions (no fetal calf serum for 24 hr), followed by the addition of fresh serum to a concentration of 10%. β-Actin mRNA was then quantified as a function of time after addition of serum, using xeno-competitive PCR (Fig. 4A) [Figure 6]. All analyses were carried out within a single batch. The results showed a time-related fluctuation in β-actin mRNA, with a rapid initial decline, followed by a gradual increase presumably reflecting disturbances in the relative rates of transcription and catabolism, after addition of serum. The same result was reproduced in a similar but separate experiment, where Hep 3B cells were cultured in partially serum-deprived conditions (1% fetal calf serum for 60 hr; Fig 4B) [Figure 6]. These results were also analyzed within a single batch, in which the volume of human mRNA was deliberately varied by 500-fold with respect to the experiment depicted in Figure 4A [Figure 6], to exclude the possibility of high- and low dose hook effects. In both cases, within 15 min after serum addition, the β-Actin mRNA fell to undetectable or

virtually undetectable levels, possibly reflecting translation-related mRNA consumption and then rose again, presumably reflecting enhanced serum-induced transcription. The conclusion that β-Actin mRNA is regulated by unidentified factors within serum is in accordance with the work of Jamal and Ziff. (8) [79]

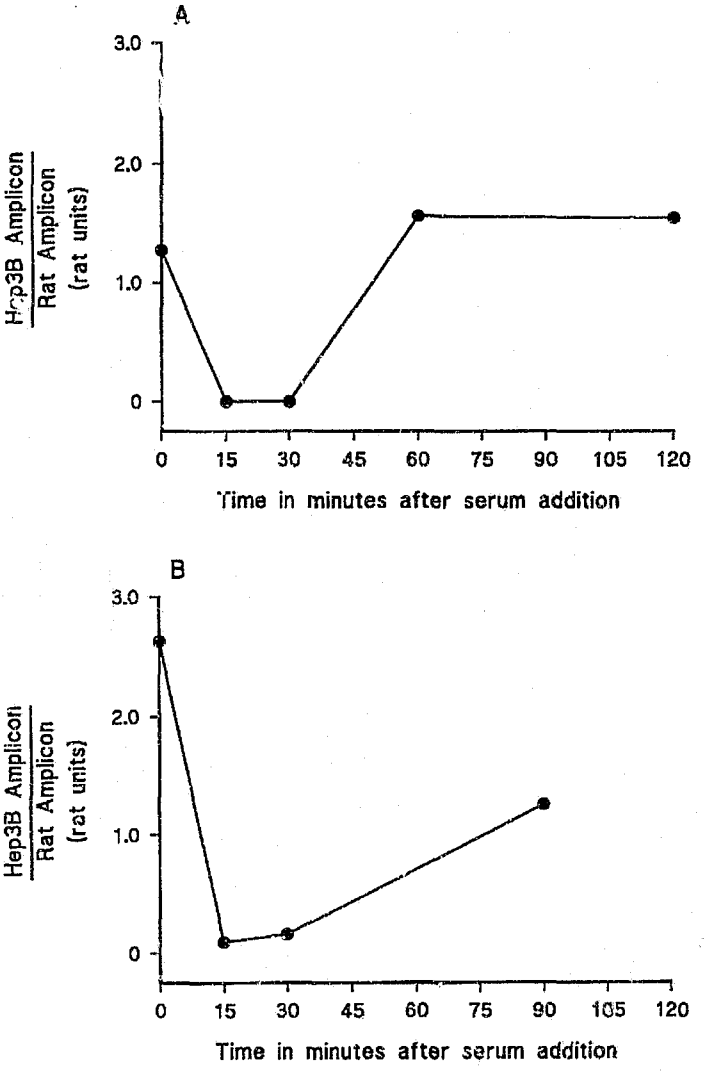

Figure 6: (A) Quantitative analysis of the effect of 10% fetal calf serum on β-Actin mRNA levels in Hep 3B cells precultured under totally deprived serum conditions for 24 hr before serum addition. (B) Similar results were obtained using Hep 3B cells partially deprived of serum for 60 hr before serum addition.

In summary, we have designed a simplified RNA quantitation approach, using xeno-competitive PCR analysis (X-PCR). The similarities between species provided us with the primers, whereas the differences provided us with a restriction site applicable to one species only, enabling us to distinguish one product from the other. We have used this method to quantify β-actin mRNA as an example. The same approach can be exploited with other mRNAs. In this connection we are using X-PCR to quantify variations in erythropoietin mRNA in response to various stimuli.

Variations of this method could use known polymorphic differences among individuals within a species to generate the restriction sites needed for allo-competitive PCR (A-PCR). X-PCR and A-PCR, therefore, alleviate the need for site-directed mutagenesis in a setting where mutations are readily available. Obviously, the same approach could be applicable to specific DNA quantitation.

ACKNOWLEDGMENTS

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2.3 Micro-arrays

- Journal article: Use of High-Density cDNA 'meso-arrays' to analyse gene expression patterns using a non-radioactive detection system: Implications for laboratories with limited resources.

Rusla Dubreuil Lastrucci, Gwynneth Stevens, Wendy Stevens, Elaine Wick Poplin*, Kel Locklar*, Carol Crowther, Lesley E Scott and Barry Mendelow.

*Research Genetics Inc, Huntsville, Alabama, USA.

- Submitted.

- Abstract: Micro-array Technology: The Analysis of Gene Expression Patterns Using a Chemiluminescent Detection Protocol.

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*Research Genetics Inc, Huntsville, Alabama, USA.

International Society of Haematology. South Africa, September 1999.

  - Presented again by request at BIOY2K Combined Millennium Meeting, Grahamstown, South Africa, January 2000.

(Appendix 1.3)

- Abstract: Micro-array technology: comparison of gene expression in B-chronic lymphocytic leukaemia and normal peripheral blood. RMD Lastrucci, WS Stevens, C Crowther, D Brittain G Stevens. BIOY2K Millennium Meeting, Grahamstown, South Africa, January 2000.

(Appendix 1.4, 1.5)

- Article in newsletter: This article was written on the request of Invitrogen/Research Genetics for their first International newsletter of 2000.

2.3.1 Journal Article (submitted)

Title Page

Use of High-Density cDNA 'meso-arrays' to analyse gene expression patterns using a non-radioactive detection system: Implications for laboratories with limited resources.

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Keywords: cDNA micro-arrays, Digoxigenin dUTP, chemiluminescence, non-radioactive detection.

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Abstract

The rapidly advancing field of array technology offers the first opportunity of providing a systematic approach to surveying RNA variation or RNA expression profiles. There are many formats of arrays available at present ranging form nylon based arrays of various sizes, to glass or even silicon-based arrays. The cost differs as well, dependent on the format, but even though membrane-based arrays are far cheaper than their glass counterparts, they still require the use of costly phosphor imagers to analyse the data generated. Arrays are available on nylon membranes from numerous manufacturers, including Research Genetics, Inc., Huntsville, Alabama. These are 7cm x 5cm nylon membranes containing approx. 5000 known genes and ESTs arrayed 750μm apart, and are designed for used with ^{33}P or ^{32}P labelled cDNA.

However, by replacing the radioactivity with DIG-dUTP and subsequently detecting the bound probe by chemiluminescence/colour precipitate, we were able to utilise and analyse these high-density membrane filters effectively, without the need for radioactivity and a costly phosphor imager.

Introduction

The rapidly advancing field of micro–array technology offers the first opportunity of providing a systematic approach to surveying RNA or DNA variation or RNA expression profiles in a parallel fashion on a large scale. Several recent review articles have suggested that they may become standard tools in molecular research as well as in

the molecular diagnostics arena (1,2,3,4) [57 58 80 81]. The most common arrays available at present, range from glass-based micro-arrays to nylon membrane-based macro-arrays. Affymetrix produces a glass-based micro-array which is comprised of tens of thousands of cDNA probes spotted onto a glass slide, which is detected using fluorochromes, and analysed with a laser scanner. This methodology is extremely costly.

Currently available commercial membrane-based-arrays monitoring RNA expression levels of 4,000-10,000 genes/EST clusters are fixed onto nylon membranes, hybridised to a radioactively labelled cDNA probe, and the resulting data is recorded with a phosphor imaging system. Reports in the literature describe non-radioactive methods for successfully detecting 23 x 23 cm macro-arrays available from Clontech (5,6) [82 83]. Intermediate density arrays, such as those available from Research Genetics, Inc., Alabama, are 7cm x 5cm nylon membrane containing approx. 5000 known genes and ESTs arrayed only 750μm apart. Although these 'meso-arrays' are cheaper than the glass-based micro-arrays, they still require the use of costly phosphor imagers to analyse the data generated.

The use of ^{32}P can cause 'blossoming' of the radioactive signal to obscure neighbouring genes and to hinder the subsequent software analysis; thus the manufacturers recommend the use of ^{33}P to reduce this phenomena.

We describe a method using a non-radioactive label combined with a chemiluminescent and/or colour precipitate to differentially screen mRNA on these high-density membrane arrays (GF211, GENEFILTERS®, Research Genetics).

Methods

Mononuclear cells were separated from whole blood using Ficoll-Hypaque (density 1.077g/l) (Sigma Diagnostics), and their mRNA was extracted using a mRNA extraction kit (Roche) via a biotin-labelled oligo(dT) probe and streptavidin magnetic particles. The integrity of the mRNA was checked using RT-PCR amplification of the HPRT mRNA.

The protocol supplied with the GENEFILTERS purchased from Research Genetics, was largely adhered to, modifications are discussed below.

Prior to proceeding with the following protocol, all the written information was cut off the membrane as it was found to generate a non-specific signal.

The GENEFILTER was prewashed in 0.5% SDS as recommended by the manufacturers, and prehybridised in a hybridisation roller tube in a roller oven (Hybaid, Inc. Roller Oven). The membrane was placed with the DNA side facing the interior of the tube so that it was not touching the glass. Five millilitres of DIG Easy Hyb hybridisation solution (Roche) containing 5.0 µl denatured Cot-1 DNA (1 µg/µl, Roche) and 5.0 µl Poly $dA_{(18)}$ (1 µg/µl, synthesised) as blocking agents, was added to the tube containing the membrane and mixed thoroughly removing any bubbles. The membrane was prehybridised for 2 hours at 42°C.

Two hundred nanograms of extracted mRNA were labelled with DIG-dUTP. Labelling took place concurrently with reverse transcription and the reagents and protocols were as follows: Two microlitres oligo $dT_{(15)}$ (Roche) was added to 200ng mRNA in 8µl H_2O in order to prime the reverse transcription reaction. The mixture was heat denatured

for 10min at 70°C and chilled on ice before addition of the enzyme. To the primed RNA, the following reagents from a Superscript II kit (GibcoBRL Life Technologies) were added: 6.0µl 5X first strand Buffer, 1.0µl DTT, 1.0µl dNTP mixture containing dATP, dGTP, dCTP at 20mM, 0.25µl dTTP also at 20mM, 6µl DIG-11-dUTP, 300U reverse transcriptase and made up to a final volume of 30µl with H_2O. All the above reagents were mixed thoroughly and incubated for 90 minutes at 37°C, followed by a 70°C incubation for 15 minutes to stop the reaction.

The labelled probe was purified of unincorporated nucleotides by passage through a G50 Sephadex spin column (Roche) as per the manufacturer's instructions.

One microlitre of the purified probe was retained and spotted onto a membrane, incubated with anti-DIG antibody conjugated to alkaline phosphatase and detected with NBT/BCIP (Roche) to check for label incorporation. The remaining purified probe was then heat denatured in a boiling waterbath for 3 minutes and pipetted into the roller bottle containing the membrane and the prehybridisation solution. Care was taken not to pipette probe directly onto the membrane. After adding the probe, the solution was mixed thoroughly by vortexing. Hybridisation occurred overnight (12-18 hours) at 42°C in a hybridisation roller oven at approximately 8-10 rpm (Hybaid).

The membrane was then washed twice in 30ml 2X SSC; 1% SDS at 50°C for 20 minutes each time in the roller oven. A final wash was carried out in 100ml 0.5X SSC; 1% SDS at room temperature for 15 minutes in a plastic container. After washing, the GENEFILTERS membrane was prevented from drying out by placing it on a piece of filter paper moistened with deionised H_2O and wrapped in plastic wrap. Creases or bubbles were avoided as they may interfere with the imaging.

Chemiluminescent detection was carried out using the CDP-Star substrate system (Roche). The light signal generated enables the detection of biomolecules, which is recorded on film or with instrumentation. On nylon membranes, the maximum light emission from the CDP-Star substrate is reached within a few minutes; therefore multiple images may be easily acquired.

After the hybridisation and stringency washes, the blots were subjected to immunological detection using anti-DIG antibody conjugated to alkaline phosphatase (Roche) followed by CDP-Star (Roche). The membrane was rinsed and 20ml dilute anti-DIG-AP conjugate (37.5 mU/ml, 1:10 000) was added and incubated for 30 min. The membrane was washed and incubated in a sealed hybridisation bag for 5 min in 1-2ml dilute CDP-Star (25mM, 1:200) solution. Excess liquid was drained off and the membrane blotted briefly (DNA-side up) on Whatman 3MM paper. At no point was the membrane allowed to dry completely.

The damp membrane was placed in a new hybridisation bag and exposed for 15s-15min to X-ray film or 15min to 35min in the Lumi-Imager (Roche).

Luminescence continued for a few hours (at least 24 hours according to the manufacturer), thus multiple exposures were taken to achieve the desired signal strength.

The images were stored directly as 16 bit Tiff files from the Lumi-Imager without any alterations. The images obtained with the chemiluminescence were satisfactory (Figure 1(a)) [Figure 7] in spite of their proximity, and compared well with the images obtained by Research Genetics using ^{33}P. In addition the images were analysable using their Pathways software (Figure 1 (b)) [Figure 7].

Without further preparation, the filters were then detected with NBT/BCIP allowing a coloured precipitate to form on the membranes (Figure 1(c)) [Figure 7]. Although not as sensitive as the CDP-Star, the coloured spots were very well contained and allowed gross, visual analysis of the filter as well as confirmation of the results obtained with the CDP-Star. Identification of the spots as to their representative gene/EST, was possibly easier on a direct visual basis, using the colour precipitate than either CDP-Star or radioactivity. By scanning the NBT/BCIP image on an inexpensive, conventional, flatbed scanner (ScanMagic 600CP) at 800dpi we were able to generate a 8-bit grayscale image of this result and also analyse it with the Pathways program.

If alkaline labile DIG dUTP was used to label the cDNA, then stripping of the GENEFILTERS could be carried out by rinsing the membrane briefly in sterile, redistilled H_2O, two washes for 15 min at 37°C in 0.2M NaOH, 0.1% SDS and a final rinse for 5 min in 2x SSC (as per the Manufacturer's instructions for alkaline labile DIG-dUTP). The membranes could be stripped efficiently and reprobed, provided that they were never dried to completion during the entire procedure. Prehybridisation and hybridisation with the next labelled cDNA could then be carried out. A colour precipitate present on the membrane could easily be stripped off first using dimethylformamide (50-60°C) as recommended by Roche.

Results

mRNA extracted from leukaemic and normal individuals, was reverse transcribed and labelled with DIG-dUTP (Roche), and hybridised to the GENEFILTERS (Research

Genetics). Using anti-DIG alkaline phosphatase (Roche) and CDP-Star (Roche), the resulting chemiluminescent signal was detected using a Lumi-Imager (Roche) available to us (Figure 1(a)) [Figure 7], and recorded in a 16 bit Tiff format. Further processing of the data was then carried out with the Pathways™ (Research Genetics) software allowing single and multiple filter analysis (Figure 1(b)) [Figure 7]. The images obtained with chemi-luminescence were more than satisfactory and allowed complete filter analysis to the full extent of the software. In addition, the filters were then directly detected with NBT/BCIP (Roche) and neat, contained, coloured spots were obtained (Figure 1(c)) [Figure 7]. This allowed immediate analysis of the membrane without the use of any instrumentation and furth(lirect visual verification of the results obtained with chemiluminescence. Method reproducibility is usually illustrated by repeated results on a single variable (in this case gene expression) for the same sample. This is not only costly but also unnecessary, since each membrane evaluates > 4000 genes at once, and a direct comparison of signal intensities between two membranes analysing the same sample would illustrate method reproducibility with the NBT/BCIP approach. Signal intensities showed good correlation ($r = 0.906$) between 100 randomly chosen paired data points across the membranes. The Bland-Altman plot in Figure 2 [Figure 8], however, best illustrates direct membrane comparison, showing 95% data points between the lines of agreement. Direct method comparison can further be highlighted by the ratio value (equal to 1) generated by the Pathways™ software. The signal intensity ratio between the two membranes ranges from –1.33 to 1.24 emphasizing their similarity and therefore method reproducibility.

Discussion

Phosphor imagers cost between $ 20 000 - $65 000 each, and as such are financially out of the reach of many laboratories especially in the developing world. Using a non-radioactive labelling and detection method for the GENEFILTERS has enabled the cost-effective use of array technology in our laboratory. Without this innovation, use of this modern technology to its fullest potential would have been out of our reach. In addition, the use of a colour detection system allowed direct analysis of the membrane, and further confirmed the chemiluminescent data.

Detection limits for DIG-labelled probes have been reported to be equivalent to those for radioactively labelled probes under optimised conditions (7) [84]. According to the manufacturers, immediately after the substrate addition, CDP-Star generates a luminescent signal of an approx. 10-fold increased sensitivity, compared to other chemiluminescent substrates, the NBT/BCIP however is less sensitive. Advantages of using a non-radioactive system include avoidance of using radioactive labels, ease of probe preparation, extended storage and multiple use of the same probe.

In summary, by replacing the radioactivity used in the protocol recommended by the manufacturers of the GENEFILTERS, with DIG-dUTP and subsequently detecting the bound probe by chemiluminesence and/or a colour reaction, we were able to utilise these high density filters effectively. Should such filters be used for the routine diagnostic analysis of expression profiles, the inherent advantages and cost effectiveness of a non-radioactive system in such a facility would obviously be realised.

Acknowledgements:

Roche, South Africa and Germany for valuable technical advice on the use of the Lumi-Imager and the DIG non-radioactive systems.

SAIMR for funding.

(a)

(b)

(c)

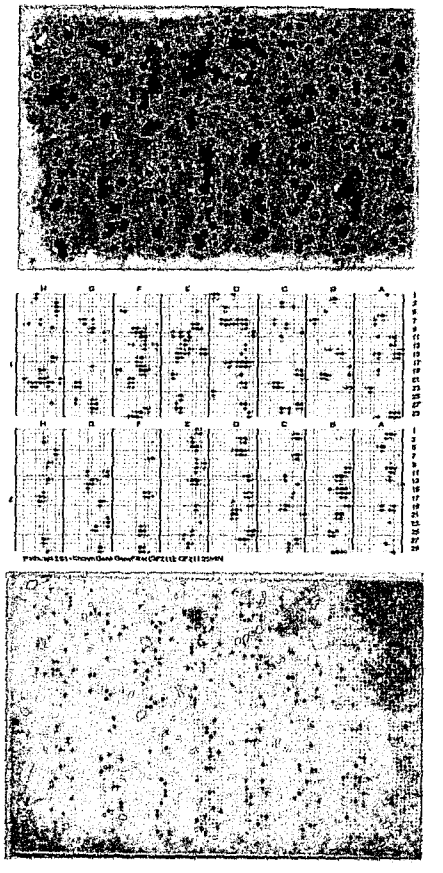

Figure 7: The figures show a GENEFILTER (GF 211) depicting the mRNA expression profile of a sample labelled with Digoxigenin. The CDP-Star chemiluminescent output was detected on a Lumi-Imager™ and image (a) was produced. This image was then processed using Pathways software to produce a computer-generated synthetic image (b). Finally the GENEFILTER was processed with NBT/BCIP colour reaction to generate image (c), which produced neat, contained dots that could either be analysed visually or with Pathways, and either way confirmed that the results obtained in (a) were correctly aligned. The latter is definitely an advantage not yet obtained by radioactive or non-radioactive means.

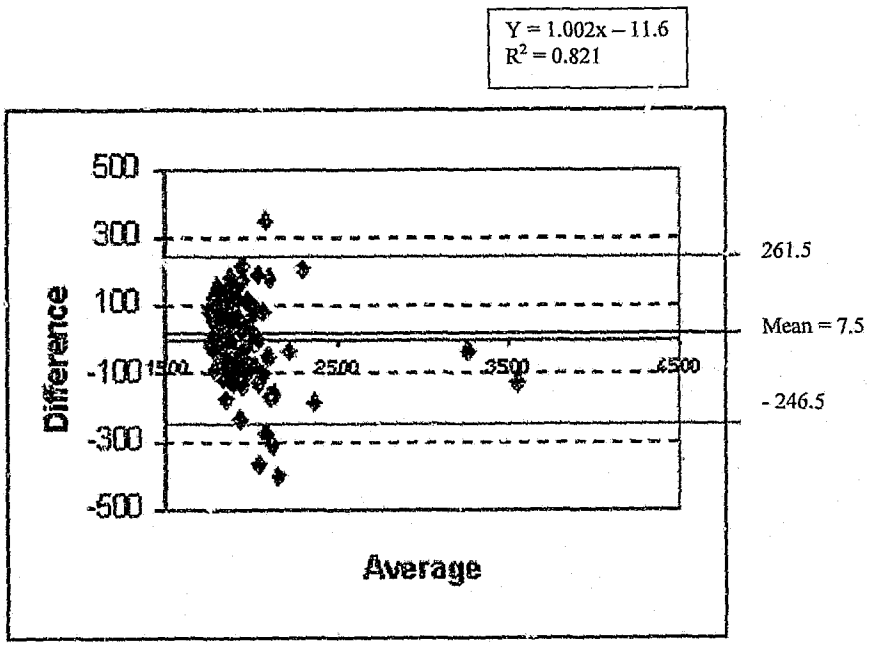

Figure 8: A Bland-Altman plot showing lines of agreement to compare the signal intensities from two GENEFILTERS.

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2.3.2 Article in newsletter

Research Y2K: Gene Expression arrays, an Application Note

GENEFILTERS® Microarrays and Pathways™ Analysis Software

Rusla M. Dubreuil Lastrucci

Research and Development Programme

Dept. Haematology, School of Pathology

Faculty of Health Sciences

University of the Witwatersrand and S.A.I.M.R.

Arrays advance technology. In recent history molecular biology has been characterized by quantum technological leaps affecting most areas of scientific research. Additionally, it has paved the way for a new dimension in our understanding of the molecular mechanisms underlying many biological processes. This new era of technological explosion has also touched the biomedical sciences with major impacts on anatomy, physiology and pathology. These new insights in turn are essential prerequisites for radically new conceptual approaches in the diagnosis and treatment of patients.

Historical Background. With their conceptual beginnings a quarter of a century ago in Edwin Southern's laboratory, gene arrays have taken the scientific world by storm. The use of array technology for gene expression profiling has rapidly become an important method for associating changes in gene expression with cellular phenotypes.

Large Scale Research Capability. The advancing field of microarray technology offers the first opportunity of providing a systematic approach to surveying RNA variation or RNA expression profiles in a parallel fashion on a large scale. Recent literature articles suggest that they may become standard tools in molecular research as well as in the clinical diagnostics arena.

Types of Arrays. Microarrays come in several different forms and are available for

gene expression and analysis or actual DNA sequence analysis. The most familiar types are those on glass slides (called gene 'chips') and those on nylon membranes, such as Research Genetics' GENEFILTERS® microarrays. Any standard molecular laboratory with existing expertise and very few additional purchases can use GENEFILTERS® microarrays.

Simple Protocol with Enormous Output. The initial profiling experiments using microarrays compare two samples (control versus test) identifying increased or decreased gene expression, and already the magnitude of the results is staggering. The database grows upon the addition of multiple time points, many types of cells and various additives. Thus an interface between computational and biological sciences is obviously a necessity in order for the initial analysis and [illegible] management of these expansive results to be administered effectively.

Cost Effective! Arrays offer a cost effective and optimized method for achieving insight into what genes are expressed or not in neoplasia, inherited disorders, and infectious disease (to name but a few). Workers have shown that there are distinct patterns of gene expression amongst the leukaemias.

Future Potential. The value of such information is phenomenal, not only as a tool to distinguish between various related disease entities by virtue of their distinct expression patterns; but a few diagnostic, prognostic and therapy related genes could potentially be spotted onto a custom made, disease-specific array, and can be used to routinely evaluate disease at the clinical level.

GENEFILTERS microarrays have been used in our laboratory to study:

1. Comparison of differential gene expression in leukemias such as CLL (chronic lymphocytic leukemia), with a view to use defined expression patterns in the diagnosis, prognosis and response to therapy of these patients.
2. Functional T-lymphocyte biology with regards to gene expression profiles and the changes that occur with HIV infection and disease progression that may result in their ultimate depletion.
3. Toxicological changes that may occur in various cell populations in response to certain herbal and traditional remedies administered to patients.
4. Changes in gene expression in hemopoietic cells related to their growth on an extracellular matrix derived from an adenocarcinoma.
5. Gene expression profiles of cell populations in patients with certain types of dementia as compared to control individuals.

2.4 PCR for B-cell (Immunoglobulin) gene rearrangements

No section on the molecular diagnostics of neoplastic disease is complete without dealing with this particular corner stone in the detection of clonal expansion. Immunoglobulin gene rearrangements were initially, and currently are still, carried out using standard Southern blotting [85], in fact many first world laboratories still use it routinely. PCR detection of immunoglobulin rearrangements is now frequently used, and although not the most straightforward of PCR methodologies, it goes a long way to illustrate how a general molecular diagnostic tool can be used very effectively indeed.

2.4.1 Introduction

Lymphocytes are unique cells in that they, unlike other somatic cells, physiologically and not pathologically, can alter the sequence of their immunoglobulin or T-cell receptor genes [52]. This is the process by which molecules of sufficient diversity are generated to enable recognition of the many antigens to which the body may be exposed. Rearrangement of the various IgH gene V (variable), D (diverse), J (joining) segments occurs in all B-cells, yielding rearranged genes which are still further varied in size due to various mechanisms including the addition of random nucleotides by terminal deoxy transferase (TdT) at the junctions. When PCR is performed using primers in consensus regions on these polyclonal populations, a smear of the many different size products is produced [11 21].

As B-cells from a clone all have the same size rearranged fragment, PCR products from these monoclonal populations appear as distinct bands ('clonal marker'). Normal and monoclonal B-cell populations can be readily amplified with consensus IgH region (VH) framework directed primers (FR 1, 2 or 3), along with a consensus IgH J-region (JH) primer. The amplification products are small, in the region of 80-120 base pairs, depending on the exact primer pair (see abstract 2.4.5) so that even highly degraded samples and archival material are suitable for analysis. PCR products are electrophoresed to distinguish polyclonal populations that appear as ill-defined smears on the gel and clonal populations that appear as narrow intense bands [11 20 33].

2.4.2 Clinical significance

The IgH gene rearranges earliest in B-cell ontogeny and is accordingly the most valuable locus to demonstrate clonality in B-cells. Approximately 98% of all B ALL (null ALL, common ALL, and pre-B ALL) and all mature B-cell malignancies have rearranged their IgH genes. Evaluation of IgH rearrangements helps to confirm clonality in the absence of more specific clonal markers or in surface immunoglobulin-negative tumours [33]. The disadvantage of this PCR is that it is necessary to detect a clonal population within a background of normal cells and their associated range of fragment sizes, if the clonal population is small it may be lost in the polyclonal background. In addition to this, documented primer sets although based on consensus regions, are at best a compromise to the extensive variability in the rearranged immunoglobulin gene. Thus the particular primer set being used may miss a particular rearrangement. Reed et al [86], using a semi-nested FR3/JH assay were able to demonstrate one or two dominant bands indicating a

clonal population, in 15 of the 23 cases (65%) of B-cell lymphomas investigated. The primers that are currently used were published by Potter *et al* [87] (Table 4). As an internal control for failed PCR the β-Globin gene was also amplified in each tube.

2.4.3 Materials and methods

2.4.3.1 DNA extraction

DNA was extracted from flow cytometric suspensions of patients and normal individuals using the method described by Talmud *et al* [88]. Four hundred microlitres of 0.17 M ammonium chloride was added to 100µl of the specimen. This was mixed well by inversion and then left at room temperature for 20 minutes before being spun down in a microcentrifuge for 30 seconds. The supernatant was then discarded and the pellet was resuspended in 200µl 0.05 M sodium hydroxide. This was boiled for 10 minutes and neutralised by the addition of 25µl 1 M Tris HCl (pH 8.0). The extracted DNA was quantitated using a spectrophotometer and stored at -20°C until further analysis.

2.4.3.2 Amplification

PCR amplification was carried out using the forward and reverse primers FR3 and LJH for the IgH gene and G gamma F and R for the β-Globin gene (see Table 4). Each 50 µl reaction contained approximately 0.5 µg genomic DNA, 5 pmoles of each primer, 1.5mM $MgCl_2$, 200µM of each dNTP and 1.25 U Taq DNA polymerase (PCR Core Kit, Roche). A first denaturation step at 94°C for 7 minutes was followed by 30 cycles of 94°C for 1 minute, 57°C for 1 minute and 72°C for 30 seconds. The VLJH (5 pmoles) nested primer was then

added to the PCR tube, and the mixture then placed for a further 10 cycles of 94°C for 1 minute, 56°C for 1 minute and 72°C for 30 seconds. Thirty microlitres of PCR product from each sample was visualised on a 3% agarose gel.

The criteria for scoring a positive result (i.e. the presence of a monoclonal population) with the PCR technique were as follows:

- Bands were taken as no more than 1mm in width with a sharp edge.
- Bands had to be within the expected size range.
- An empty lane without background smear or primer-dimer artefact was regarded as a failed PCR rather than a true negative and repeated.
- Polyclonality was reflected as a smear.
- The internal PCR control product (323 bp), β-globin (G gamma), was visible.

Table 4: Nucleotide sequences of the primers used in the immunoglobulin gene rearrangement PCR [87]

	Primer Sequence
FR3	5' aca cgg cYS tgt att act gt 3'
LJH	5' tga gga gac ggt gac c 3'
VLJH	5' gtg acc agg gtn cct tgg ccc cag 3'
G gamma F [89]	5' agt gct gca aga aga aca act acc 3'
G gamma R	5' ctc tgc atc atg gtc act gag ctc 3'

Y = c/t S = g/c

2.4.4 Results and discussion

In Figure 9, the results of the PCR analysis can be seen. In lanes (i) and (vi) is a 100 bp molecular weight marker (Roche), lane (ii) depicts a patient with a monoclonal band and lane (iii) a polyclonal or negative result. Lane (iv) is the positive control and lane (v) is the reagent blank. These results clearly show the results expected with various populations of cells. The 323 bp internal control PCR product (β-globin) is visible in lanes (ii) and (iv).

IgH gene rearrangement studies are useful in the detection of a monoclonal population of cells in B-cell malignancies and is used routinely in the PCR laboratory in conjunction with and in the absence of other more specific clonal markers.

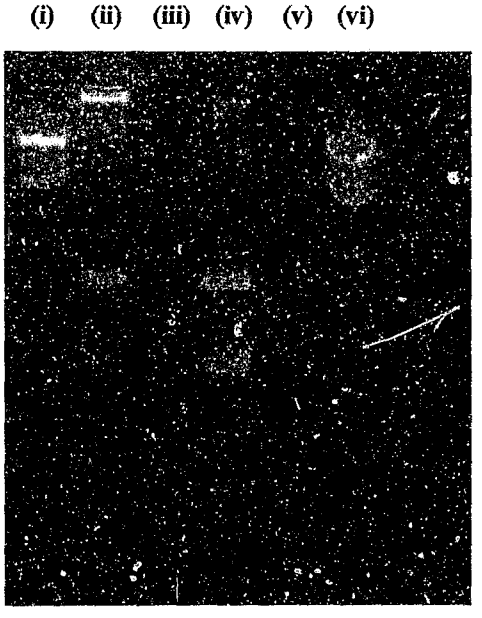

Figure 9: A 3% agarose gel depicting an immunoglobulin gene rearrangement PCR. Lanes (i) and (vi) show a 100 bp molecular weight marker (Roche), lane (ii) depicts a patient with a monoclonal band and lane (iii) a polyclonal or negative result. Lane (iv) is the positive control and lane (v) is the reagent blank. The 323 bp internal control PCR product (β-globin) is visible in lanes (ii) and (iv).

2.4.5 Immunoglobulin Gene PCR: Diagnostic Application and Interpretation (abstract)

- Abstract: N. Sioutos, A. Bagg, R. Dubreuil Lastrucci*, W.C. Pugh, J. Locker, J. Cossman. Georgetown University of Washington D.C., USA, *University of the Witwatersrand, Johannesburg South Africa. M.D. Anderson Cancer Centre, Houston TX, University of Pittsburgh, PA.

International Academy of Pathology, San Francisco, March 1994.

(Appendix 1.6)

2.5 Oligo extension in follicular lymphoma, t(14;18), bcl2/Jh fusion

- Journal Article and citation: Extension of a Cold - Labelled Oligoprobe for the Analysis of PCR Products.

Rusla M Dubreuil Lastrucci, Wendy S Stevens, Barry V Mendelow.

Technical Tips Online. 5/8/98. http://tto.trends.com.

Cited in: Trends in Cell Biology, 1998, 8, 387

- Abstract: Extension of a Cold Labelled Oligoprobe to Analyse Polymerase Chain Reaction Products.

RMD Lastrucci, W Stevens, BV Mendelow.

Outreach into Africa, Federation of South African Societies of Pathology Congress, Cape, July, 1997.

(Appendix 1.7)

2.5.1 Journal Article

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5/8/1998

Extension of a cold-labelled oligoprobe for the analysis of PCR products

Author:

Rusla M. Dubreuil Lastrucci, Wendy S. Stevens, Barry V. Mendelow

Affiliations

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DNA fragment size is often not sufficient to confirm the legitimacy of a PCR product, especially when there is no expectation of specific size but rather of a size range. Further manipulation of the PCR product, such as nuclease restriction or secondary PCR, can be used to confirm that the observed PCR product is real. One approach to the definitive analysis of PCR products is the traditional 'Hot Blot' described by Parker *et al.* (Ref. 1) [90]. In this study we describe a new method which achieves the same objective, but without the use of radioactive probes. DNA was extracted from patients with follicular

lymphoma, as an example, and PCR was performed using primers designed to amplify the t(14;18) translocation characteristic of this disease (Ref. 2) [91]. An oligonucleotide probe for the major breakpoint cluster (mbr) region of *bcl2* was end-labelled with biotin and used to probe the PCR product. The product was run on an electrophoretic gel, electroblotted onto a nylon membrane and detected using streptavidin alkaline phosphatase. A positive result was interpreted if the oligonucleotide probe was able to bind internally to the PCR product and was extended by the Taq polymerase, thus incorporating the gold label into the PCR product. It was found that this method was able to successfully distinguish between legitimate and illegitimate product bands.

Protocol

Template isolation

DNA was extracted from paraffin embedded tissue, blood or marrow of patients with Follicular lymphoma and normal controls according to standard methods (Ref. 3, 4) [92 27]. The oligonucleotides used are described in (Table1) [Table 5].

PCR procedure

A 35-cycle amplification was carried out at 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute, followed by a final elongation at 72°C for 10 minutes using a PCR core kit (Boehringer Mannheim), according to the manufacturer's instructions.

Standard product analysis

Amplification products were electrophoresed in a 2% agarose gel, stained with ethidium bromide and visualised on a UV transilluminator.

Oligoprobe extension (Ref. 1) [90]

Fifteen microliters of PCR product was added to 50-100 ng of labelled oligonucleotide probe, together with an additional 0.5 µl buffer/$MgCl_2$, 0.3 units Taq DNA polymerase and 0.6 µl of dNTPs, in 20 µl total volume. This mixture was carried through 1 or 2 PCR cycles of 98°C for 1 minute, 55°C for 1 minute and 72°C for 3 minutes.

The appropriate known positive and negative controls, as well as a reagent blank, were incorporated in each PCR run. The products of the extension reaction were electrophoresed as before. A molecular-weight ladder molecular weight marker XIV (Boehringer Mannheim)] was run on each gel to enable accurate size determination. The gel was then visualized, its dimensions measured and transferred onto a positively charged nylon membrane (Boehringer Mannheim), using a Trans-Blot Semi-dry transfer cell (Bio-Rad), or conventional Southern blotting (Ref. 5) [24]. The blotted gel and the membrane were checked on the UV transilluminator for efficient transfer and the membrane was air-dried and fixed by exposure to UV for 45 seconds.

Immunological detection of extended oligonucleotide probe

Standard protocols enclosed in the DIG Nucleic Acid Detection Kit (Boehringer Mannheim) were followed, except for the replacement of anti-digoxigenin Fab-fragments conjugated with alkaline phosphatase by streptavidin alkaline phosphatase conjugates (Boehringer Mannheim), where appropriate. The nylon membrane was washed, blocked and incubated with the appropriate alkaline phosphatase conjugate. The membrane was again washed and subsequently incubated with NBT/BCIP, producing an enzyme-catalyzed, insoluble, coloured precipitate.

The oligonucleotide extension method showed a high degree of specificity, as certain bands failed to produce a result post extension. The agarose gel illustrated in (Fig. 1) [Figure 10] shows an example of a positive reaction in a characteristic follicular lymphoma, with negative reactions in other lymphomas and the negative control. Experiments conducted to optimize the oligonucleotide extension protocol (agarose gel and blots not shown) demonstrated the following. (a) Comparison of the two different annealing temperatures, of 43°C and 55°C, during the oligonucleotide extension, showed no difference. The latter temperature was subsequently used as it afforded a higher specificity. The sensitivity of the technique proved to be good, as faint positive bands on PCR became more clearly positive after oligonucleotide extension. (b) The use of two oligonucleotide extension cycles, instead of one, showed no apparent increase in signal. Any more than two cycles is unadvisable because of the possible incorporation of non-specificity at this stage. (c) The use of capillary blotting, instead of electroblotting, showed no significant difference in signal on the nylon membrane. (d) Although the method required the addition of more Taq, nucleotides and buffer, we tried to ascertain whether it would still be possible to carry out the oligonucleotide extension, relying on residual reagents present from the amplification, if only the labelled oligoprobe was added. It was found that although oligonucleotide extension did occur, it was more efficient when oligonucleotide extension was performed sooner after amplification. Optimal oligonucleotide extension was, however, still obtained when fresh reagents were added.

This technique allowed oligonucleotide extension and detection, without the use of radioactivity, quickly and easily. Ample labelled probe was obtainable. This could be stored indefinitely, without requiring repeated and expensive probe labelling. The method required that the molecules be transferred to a gel before detection; transfer of the attached digoxigenin and biotin moieties appeared to present no problem, and was quickly and easily achieved. The method still has the advantage of fragment size as a crosscheck, unlike some of the techniques that rely exclusively on the presence of a signal.

In short, this method was found to successfully and repeatedly distinguish between legitimate and illegitimate PCR product bands, in a simple, safe and easily executable manner, rendering PCR results reliable. Studies are under way to further explore the specificity of this technique for variants of the t(14;18) and various other translocations.

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Table 5: The primers used in this study spanned the t(14;18) translocation and an amplicon size of 80-300 bp was expected (Ref. 2) [91]

Primer	**Sequence**
Jha - Jh consensus sequence	5' acc tga gga gac ggt gac c 3'
mbr - major breakpoint region	5' gag ttg ctt tac gtg gcc tg 3'
Probe - *bcl2* homologous	5' biotin-gcc tgt ttc aac aca gac c 3'

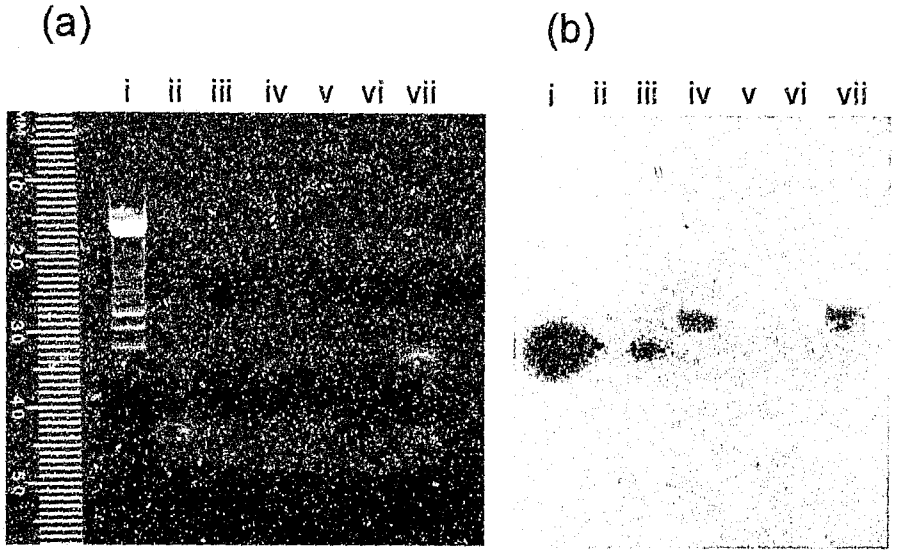

Figure 10: Agarose gel (a) and oligonucleotide extension blot (b). (i) Molecular weight ladder, (ii) marrow infiltrated by diffuse large cell lymphoma, (iii) marrow-derived $CD5^{+}CD19^{+}$ cells (probable mantle-cell lymphoma), (iv) lymph-node tissue derived from a patient with lymphadenopathy of undetermined aetiology, (v) normal DNA, (vi) reagent blank, (vii) characteristic follicular lymphoma. As a result of this analysis, lane (iv) was interpreted as follicular lymphoma.

PRODUCTS USED

positively charged nylon membrane	from Boehringer Mannheim
Trans-Blot Semi-dry transfer cell	from Bio-Rad
molecular weight marker XIV	from Boehringer Mannheim
DIG Nucleic Acid Detection Kit	from Boehringer Mannheim
streptavidin alkaline phosphatase conjugates	from Boehringer Mannheim
PCR core kit	from Boehringer Mannheim

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(top of page)

2.6 Boolean principles to identify chromosomal translocations

- Abstract: Identification of cancer associated chromosomal translocations using differentially labelled chromosomes and Boolean logic.

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*Sanger Centre, Wellcome Trust Genome Campus, UK. American Society of Hematology, 39th Annual Meeting & Exposition, December 1997, San Diego CA, USA.

Abstract Published: Blood, November, 1997, 90 (10) supplement 1, 217b.

(Appendix 1.8)

2.6.1 Introduction

2.6.1.1 Chromosomal breakpoints

Breakpoints in chromosomes, the evidence of which are deletions, inversions and translocations, have highlighted the role of genes in neoplasia. It was not long after workers visualised these breakpoints that they identified non-random associations between these microscopic lesions and specific disease phenotypes. With the advent of molecular methodology, the molecular makeup of these breakpoints was elucidated and the specific genes resulting in a particular disease phenotype, identified. The harbinger of these phenotypic, microscopic and molecular associations was chronic myeloid leukaemia (CML), the Philadelphia chromosome (Ph), and the BCR/abl gene fusion. The Ph chromosome in CML was found to involve a reciprocal translocation t(9;22)(q34;q11), bringing the 3' c-abl proto-oncogene sequences of chromosome 9 adjacent to the 5' sequences of the 5.8 kb breakpoint cluster region (BCR) on chromosome 22. This hybrid gene transcribes into two varieties of 8.5 kb chimeric BCR-abl mRNA which differ by 75 bp. These in turn both translate into a chimeric 210 kd-protein product that is considered essential to the pathogenesis of CML [93 94 95]. As CML progresses, further chromosomal abnormalities often arise and particularly the occurrence of trisomy 8 has been non-randomly associated with blastic transformation in CML [53 96].

The Department of Molecular Medicine and Haematology has been involved in the further collection of cytogenetic (classical and FISH) data from well known breakpoints,

as well as original contributions to identify new non-random associations. Examples are work carried out by Rosendorff *et al* in the chromosomal localisation of c-*mos* [97] in acute nonlymphocytic leukaemia; Keene *et al* [98] identifying translocations involving 12p13 associated with malignant eosinophilia; and Bernstein *et al* who described inversions and translocations involving 3q21 in CML and ANLL associated with hyperactive thrombopoiesis [99 100], as well as describing variant t(9;22), t(8;21) and t(15;17) translocations in the local population [61 62 101 102]. These publications serve to illustrate the tremendous amount of work, such as chromosome walking, DNA library generation, cloning and sequencing, involved in successfully generating information on these molecular lesions. However, the resources that were required to generate this data are no longer accessible and available to continue this kind of health care and research in the Department.

The mechanism underlying the translocation phenomenon is often not clear, but there are hints as to what might be precipitating factors. Certainly, in any of the translocations involving the immunoglobulin genes (chromosome 14), it appears that the 'controlled promiscuity' that allows for IgH gene rearrangement itself sometimes accidentally incorporates another partner in the form of an oncogene (e.g.: t(14;18) – bcl2, t(11;14) – cyclin D1, and t(8;14) – myc) [11 33]. Workers have also speculated chromosome fragility, *alu* recognition sequences, topoisomerase recognition sites and spatial dynamics [33]. Although we have come a long way there still remain many translocations that need to be detected or that need further characterisation.

The processes currently involved in the detection of a translocation are demonstrated below when a CML patient was analysed cytogenetically and molecularly for the presence of the t(9;22) translocation and other chromosomal abnormalities which are definitive of this disease and its progression. Patient bone marrow or peripheral blood samples were obtained for mononuclear white blood cells and separated on Ficoll/Hypaque. Chromosomal preparations were made from bone marrow and peripheral blood cell cultures. Standard GTG banding techniques were carried out and the karyotypes analysed microscopically [103] (Figure 11). FISH, to quantitate the number of chromosome 8 copies present in the cells, was carried out using a chromosome 8 library when standard cytogenetic results were inconclusive (Figure 12) [104]. Molecular analysis was carried out using RT-PCR (Figure 13) on RNA extracted from patient white blood cells and stored in guanidium isothiocyanate [77].

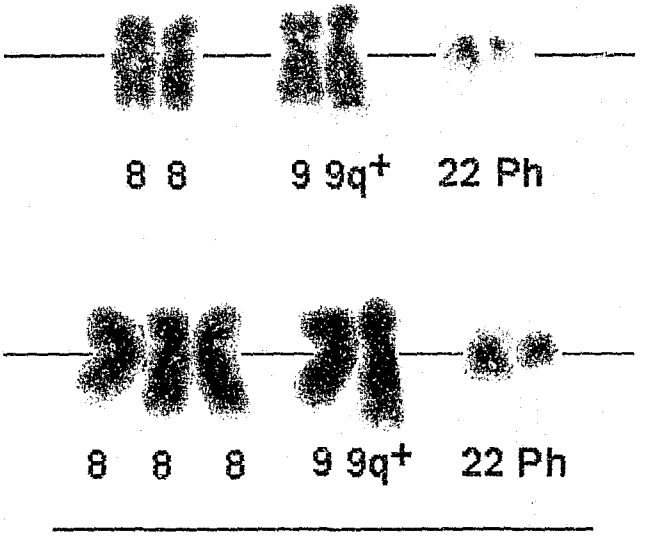
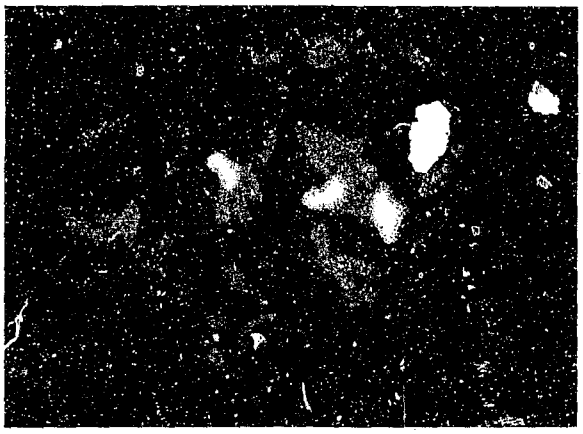


Figure 12: GTG banding of a bone marrow specimen obtained from a patient showing both a Ph^+ (a) and a Ph^+, +8 clone (b).

(a)



(b)

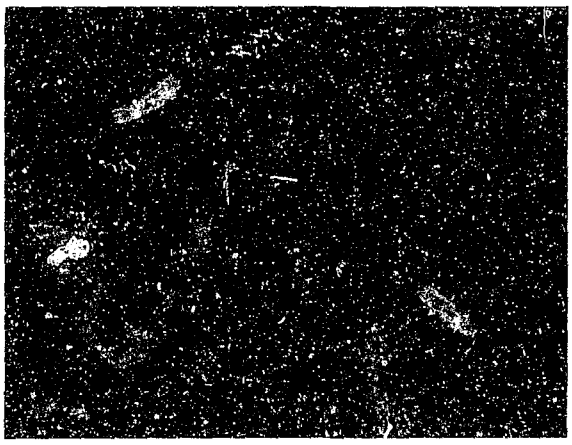


Figure 12: FISH for chromosome 8 showing (a) trisomy 8 in a patient (b) and two chromosome 8's in a normal control. The chromosome 8 library was labelled with biotin and detected using fluorescein-conjugated avidin.

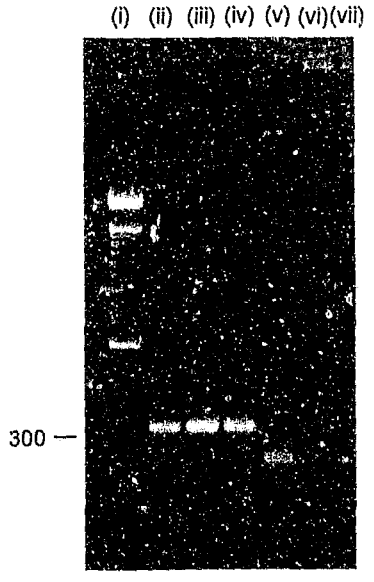


Figure 13: A 2% agarose gel depicting RT-PCR products. Lane (i) 100 bp molecular weight ladder; (ii) a CML patient with both sizes of message; (iii) a patient with the larger (325 bp) message; (iv) positive control for larger (325 bp) message; (v) positive control for smaller (250 bp) message; (vi) negative control (mRNA from normal peripheral blood); (vii) blank (no mRNA).

[RT-PCR was performed to detect the BCR-abl translocation using a downstream abl primer (5' tgt gat tat agc cta aga ccc gga g 3') and a upstream BCR primer (5' gtg aaa ctc cag act gtc cac agc a 3')[105]. Amplification of the chimeric BCR-abl mRNA resulted in one or two sizes (325 bp and 250 bp) of product.]

2.6.1.2 Problems and shortfalls of classical cytogenetics, FISH and PCR

Classical, banded metaphase cytogenetics can still have an advantage over the newer technologies, in that all gross chromosomal aberrations can be seen at one time. This is of course dependent on the quality of the metaphase, which is often sub-optimal with leukaemias (as can also be seen in Figure 12, in the difference in quality between the normal and the CML). Certain chromosomal aberrations are too small or obscure to be picked up using conventional cytogenetics. Microscopically identified breakpoints do not supply sufficient information as to which genes or regulatory elements are involved at the molecular level, and identification of the molecular pathology often involves a vast amount of work. Solid tumours are only now being successfully processed in the type of cell culture and generation of metaphase and interphase nuclei needed for cytogenetics and FISH to be carried out, and thus only now are the involved translocations being identified. FISH and PCR based methods rely on prior knowledge of what the disorder may be in order to choose relevant PCR primers or FISH probes with which to further carry out the diagnosis. Multicolour FISH, although detecting many more chromosomes in one metaphase, requires many expensive probes. Cytogenetics and FISH, are labour intensive and relatively expensive technologies (section 1.2.3), are not appropriate for the widespread detection of translocations in a developing country.

Apart from relying on some indication as to what the molecular lesion may be, in order to choose the correct primers, PCR does have other limitations. The majority of PCR assays used to detect chromosomal translocations are RT-PCR based, due to the large introns that

are involved in the breakpoints [33]. This is acceptable as long as the sample is fresh, however most incorrectly stored or archival material precludes the use of RT-PCR. DNA PCR (and sometimes RT-PCR), where used for translocations, often does not include the breakpoints that occur outside of the known breakpoint cluster regions, and thus only detects a percentage of the translocations. For example, in the t(14;18) major breakpoint cluster region, only 80% of the translocations fall into the range of these primers [106].

Thus a cost effective method capable of detecting **any** unspecified translocation on the molecular level would be of use both diagnostically and in the research arena, especially if such a technique is placed in a user-friendly array format that would require minimal skill to interpret the results.

2.6.1.3 Definition of Boolean principles

George Boole (1815-1864) was a British mathematician and logician, who developed Boolean algebra. In Boolean algebra, logical propositions are denoted by symbols and can be acted on by abstract mathematical operators that correspond to the laws of logic. Boolean algebra is of prime importance in the study of pure mathematics and in the design of modern computers [107]. Boolean Algebra is concerned with propositions and their *truth-values* rather than variables and their numerical values. The elements that are contained in a 'set' in Boolean algebra may be abstract objects, or concrete things such as numbers, propositions, or electrical networks. Boole originally defined the elements

of a Boolean algebra as a collection of propositions, or simple declarative sentences having the property that they were either true or false but not both. The operations were essentially conjunction and disjunction. If x and y represent two propositions, then the expression "x *or* y" would be true if and only if either x or y or both were true. The statement "x *and* y" would be true if and only if both x and y were true. In this type of Boolean algebra, the complement of an element or proposition is simply the negation of the statement (*not*). For example, let p be the statement "The object is blue", and let P be the set of all elements for which the statement p is true, that is, the set of all blue objects. P is called the *truth set* for the proposition p. Let q be the statement "The object is red", and let Q be the set of all elements for which the statement q is true, that is, th set of all red objects. Q is called the *truth set* for the proposition q. The Boolean operatives *and*, *or* and *not* are illustrated in Figure 14 as the following statements: (a) blue objects; (b) red objects; (c) blue *and* red objects; (d) blue *or* red objects; (e) blue *and not* red objects; (f) red *and not* blue objects.

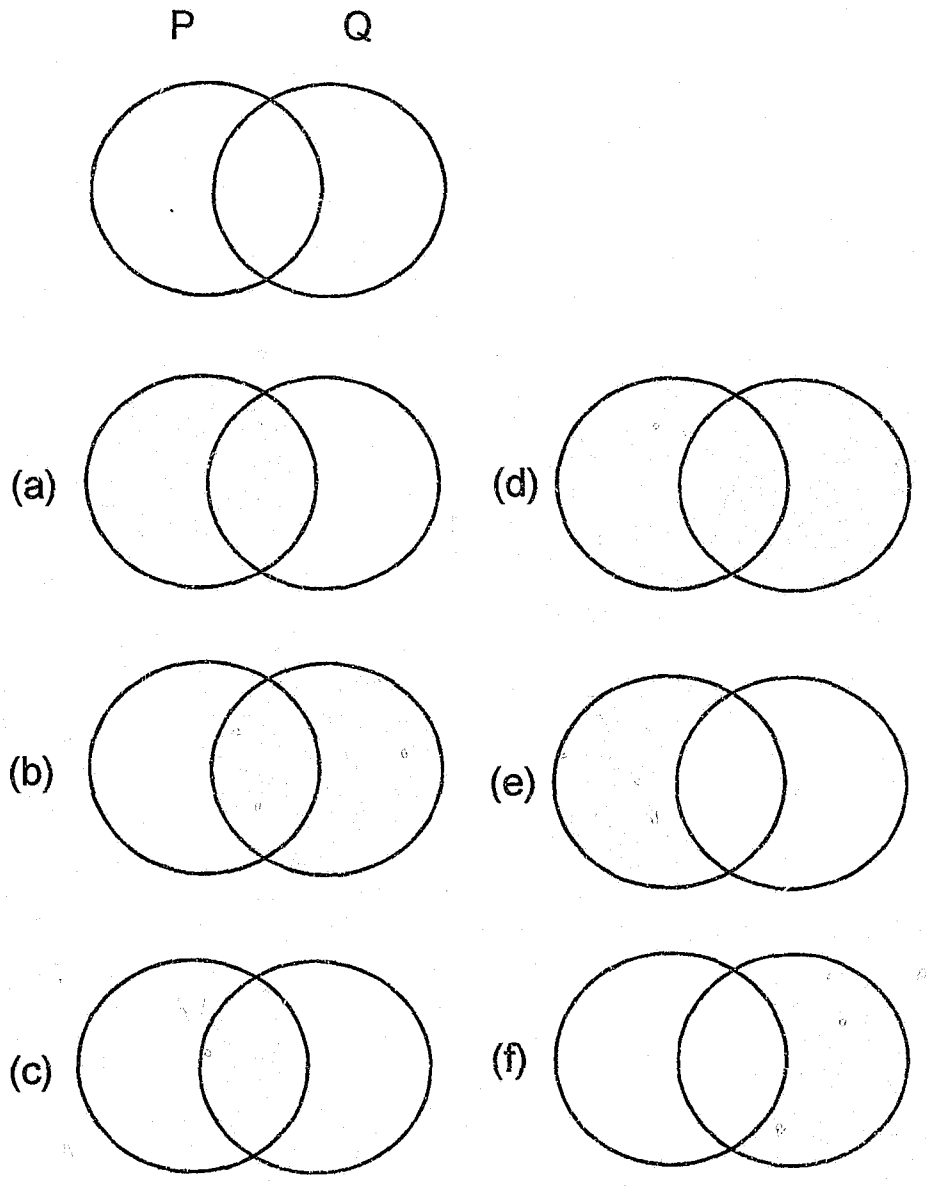


Figure 14: Boolean principles of logic

2.6.1.4 Experimental Logic

The main objective of this project was to develop a molecular technology that would facilitate the molecular identification and characterisation of chromosomal breakpoints involved in cancer. The methodology used makes use of Boolean logic. If, to further the analogy in 2.6.1.3, there were two 'sets' of molecules, one labelled blue and the other labelled red, any molecule that we discovered that was both blue *and* red (Figure 14 (c)) would be a mixture (chimera) of both molecules.

It is possible using nucleic acid labelling technology to label molecules with different moieties such as biotin or DIG (Roche). Were chromosome A labelled with biotin and chromosome B with DIG, then any fragment of DNA or RNA presenting both biotin *and* DIG labels could be assumed to be a hybrid molecule between chromosomes A *and* B, and thus by conventional wisdom and definition, a **translocation** between chromosomes A *and* B.

2.6.1.5 Conceptual outline of protocol

In order to demonstrate the feasibility of the Boolean system's ability to detect a chromosomal translocation, it was decided to use follicular lymphoma patients known to have a translocation between chromosome 14 and chromosome 18, viz. t(14;18) translocation (bcl2/Jh fusion). Firstly, material from each chromosome had to be individually obtained either by employing a chromosome library, or from a preparation of flow sorted chromosomes. The latter method is advantageous as there would be no contaminating plasmid material and the entire chromosome would be represented. Due to

collaboration with Dr Nigel Carter, at the Sanger Centre, Cambridge, flow sorted chromosomes 14 and 18 were obtained from that facility. The chromosome DNA was either extracted from the relevant chromosome library or used directly in the form of flow sorted chromosomes. This chromosomal material was then labelled with either biotin-16-dUTP (biotin dUTP) or DIG-11-dUTP (DIG dUTP) using either DOP-PCR (Roche) or any other random PCR thereby incorporating the labelled dUTP.

Since the human genome contains 3-6% repetitive sequences, it becomes necessary to rid the chromosome preparations of these repetitive and common sequences. The former was achieved using Cot 1 DNA (Roche), as it represents all *alu* and *kpn* family sequences [108]. The Cot 1 DNA was immobilised on magnetic beads (Roche) and then used to extract the repetitive sequences in a solution of the chromosome preparations. What remained in the supernatant fluid (SNF) was representative of non-repetitive (*not* cot), labelled, chromosomal DNA (Figure 14 (e) and (f)).

The direct use of the labelled chromosome specific fragments as primers in a straightforward PCR reaction, allows the extension of these fragments across the breakpoint, if a translocation were present. If two different chromosomes were involved in the translocation, automatically the chimeric fragment of amplified DNA would be labelled with both labels. This fragment would then be isolated and/or detected by means of these two labels. When the two involved chromosomes have been identified, this technique could be carried still further to fully characterise and sequence the breakpoint.

In summary, conceptually this technique could be used to isolate new or variant translocations and to identify the genes involved in the malignancies.

2.6.2 Materials and methods

Figure 15 depicts a simple flow chart of the protocol outline, detailed diagrams appear latter to clarify individual sections.

2.6.2.1 Chromosomal material

2.6.2.1.1 Flow sorted chromosomes

Flow sorted chromosomes were obtained from Dr Nigel P Carter, Sanger Centre, Wellcome Trust Genome Campus, Cambridge, UK. There are approximately 500 chromosomes per 33µl, and 33µl per 500µl tube.

2.6.2.1.2 Plasmid preparations

Chromosome 14 and 18 libraries [109], were cultured overnight in 4-8 ml LB (Luria Broth) containing 50µg/ml ampicillin, at 37°C with agitation. The media were either inoculated from the agar plates or 15 % glycerol stocks using sterile techniques. The cells were pelleted in a microfuge (4°C) for 5 minutes. A High Pure Plasmid Isolation Kit (Roche) was used to isolate the plasmid DNA. The DNA was then stored at –20°C. A 5.6 kb immunoglobulin probe (Jh) [110 111], was also isolated in the above manner.

2.6.2.2 DOP PCR

DOP PCR (Roche kit) allows the statistical and representative amplification of an uncharacterised or unknown DNA template. The reaction utilises a universal primer possessing a 6-nucleotide long degenerate region (N^6) that statistically represents all possible combinations of 6 nucleotides. At the 3' end of the primer is a GC rich 6-nucleotide stretch, which theoretically occurs every 4 kb in the genome. This facilitates efficient primer hybridisation and start of the polymerisation reaction. Without these 3' nucleotides, the primer would bind to virtually every 6 nucleotides in the target, rendering the PCR reaction inefficient due to competition for space and reagents. At the 5' end of the DOP primer is a linker region containing a *Xho*I restriction site, for possible cloning purposes (Table 6). The kit protocol was followed. Low specificity primer annealing was achieved in the first 5 cycles of DOP PCR through amplification at a relatively low annealing temperature of 30°C. Subsequent to primer annealing the temperature was increased over a time range of 3 min to the polymerisation temperature of 72°C. The amplification products of these low specificity cycles have the DOP primer sequence incorporated at their respective termini that are then utilised in the subsequent, more specific thermo-cycles [112].

Table 6: Sequence of the DOP primers

Primer	Sequence
DOP (Roche kit)	5' ccg **act cga g**nn nnn nat gtg g 3'
DOP 2	5' t**gg cgg ccg c**nn nnn nac gtc g 3'

In order to perpetuate the Boolean logic on which this methodology is based and due to a concern that the DOP region may cross react between the two chromosomes at a later stage. A second, different DOP primer (DOP2 with a *Not* I restriction site) was designed and synthesised (Table 6). This was used on chromosome 18, while the commercial DOP primer was used on chromosome 14.

A second round of DOP PCR was used to incorporate biotin or DIG labels into the amplified chromosomes 14 and 18 respectively.

The resulting combinations were as follows:

Chromosome 14, amplified with DOP and labelled with biotin, and

Chromosome 18, amplified with DOP2 and labelled with DIG.

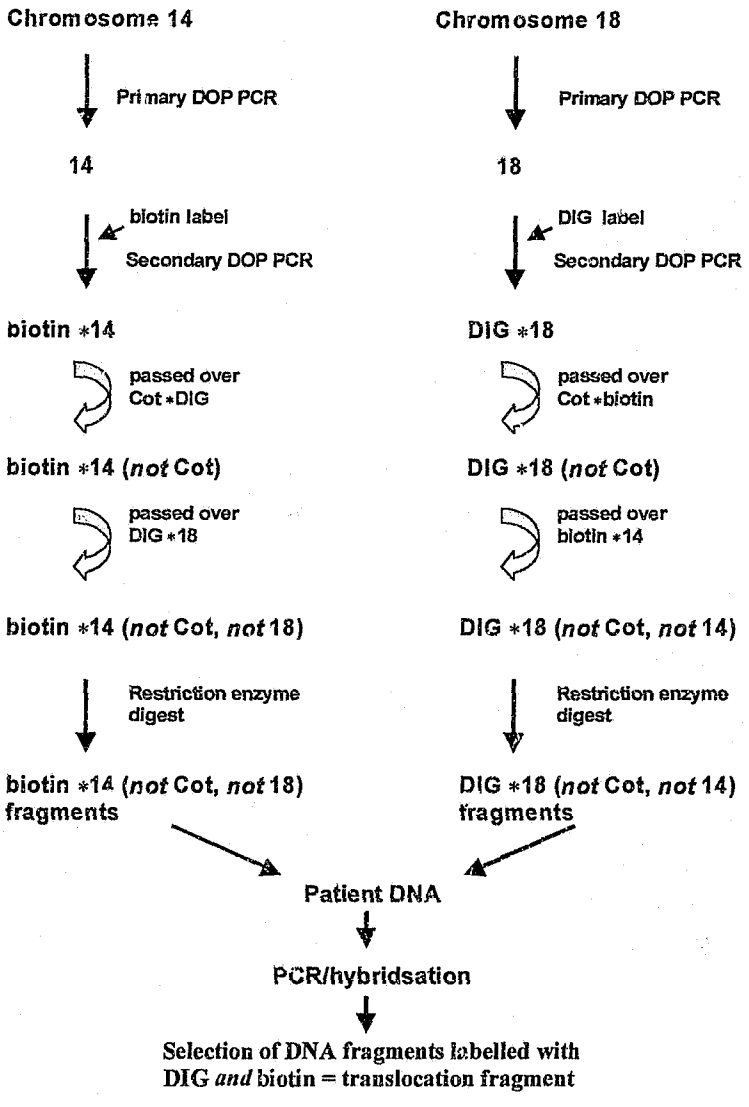


Figure 15: Flow chart of the protocol outline, demonstrating the Boolean principles used to isolate translocation-bearing patient DNA

2.6.2.2.1 Primary DOP PCR

DOP PCR was carried out using 33µl of flow sorted chromosomes, which corresponded to approximately 500 chromosomes. Using a set of pipettes reserved for PCR only, to the tube containing the sorted chromosomes 14 or 18, the following was added: 3.75µl of DOP or DOP2 primers (respectively), 37.5µl kit master mix (Roche, DOP PCR kit) and H_2O to a final volume of 75µl. The mixture was processed in a Perkin Elmer 2400 thermal cycler using the following protocol:

94°C for 9 minutes

9 cycles of

94°C for 1 minute

30°C for 1.5 minutes, ramping at 0.23 °C /second to 72°C

72°C for 3 minutes

30 cycles of

94°C for 1 minute

62°C for 1min

72°C for 1 minute

72°C for 9 minutes

At this stage there was a choice to either generate fragments 300-500 bp in length or 300-3000 bp by varying the cycling protocol. The shorter protocol was chosen. The primary PCR products were analysed on a 2% agarose electrophoretic gel.

2.6.2.2.2 Secondary DOP-PCR (labelling)

Using a set of pipettes reserved for PCR use only, the following components were added to a sterile 200 µl PCR tube: 14.5µl of primary PCR product, 2.5µl DOP (2 µM) or DOP2 (2 µM) primer, 200µM of dCTP, dATP and dGTP, 66µM of dTTP and 134µM of labelled (biotin or DIG) dUTP (Roche).

The mixture was vortexed, spun in a microfuge briefly and processed in a thermal cycler using the following protocol:

94°C for 4 minutes

35 cycles of

94°C for 1 minute

62°C for 1 minute

72°C or 1 minute

72°C for 9 minutes

The DOP PCR product was analysed by running 5 – 10µl on a 2% agarose gel.

2.6.2.3 Sephadex columns

Both chromosome products were passed through a Sephadex column (G50, molecular weight cut off >72 bp (Roche)) to remove all unincorporated, labelled nucleotides that could otherwise later be incorrectly incorporated into products. The molecular weight cut off should be kept > 50 bp, as these fragments have to serve as primers and probes at a later stage.

2.6.2.4 Removing repetitive and common sequences

As stated previously, the human genome contains 3-6% repetitive sequences, it was thus necessary to rid the chromosome preparations of these repetitive sequences as they could generate a false positive signal in the final step. Additionally there may have been other regions homologous between the two chromosomes.

2.6.2.4.1 Removal of repetitive sequences

Six µg of Cot 1 (Cot) DNA (Roche), was randomly primed and labelled, using a random prime labelling kit (Roche), following the manufacturer's instructions and either using biotin dUTP or DIG dUTP, in a 1:2 ratio with dTTP (Figure 16 (a)). The labelled Cot DNA was then passed over a Sephadex G50 column to remove the unincorporated nucleotides. Cot 1 DNA in excess would rapidly hybridise to repetitive sequences in the target molecules, allowing most of the specific sequences to remain single stranded and to then bind to their chromosomal targets.

The biotin labelled Cot DNA was immobilised onto streptavidin magnetic beads (Roche) and used to 'fish-out' the repetitive sequences in the DIG labelled chromosome 18 preparation (Figure 16 (b)). What remained in the supernatant was representative of non-repetitive (*not* cot), labelled, chromosomal DNA. The kit protocol (Roche) was followed with a few modifications. Two hundred µl of streptavidin coated magnetic beads were washed twice with TEN_{100} buffer (10mM Tris-HCl, 1mM EDTA, 0.1M NaCl, pH 7.5), and placed in a final volume of 200µl TEN_{100}. The labelled Cot DNA was denatured at 94°C for 5min, flash cooled on ice and made up to a volume of 100µl with TEN_{100}. The Cot DNA was added to the magnetic beads and left at room temperature for 30 minutes in order to allow the streptavidin and biotin to associate. The magnetic beads were separated on a magnetic stand/separator (Roche), the supernatant fluid removed and the beads washed well with TEN_{500} (as above but with 0.5M NaCl) and placed in a 200µl volume of TEN_{500} (off the magnetic separator).

Two hundred µl of the DIG labelled chromosome 18 was denatured at 94°C, flash cooled on ice and added to the streptavidin magnetic beads, biotin labelled Cot mixture and placed at room temperature for 30 minutes to allow all Cot-like sequences in the chromosome 18 preparation to associate with the biotin labelled Cot. The magnetic beads were again separated on a magnetic stand and the supernatant fluid removed into a clean tube. This supernatant fluid was now largely free of repetitive sequences and could be termed chromosome 18 *not* Cot (Figure 16 (b)).

The procedure was repeated for biotin labelled chromosome 14, using DIG labelled Cot and anti-DIG (<DIG>) magnetic beads, otherwise the protocol remained exactly the same (Figure 16 (c)). At all stages the fraction bound to the magnetic beads could be removed with heat (94°C) and pure H_2O to dissociate the hybridised chromosomes 14 and 18; and 6M guanidine HCl to break the bonds between the streptavidin and biotin or the anti-DIG and DIG moieties. These fractions were spotted onto a nylon membrane and their label detected enabling one to check that the correct fraction, if any, had bound.

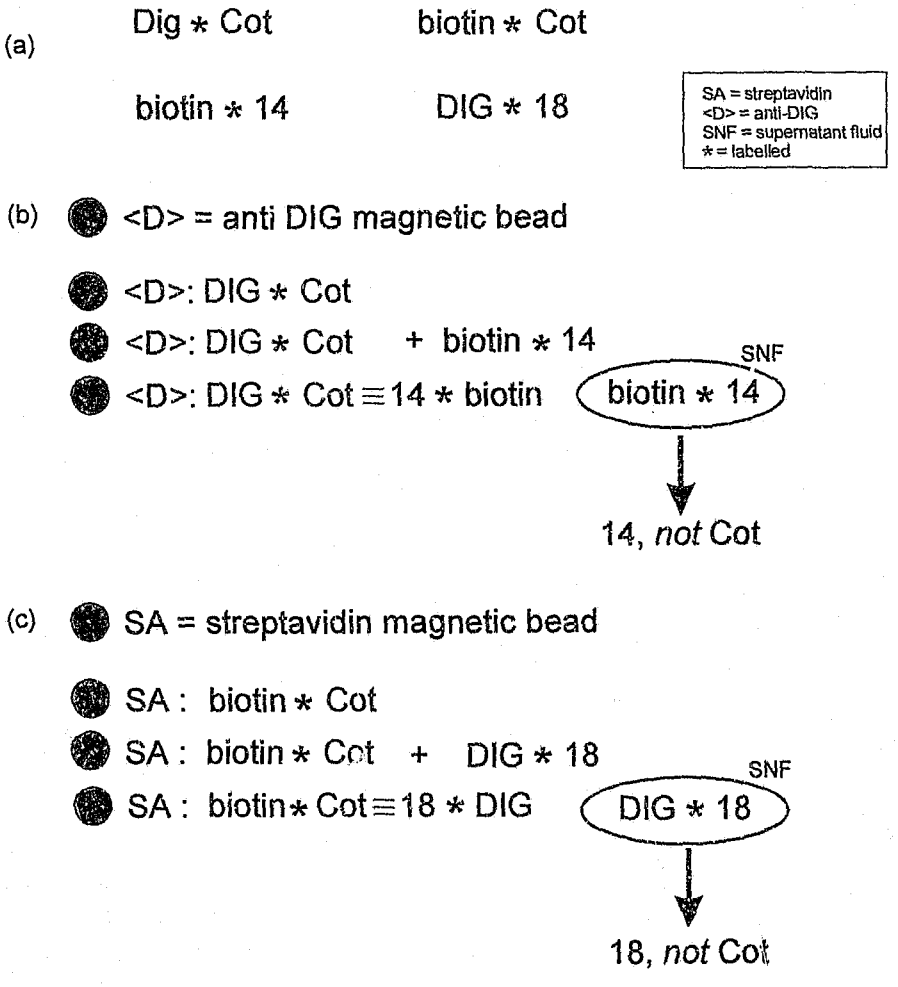


Figure 16: Removal of repetitive sequences (a) Labelled Cot DNA, (b) Immobilisation of DIG labelled Cot onto magnetic beads and 'fishing out' of chromosome 14 repetitive sequences, (c) Immobilisation of biotin labelled Cot onto magnetic beads and 'fishing out' of chromosome 18 repetitive sequences.

2.6.2.4.2 Removal of sequences common to both chromosomes

Following the same logical principles as for the removal of repetitive sequences (Boolean *not*), an aliquot (one third of the total volume) of DIG labelled chromosome 18 (*not* Cot) was immobilised on <DIG> magnetic beads and used to 'fish out' common sequences from the biotin labelled chromosome 14 (*not* Cot). The same was done for the remaining two thirds of DIG labelled chromosome 18, by immobilising an aliquot of biotin labelled chromosome 14 (one third) on streptavidin magnetic beads. The whole procedure could be repeated a second time if deemed necessary (Figure 17).

The resulting SNF could thus be termed:

DIG labelled chromosome 18, *not* Cot, *not* 14 and

Biotin labelled chromosome 14, *not* Cot, *not* 18.

The SNFs were then precipitated with 10% (v/v) 3M NaOH, pH 5.2 and 2.5 (v/v) 100% ethanol, at -70°C.

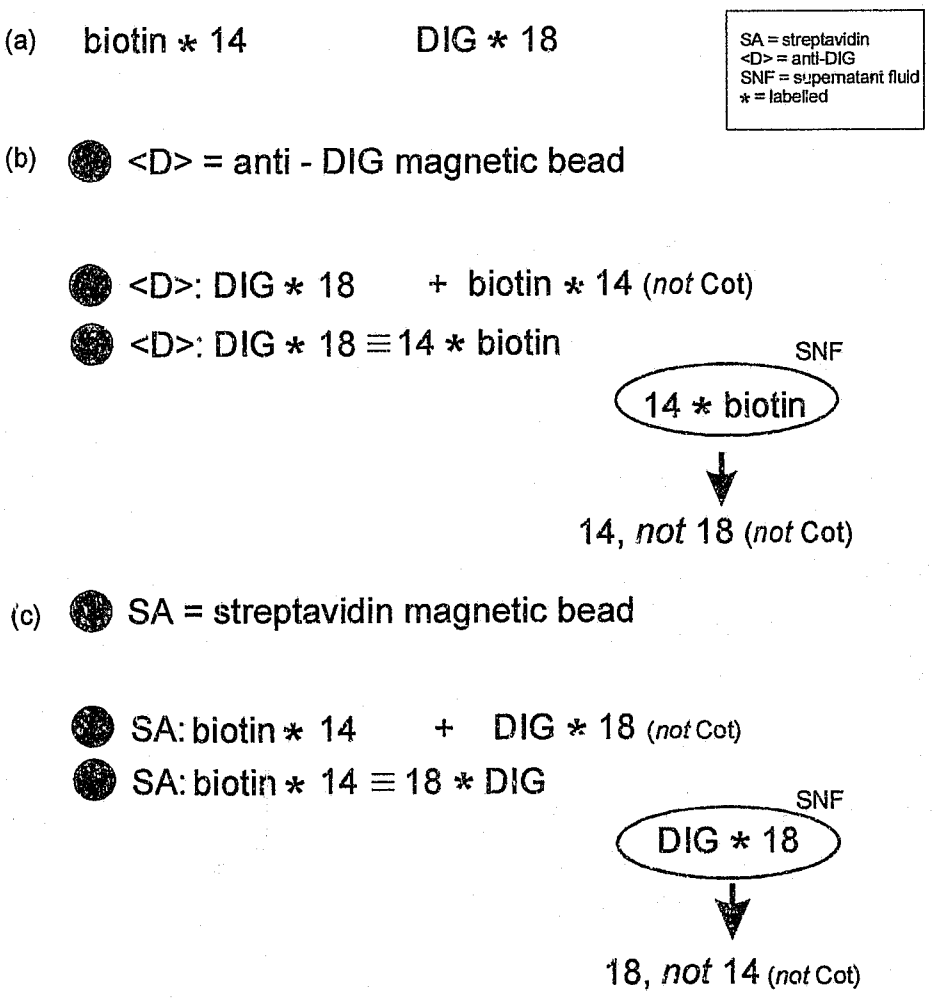


Figure 17: Removal of sequences common to both chromosomes (a) Labelled chromosomal DNA (b) Immobilisation of DIG labelled chromosome 18 onto magnetic beads and 'fishing out' of common sequences from chromosome 14 (c) Immobilisation of biotin labelled chromosome 14 onto magnetic beads and 'fishing out' of common sequences from chromosome 18.

2.6.2.5 Digestion of product with restriction enzymes

The selected (*not* Cot, *not* 14/18) DOP PCR chromosomal products generated above would have 3' ends complementary to the DOP primer sequence. As these could hybridise to one another it was necessary to generate 3'ends that were complementary to chromosomal sequences instead. This was achieved by cutting the products with frequent (tetranucleotide) cutters (*Rsa* I and/or *Hin*d III) or hexanucleotide cutters, thus generating smaller fragments with 3' hydroxyl ends complementary to chromosomal DNA and thus the patient's DNA. Assuming that restriction endonuclease sites are distributed randomly along the DNA, a tetranucleotide target can be expected to occur once every 4^4 (256) nucleotides and a hexanucleotide target every 4^6 (4096) nucleotides.

The labelled chromosome material was resuspended in 8µl of H_2O, 1µl (10U) of enzyme and 1µl enzyme-buffer was added, and the mixture was placed at 37°C overnight.

2.6.2.6 Patient DNA extraction

Follicular lymphoma patients were chosen who were known to have a classical t(14:18) translocation, as defined by classical t(14:18) PCR and oligo extension (see 2.5), and additionally one patient (3) had been shown cytogenetically to have a t(14;18) translocation. The normals were negative using t(14;18) PCR. Patients were also shown to have a monoclonal band with the IgH PCR (see 2.4).

DNA was extracted from paraffin embedded tissue, blood or marrow of the patients with follicular lymphoma (t(14:18)) and known normal controls according to standard methods

(see 2.4.3). The DNA concentration was analysed spectrophotometrically and the DNA was run on a 2% agarose gel.

2.6.2.7 Translocation isolation

There were two ways that the Boolean fragment could be isolated. The first involved a PCR reaction and the second was based exclusively on hybridisation technology.

2.6.2.7.1 Boolean PCR

The first method designed to isolate the t(14;18) translocation was the amplification of the patient DNA with the two sets of primers finally generated in 2.6.2.5. Theoretically the sets of primers should randomly amplify all areas of chromosomes 14 and 18, however only where there is a translocation between the two chromosomes will the resulting molecule be a product of both primers and thus have incorporated both biotin *and* DIG labels.

A PCR reaction was made up with 1.0µg of patient (or normal) DNA, 0.1µg of DIG labelled chromosome 18 and 0.1µg of biotin labelled chromosome 14 as primers, 0.2µM of dNTPs, 1.25U of Taq DNA polymerase (Roche) or 0.25µl Expand high fidelity mixture (Roche), and 1.5mM $MgCl_2$.

The reaction was placed at:

94°C for 4 minutes

40 cycles at:

94°C for 1 minute

62°C for 1 minute

72°C for 1 minute

94°C for 7 minutes.

The products were run on a 2% agarose gel to see if amplification had occurred, as indicated by a smear.

This PCR product was purified using the magnetic beads either coated with <DIG> or streptavidin. The <DIG> beads used first select out all PCR products that had incorporated DIG and biotin-only labelled products are discarded - primary selection (Figure 18 (b)). The <DIG> selected products were then be eluted from these beads (the fraction bound to the <DIG> magnetic beads can be removed with heat (94°C) and pure H_2O), and added to the streptavidin beads which would select out all PCR products that had biotin incorporated (secondary selection), viz., only the hybrids containing both DIG and biotin (Figure 18 (c)), as the biotin-only products have already been discarded. Alternatively the primary selection above could have been carried out as described, but the secondary selection occurs *in situ* and takes the form of a colour reaction, using streptavidin alkaline phosphatase. We used NBT/BCIP (Roche), which formed a blue/purple colour precipitate only if the translocation was present (Figure 18(d)). NOTE: Both the 14-18 and the 18-14 hybrids should be present.

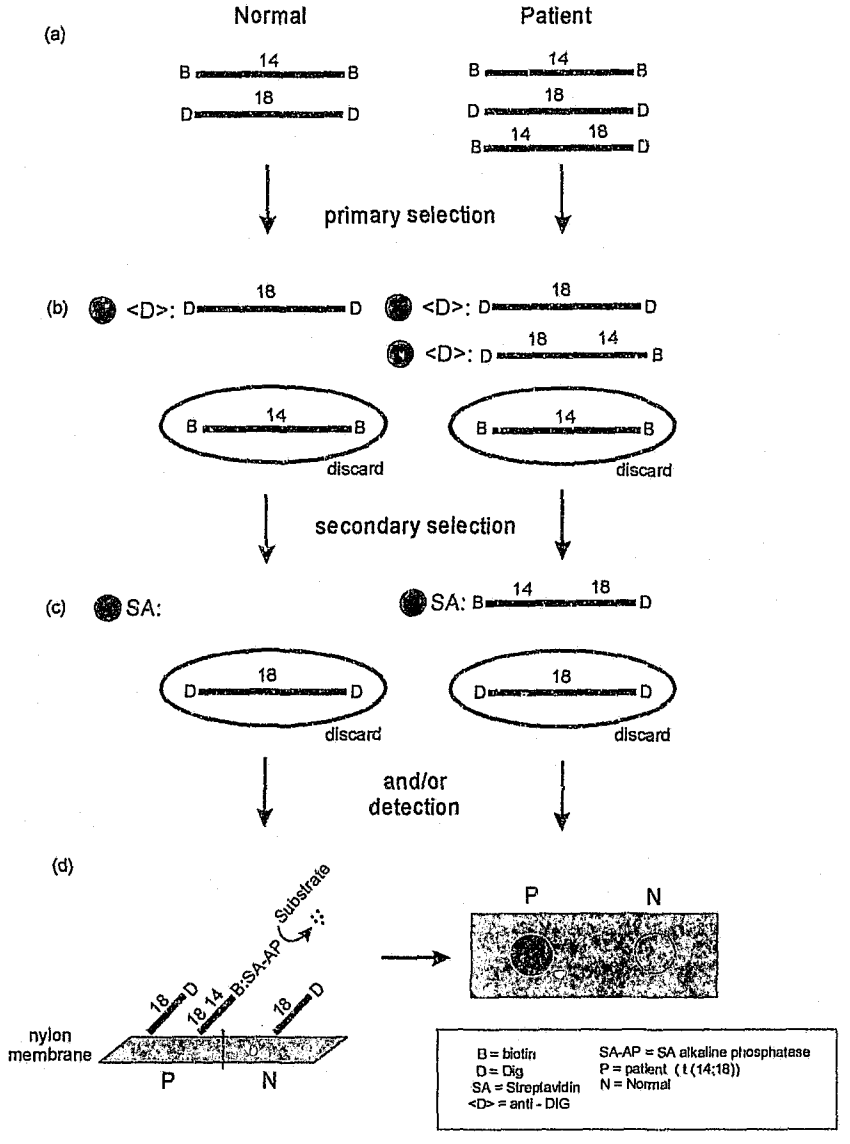


Figure 18: Isolation and detection of the DNA fragment bearing the chromosomal translocation using Boolean PCR.

(a) Boolean PCR products

(b) Primary isolation

(c) Secondary isolation

(d) Secondary isolation as the detection step

2.6.2.7.2 Dot blotting

All samples that needed to be visualised, including those required to check the binding and elimination processes, were reduced in volume (ethanol precipitation) and approx. 5µl was spotted onto a nylon membrane, allowed to air dry and UV-nicked (320nm, 45 seconds) to fix the DNA to the membrane. The presence of either DIG or biotin was then detected using either <DIG> or streptavidin alkaline phosphatase and NBT/BCIP (Roche) according to the manufacturer's instructions, which formed a blue/purple colour precipitate only if the correct moiety was present (Figure 18 (c)).

2.6.2.7.3 Micro-titre plate (MTP) analysis

Alternatively to being blotted on a membrane, the Boolean products were isolated in streptavidin or <DIG> MTP wells. These replaced the magnetic beads in the primary and secondary selection processes. The detection step took place in the MTP using <DIG> or streptavidin horseradish peroxidase (HRP) and ABTS (Roche). A soluble substrate was detected at 405nm (reference filter, 492nm), at 15minutes, in the MTP reader. If an <DIG> MTP was used to capture the Boolean products then the presence of the hybrid molecule was detected with streptavidin HRP. If a streptavidin MTP was used then the detection was carried out using <DIG> HRP (Roche kit).

2.6.2.7.4 Boolean hybridisation

An alternative method that can be used to isolate the translocation was based on hybridisation alone. The principle of this method was almost identical to that of the

Boolean PCR but left out the PCR step. It relied exclusively on the capture of the translocation bearing DNA onto fragments of the one chromosome (e.g. 14) which had been immobilised onto the magnetic beads/solid support by virtue of their biotin label. The selected fragments were in turn hybridised to the labelled chromosome 18 fragments ('primers' generated in 2.6.2.4) (Figure 19). The presence of the final molecular layer was then detected by colourimetric means. Again only fragments positive for both chromosome 14 *and* 18 results in a signal (Figure 19).

Instead of the whole of chromosome 18, a section of that chromosome known to encompass all the translocations described that area can be used; such as a DIG labelled immunoglobulin gene probe (Jh), isolated in (2.6.2.1.2). This would result in fewer fragments to complicate the reactions, but would be less 'universal' than if the whole chromosome was to be used.

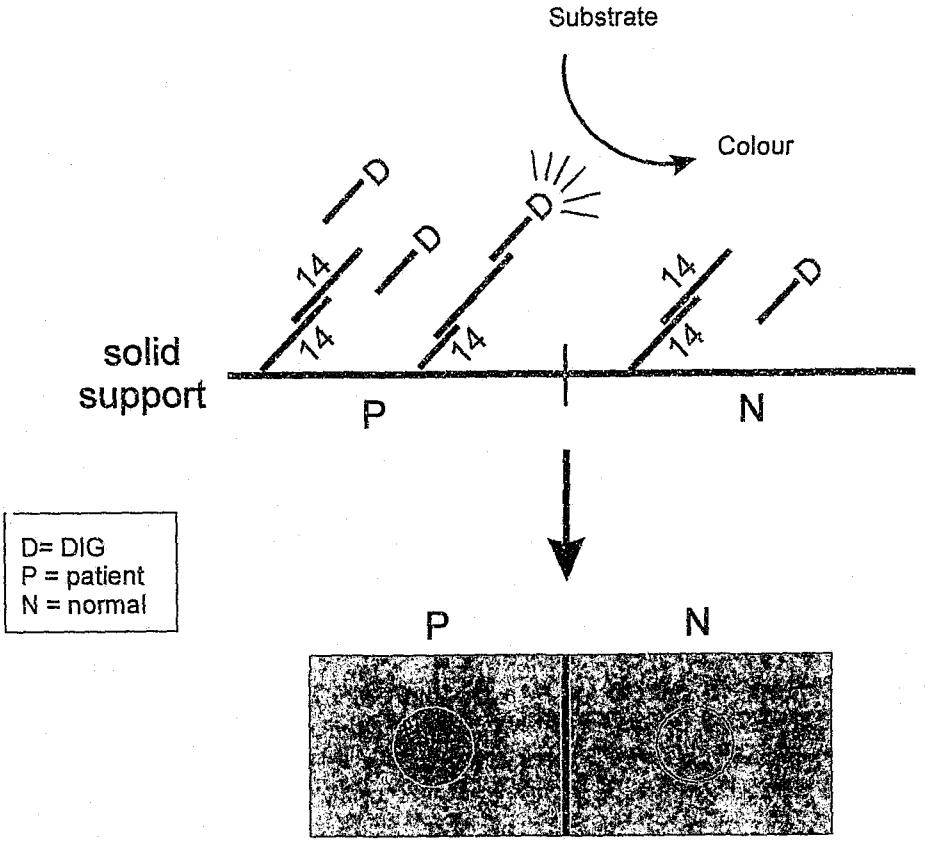


Figure 19: Isolation and detection of the DNA fragment bearing the chromosomal translocation using Boolean hybridisation.

2.6.3 Results and discussion

2.6.3.1 Patient samples

Patients (n=5) and normal controls (n=4) were checked for their monoclonal (or lack thereof) status (2.6.2.6) using the t(14;18) PCR and oligo-extension (2.5), and/or the IgH gene rearrangement PCR (2.4). All five patients showed a positive bcl2/Jh result with PCR and subsequent oligo-extension confirming that the patients were all positive for the t(14;18) translocation. Patients 1, 2, 3 and 4 showed monoclonal B-cell populations. Patient 5 was not done (as the patient is positive for the t(14;18) PCR, this is not a concern). The controls samples were negative for the above t(14;18) PCR.

2.6.3.2 Chromosomal preparative steps

Figure 20 shows the DNA gels with flow sorted chromosome 14 and 18 fragments post-DOP PCR, a smear can be seen between 200 and 2000 bp, although larger than expected at the top of the range.

At each selection step, aliquots of captured and supernatant fluid fractions were spotted onto nylon membranes in duplicate and were analysed for which label was present (DIG, biotin or both) throughout the repetitive sequence selection processes (see p131). There was no cross contamination visible and the results corresponded to the expected labels.

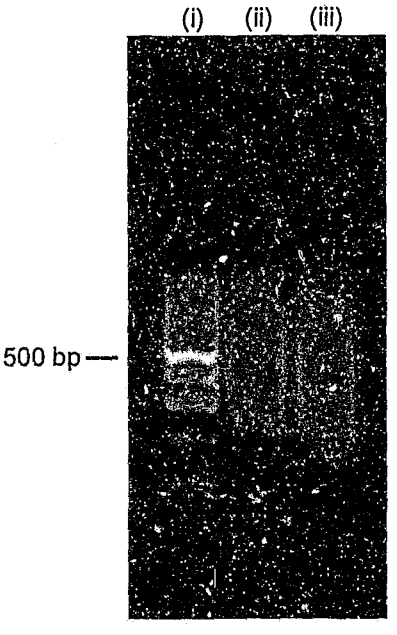


Figure 20: A 2% agarose gel showing amplified DNA post DOP PCR on flow sorted chromosome 14 (lane (ii)) and 18 (lane (iii)) fractions. Lane (i) is a 100 bp ladder (Roche).

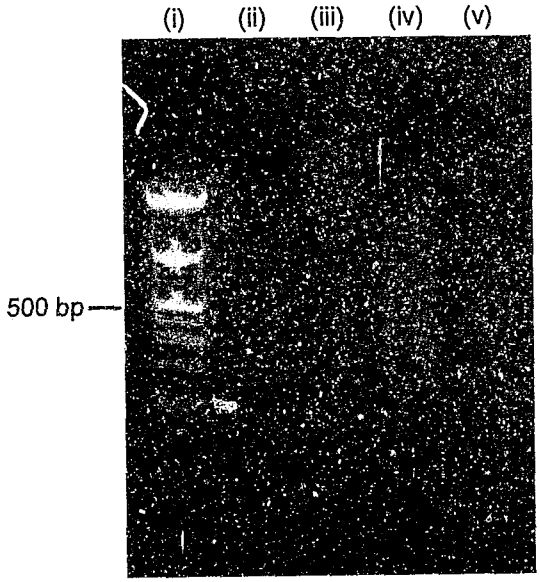


Figure 21: A 2% agarose gel showing amplified DNA post Boolean PCR on patient and normal DNA. Lane (i) is a 100 bp ladder (Roche), lane (ii) patient 1 lane (iii) normal 1 lane (iv) patient 2 and lane (v) normal 2.

2.6.3.3 Boolean PCR/hybridisation

Figure 21 shows DNA post Boolean PCR of patient and normal samples. Again the expected smears were present. It is interesting to see that the patient smears seem somewhat more limited in size compared to the normals.

Often the primer yields from the flow sorted chromosomes (after all the repetitive and common sequences were removed) were very low and could result in only two runs, one patient and one normal. In Figure 22 (a) patient 1 was positive after both primary and secondary selections and normal 1 remained negative. In Figure 22 (b), all three patients (1,2 and 3) were positive, and normal 2, although there was a faint signal, appeared to be negative. In Figure 22 (c) the dot blot depicted that patient 4 had a positive signal compared to normal 3.

Thus a positive reaction, identifying exclusively hybrid molecules (t(14;18)), was found in 4/4 patients (1,2,3 and 4) and 0/3 normal controls 1, 2 and 3.
Using the hybridisation method with the Jh probe, a positive signal was obtained with patient 5 and no signal with normal 2 (Figure 22 (d)).

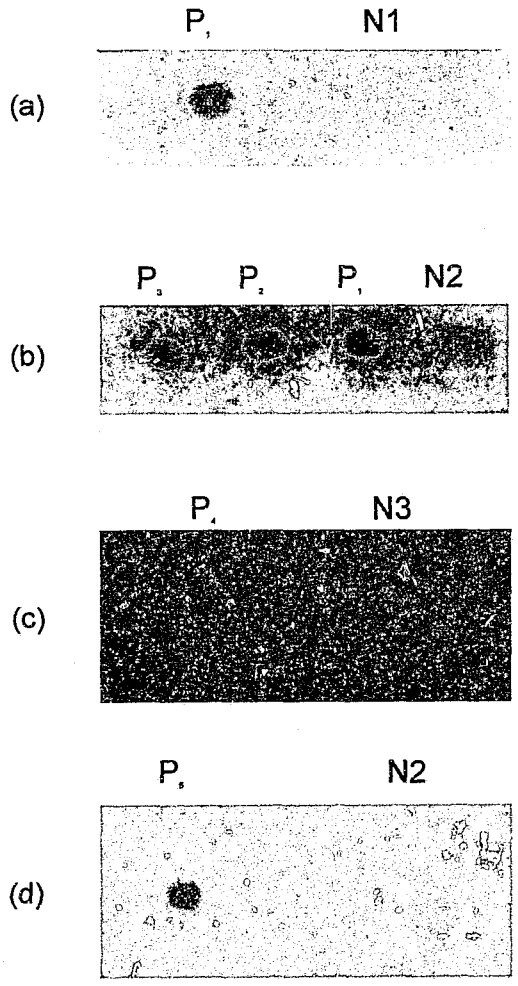


Figure 22: Dot blot results of Boolean PCR (a, b and c) and Boolean hybridisation (d). It can be seen in all the above that the patients (P) are all positive whereas the normal (N) individuals have either no signal or a very faint one.

2.6.3.4 Micro-titre plate (MTP) readings

Preliminary results obtained using soluble colour reactions and a MTP reader were promising. Signals approximately twice that of normal 4 were obtained for patient (Table 7), however background was evident for the normal control. The kit positives and negatives were all within the expected range. These results served to confirm that the methodology based on Boolean principles was able to repeatedly detect translocations, in a variety of forms.

Table 7: MTP readings for the well representing patient 1 compared to a normal control (N4). These readings were obtained using <DIG> MTP for the primary selection (1°) and detecting the hybrid molecule with streptavidin HRP at 405nm at 15min after substrate addition. The secondary selection (2°) was performed on a streptavidin MTP, and the readings were obtained using <DIG> HRP

Selection	Kit Positive	Kit negative	Patient (well)	Normal (well)
1°	0.353	0.014	0.468	0.112
2°	0.375	0.008	0.233	0.064

2.6.4 Discussion and conclusion

Positive results were obtained in all the variations of the method tried. It appears that Boolean logic will enable the ultimate goals to be achieved: (a) to identify known and novel breakpoints outside of existing molecular protocols and (b) to ultimately design a

breakpoint matrix or array to randomly identify breakpoints in patients. One of the major problems encountered was the small quantity of chromosome fragments we were able to generate after all the procedures had been executed. At each step aliquots were checked for content, thus depleting the amount of material. Flow sorted chromosomes, by their nature, are few (500 chromosomes 14 is equivalent to approx. 65pg of DNA [35]) and thus the starting material was relatively scarce. There is a certain amount of positivity with the normal controls which may be due to incomplete removal of repetitive sequences or due to DOP : DOP cross reactivity. The protocol showed a high degree of reliability, and when a step did not produce the expected fragments/result, this could always be explained by going back and examining the results and technique up to that point. The necessary use of a random PCR system to generate the chromosome specific fragments, by its nature generated different 'batches' of fragments, some better than others.

2.6.5 Future prospects

Although a complex procedure to generate the chromosome specific molecules, with more starting material and once an optimum set of chromosomal fragments (free of repetitive and common sequences), has been obtained, they would be cloned into a library for immediate future use to avoid going through the above process every time. Collaboration with Research Genetics is underway to achieve this goal. The final product would be a kit-based method, that can be carried out in less than a day.

Ultimately this novel approach would entail two panels of differentially labelled chromosomes:

Panel 1: chromosomes 1-22, X and Y labelled with biotin,

Panel 2: chromosomes 1-22, X and Y labelled with DIG placed in an array.

For diagnostic purposes it may be sufficient just to establish the presence of a chromosomal translocation. This could be achieved by firstly immobilising the biotin-labelled panel of chromosomal DNA (chromosomes 1-22, X, and Y) onto streptavidin solid supports, one 'column' for each biotin labelled-chromosome (↓) (Figure 23). These solid supports are then exposed to the patient DNA. Each immobilised chromosome will bind the relevant patient DNA as well as any hybrid DNA containing material homologous to that chromosome. The immobilised chromosomes and their captured DNA would then **each** be further hybridised with a DIG-labelled panel of chromosomal DNA (chromosome 1-22, X and Y), one 'row' for each DIG-labelled chromosome (→), generating a matrix/array of 576 potential points. At points where both the biotin-labelled chromosome (↓) and the DIG-labelled chromosome (→) are the same, a signal will also be generated (Figure 23), and this would serve as convenient experimental controls. A signal obtained implicating two different chromosomes indicates the presence of a translocation involving those two chromosomes (Figure 23 insert). It would then be possible to pick up any translocation between any two chromosomes in any tissue (Figure 23).

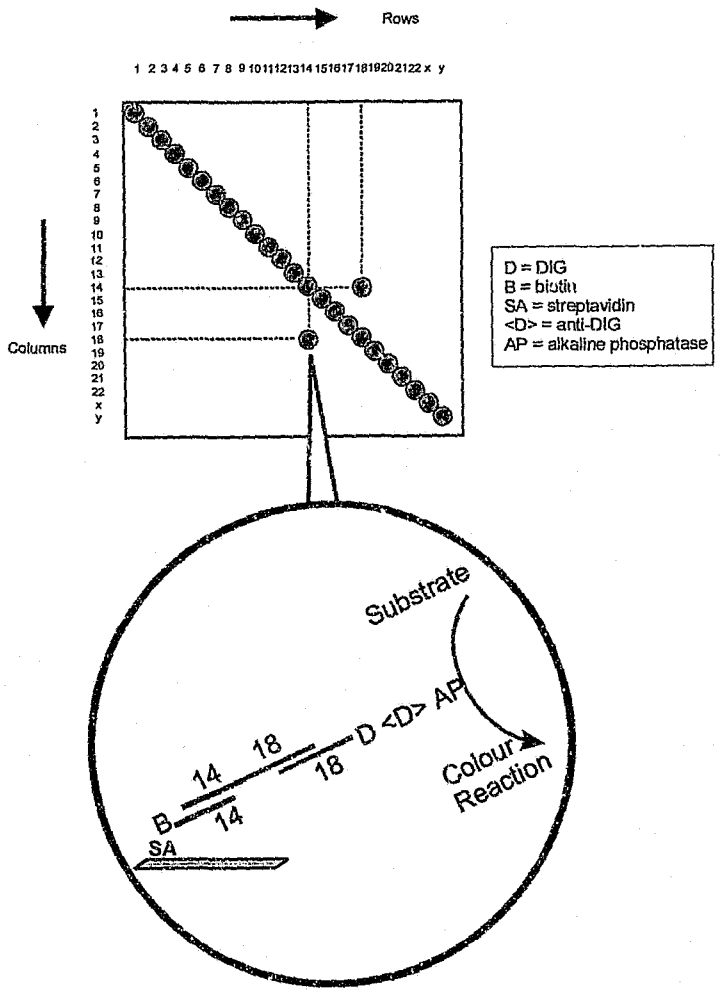


Figure 23: Chromosomal translocation matrix: biotin-labelled chromosomes each immobilised in their own 'column' (↓) of the matrix/array, the patient's DNA is then hybridised to all immobilised chromosomal fractions. The DIG-labelled chromosomes are then hybridised each to its own 'row' (→). A translocation is identified and a signal generated when a molecular 'sandwich' is formed by the presence of a hybrid DNA (see insert). The same chromosome in a row and a column will also generate a signal and thus serve as controls.

When the two involved chromosomes have been identified, this technique could be carried further to fully characterise and sequence the breakpoint to establish the involved genes causing the neoplasm. The isolated DNA could be released from the secondary solid support and cloned into a sequencing vector.

Band specific Boolean

A lot can still be achieved with more abundant chromosome material. Portions of chromosomes such as Band Specific Probes ® (Research Genetics) could be used in a microarray type format (see 2.3), i.e. in a more complex matrix than represented in Figure 23. A Boolean selection based on the chromosome bands would encompass all the translocations involving those two bands. Additionally, there is a possibility that band based Boolean logic could be of use in the detection of chromosomal inversions or insertions as well as interchromosomal translocations.

In summary, Boolean logic is a sound way to look for chromosomal translocations in neoplastic disease, either very generally or more specifically. The preliminary studies have shown encouraging results and the future prospects of this method look promising, not only as an invaluable research tool, but also as a diagnostic method able to cope with a larger repertoire of translocations at the molecular level.

3 MOLECULAR DIAGNOSIS OF INHERITED THROMBOEMBOLIC DISORDERS

3.1 Synopsis

The detection of inherited disorders involving haemostasis is another area that a haematological laboratory would concern itself with. In setting up the technology for the diagnosis of haematological malignancies, it was found that the same facilities and expertise could be employed to address molecular diagnosis in thromboembolic disorders. In this section of the thesis, the focus of the development of molecular techniques for the diagnosis and detection of inherited thromboembolic disorders has been (a) to address the issues of partial restriction enzyme digestion and (b) the multiplexing of related molecular diagnostic techniques.

The molecular diagnostics of thalassemias, sickle cell anaemia and other haemoglobinopathies has received much attention in recent years [11 16] and the appropriate molecular tests for these conditions are available and currently carried out at centres such as the South African Institute for Medical Research (SAIMR), Human Genetics Department. As South Africa is largely outside of the malaria belt, the associated protective haemoglobinopathies are not prevalent amongst the population. The X-linked bleeding disorder haemophilia, although distressing for the few individuals found in the South African population, is also not considered a major public health risk [19 113]. None of

the above are dealt with by the Haematology Department at the SAIMR and are thus not included within the scope of this thesis.

The past few years have witnessed significant advances in our knowledge of inherited thrombotic disorders [63]. South Africa is a heterogeneous population as well as individuals of mixed ancestry. Inherited disorders such as the presence of the factor V Leiden [66 114 115 116], prothrombin G20210A [68 117] and the methylenetetrahydrofolate reductase (MTHFR) C677T [69 118] mutations are present in the people of Caucasian ancestry, placing these individuals at risk of thromboembolic disease [64]. The Department of Haematology at the SAIMR deals with these disorders on a daily basis and have thus implemented molecular diagnostic procedures combined with existing laboratory techniques to diagnose them.

A good example of where diagnosis has recently become digitised is the factor V Leiden mutation associated with activated protein C resistance. The detection of a single point mutation via PCR has replaced the protein bioassay where a activated protein C sensitivity ratio (APC-SR) of > or < 2 determined respectively whether the patient was normal or not [114] (see Figure 2).

With the use of PCR and restriction enzyme technology, molecular tests are available to routinely screen for the known inherited thrombophilias. The PCR analyses of these mutations are amongst the simplest to work with as a product is always generated, thus a

failed PCR amplification would be instantaneously detected. It is the subsequent analysis (viz. enzyme restriction/digestion) of the PCR product that is diagnostic, and this is where further control is necessary. As an example, for all three of the above analyses, a restriction site is either created or abolished by the presence of the mutation (e.g. factor V Leiden; Figure 24). Without a restriction control, in the event of a failed or partial restriction, the patient may be falsely interpreted as having the allele that does not digest. By employing similar molecular strategies as described by Bertina *et al* in 1994 [115] for the PCR and restriction analysis of the factor V Leiden mutation (see Figure 24), we were able to control for possible partial digestion in both the prothrombin G20210A and MTHFR C677T PCR mutation analyses (see publications in sections 3.2 and 3.3). The scenario in these cases was slightly different, in that in both these restriction digests, the mutated allele was digested. However neither of the original PCR analyses described in the literature had incorporated restriction controls into their methodology. To incorporate a restriction control site into the MTHFR analysis, it was necessary to induce a restriction site by using a mutated primer.

Consideration of the expertise available to troubleshoot any particular protocol is required. A procedure can be designed so that inbuilt controls immediately alert the technician of any possible error in the result. These controls are often easily incorporated by merely redesigning the experiment.

When a full thrombotic screen is carried out on a patient, it can often involve between two and three PCR runs, which in realistic terms could take between 3 and 7 days before a result can be issued. Multiplexing of PCR reactions (amplifying several targets in a single reaction) is a potential solution, but it is a demanding PCR technique. It often requires extensive optimisation because primer-dimers and other non-specific products may interfere with the amplification of specific products [119]. In this thesis, a multiplex PCR reaction for the factor V Leiden and prothrombin G20210A mutations is described (see publication in section 3.4). By multiplexing the factor V Leiden and the prothrombin G20210A analyses, it was not only possible to reduce all the procedures and expenses dramatically, and use the same inexpensive restriction enzyme; but also to 'lend' the prothrombin's *Hin*dIII restriction control to the factor V Leiden's digestion.

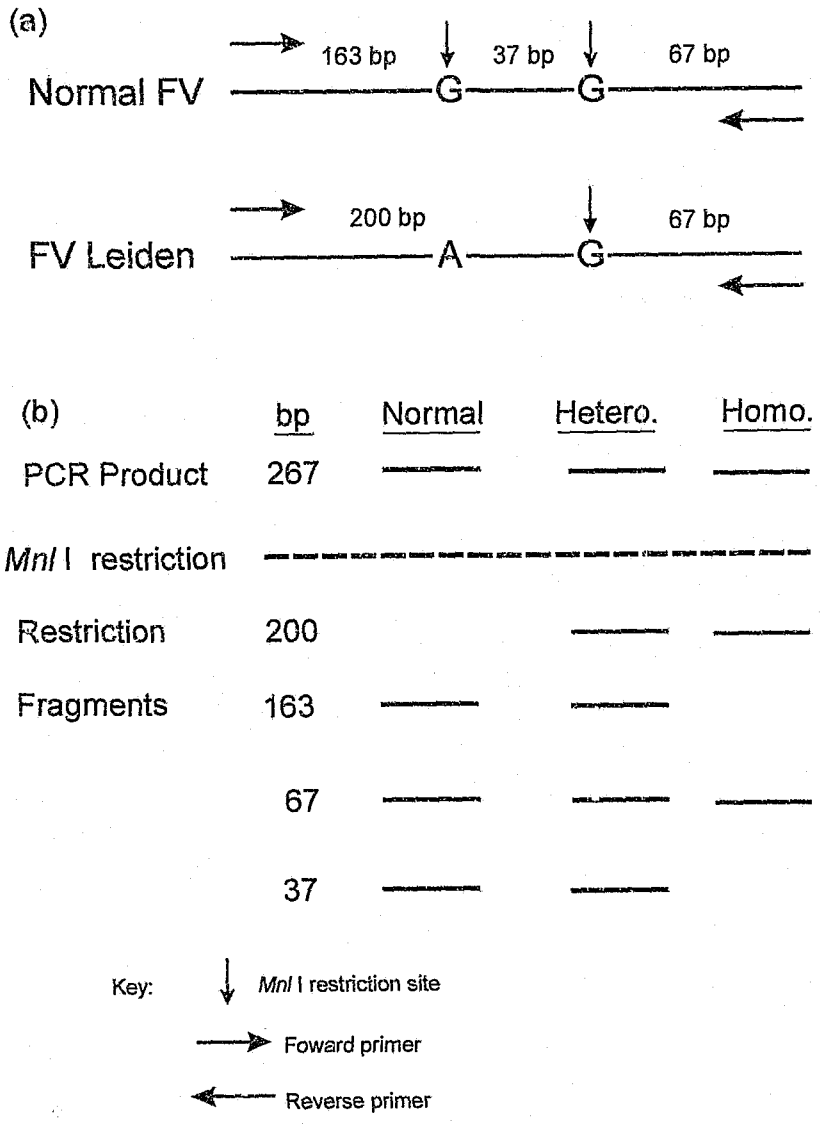


Figure 24: Schematic representation of (a) the factor V Leiden mutation (G1691A) and (b) its restriction pattern with *MnlI* post PCR.

3.2 The prothrombin G20210A mutation

- Journal article: *Development of an Internal Restriction Control in the PCR Detection* of the Prothrombin 20210A Mutation.

R M Dubreuil Lastrucci, Debbie Dawson, Marion Münster.

Clinical and Laboratory Haematology (1999) 21 (4) August 281-283.

- Abstract: Development of an Internal Restriction Control in the PCR Detection of the Prothrombin 20210A Mutation.

R M Dubreuil Lastrucci, Debbie Dawson, Marion Münster.

Association for Molecular Pathology Annual Meeting, Arlington, VA, USA, November 1998.

(Appendix 1.9)

Abstract published: American Journal of Pathology, 1998. 153 (5), 1648, G2.

- Abstract: Prothrombin 20210A Mutation is Absent in the South African Black Population.

M Münster, DA Dawson, RMD Lastrucci.

International Society of Haematology, Durban, South Africa, September 1999.

(Appendix 1.10)

3.2.1 Journal article

Development of an Internal Restriction Control in the PCR Detection of the Prothrombin 20210A Mutation.

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Accepted for publication 24th March 1999

Running Title: A Restriction Control for the 20210A Prothrombin PCR

Clin. Lab. Haem. **21**, 281-283

Summary

Detection of the presence of the 20210A/G allele in the human prothrombin gene is easily achieved by amplification using primers designed to span this region. The downstream primer creates a *Hin*dIII restriction site if the 20210A variant is present. A new forward upstream primer was designed to incorporate a naturally occurring *Hin*dIII site that, as it is present in both alleles, serves as an internal restriction control. Using this technique, the DNA of 292 unselected patients with venous thromboembolic disease was analysed. Of the 149 white patients, 4.7% were heterozygous for this mutation but none of the African Black patients were positive.

Key Words: Prothrombin 20210A mutation, restriction control, Polymerase Chain Reaction.

Introduction

The 20210A variation in the 3 ' untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increased risk of venous thrombosis. Poort *et al.* (1997) [68] showed that in unselected patients with deep vein thrombosis the prothrombin gene mutation prevalence was 6.2% whereas it was found in only 2.3% of healthy control subjects. Using the primers described by the above authors (Table 1) [Table 8], a 345 bp polymerase chain reaction (PCR) product is obtained. Their reverse primer was designed such that when the mutated allele is amplified, a *Hin*dIII restriction site is generated. The normal allele would therefore remain undigested.

In the event of a failed digestion, a patient may be falsely interpreted as normal. The presence of a second identical restriction site, unrelated to the mutation, would serve as a restriction control, as demonstrated by Bertina *et al* (1994) [115] in the detection of the factor V Leiden mutation using *Mnl*I.

Using the original reverse primer (Poort *et al,* 1997) [68], a new forward primer (Table 1) [Table 8] derived from the prothrombin gene sequence reported by Degen & Davie (1987) [120] was developed, which incorporates an additional, natural *Hin*dIII site starting at nucleotide 19826 to act as a restriction control [Figure 26].

Materials and Methods

DNA extraction

DNA was extracted from whole blood collected in EDTA from 292 consecutive patients presenting with venous thromboembolic disease, using the method described by Talmud *et al* (1991) [88]. The samples were frozen and then thawed to facilitate lysis of contaminating red cells prior to DNA extraction. Four hundred microlitres of freshly prepared 0.17M ammonium chloride was added to 100 µl of thawed buffy coat specimen. This was mixed well by inversion and then left at room temperature for 20 minutes before being spun down in a microcentrifuge for 30 s. The supernatant was then discarded, the pellet resuspended in 0.9% solution of sodium chloride by vortexing and then centrifuged again. This washing process was repeated three times. After the third wash, the pellet was resuspended in 200 µl 0.05 M sodium hydroxide. This was boiled for 10 minutes and neutralised by the addition of 25 µl 1 M Tris hydrochloric acid (pH 8.0). The extracted DNA was approximated by spectrophotometric means and stored at -20 °C until further analysis.

Amplification

PCR amplification was carried out using the proth:r and proth:x primers (Table 1) [Table 8] in the Expand High Fidelity PCR system (Boehringer Mannheim) as per the manufacturer's instructions. Each 50 µl reaction contained ~ 0.5 µg genomic DNA, 0.5 µM of each primer, 1.5mM $MgCl_2$ and 0.25 µl Expand enzyme mix. A first denaturation step at 94°C for 3

minutes was followed by 35 cycles of 94°C for 1 minute, 63°C for 1 minute and 72°C for 2 minutes followed finally by 4 minutes at 72°C.

Digestion and analysis

Twenty microlitres of PCR product from each sample was electrophoresed on an ethidium bromide impregnated 2% (w/v) agarose gel and visualised under UV light to ascertain whether successful amplification had occurred (Fitsch *et al*, 1985; Ausubel *et al*, 1988) [121] [122]. Ten units of *Hin*dIII (Boehringer Mannheim) was directly added to the remaining 30 µl and incubated at 37°C overnight (Ausubel *et al*, 1995) [123]. The restricted products were visualised on a 3% (w/v) agarose gel.

Results

The expected product of 473 bp was obtained post PCR but pre-restriction (Figure 1, lane ii) [Figure 25.] The disappearance of this original parent band and the subsequent generation of a 407 bp and a 66 bp band (Figure 1, lanes iii, iv, v and vi) [Figure 25] indicates that complete restriction has occurred in all these samples. Additional restriction generating a 384 bp fragment in lanes v and vi identify these two patients as heterozygous carriers of the 20210A prothrombin gene mutation.

Of the 292 patients investigated for thromboembolic disease, 149 were Caucasians and 143 were African Blacks. The prothrombin 20210A was detected in heterozygous form in 7 Caucasians (4.7%) but was absent in the African Black population. Although no

homozygous 20210A individuals have yet been found in our laboratory, the expected digestion pattern would be the presence of 384 bp, 66 bp and 23 bp bands.

Discussion

In the detection of the 20210A prothrombin allele, the PCR amplification is automatically controlled for as the absence of the 473 bp product implies a failed reaction. In the absence of an internal restriction control site however, the prothrombin genotype based on digestion patterns could be incorrectly interpreted in the event of a partial or failed digestion. By moving the forward primer further upstream we have incorporated an additional, naturally occurring *Hin*dIII restriction site into the PCR product. The use of this new forward primer, directly controls for the diagnostically relevant *Hin*dIII restriction digestion of the mutant allele, by allowing an additional restriction to take place in both the normal and mutated alleles. Other authors have employed methods not relying on RFLP analysis, such as allele-specific PCR by Poort *et al* (1997) [68], these however require the execution of two amplification reactions. If the aim is to investigate for multiple prothrombotic lesions, then the method by Bowen *et al* (1998) [118] would be ideal. However, in instances where the prothrombin gene is the only locus of interest, the incorporation of an additional *Hin*dIII restriction site within the PCR product, enables one to simply and confidently screen patients for the presence or absence of the 20210A prothrombin gene mutation.

Our finding of a prevalence rate of 4.7% heterozygosity for the prothrombin 20210A mutation amongst South African Caucasian individuals with venous thromboembolic disease

is similar to the data of others (Poort *et al*, 1996 [68]; Arruda *et al*, 1997 [124]; Cumming *et al*, 1997 [125]; Hillarp *et al*, 1997 [126]; Leroyer *et al*, 1998 [127]). The absence of this mutation in African Blacks is in keeping with the published data of Rosendaal *et al* (1998) [128] suggesting a European founder effect. Our findings confirm that the prothrombin 20210A mutation is an important risk factor for venous thrombosis in certain population groups and hence its inclusion in the laboratory investigation of such patients is well justified. As the demand for thrombophilia screening is on the increase and molecular techniques are now being employed routinely, the necessity for strict quality control is of paramount importance.

Table 8: Primers used in the PCR spanning the 20210A/G allele of the prothrombin gene

Primer name	**Nucleotide Co-ordinates**	**Primer sequence**
Proth:f (original forward primer)	19889 to 19908*	5' tct aga aac agt tgc ctg gc 3'
Proth:x (new forward primer)	19761 to 19781#	5' cct gat gaa ggg aaa cga ggg 3'
Proth:r (reverse primer)	20212 to 20233*	5' ata gca ctg gga gca ttg aag c 3'

* Poort *et al* 1996 [117]

Derived from Factor II gene sequence published by Degen and Davie 1987 [120]

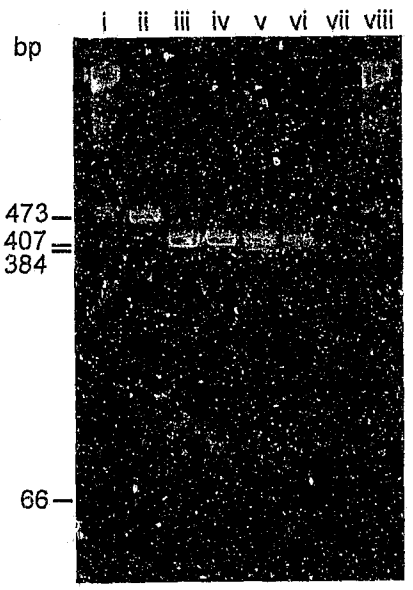

Figure 25: A 3% (w/v) agarose electrophoretic gel of the PCR products of the prothrombin gene pre- and post restriction. Lanes i and viii, 100 bp molecular weight ladder (Boehringer Mannheim. Germany); Lane ii, unrestricted PCR product; lanes iii and iv, normal individuals; lanes v and vi, individuals heterozygous for the 20210A allele; and lane vii is a reagent blank. The 66 bp fragment confirms that restriction has occurred.

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Additional information:

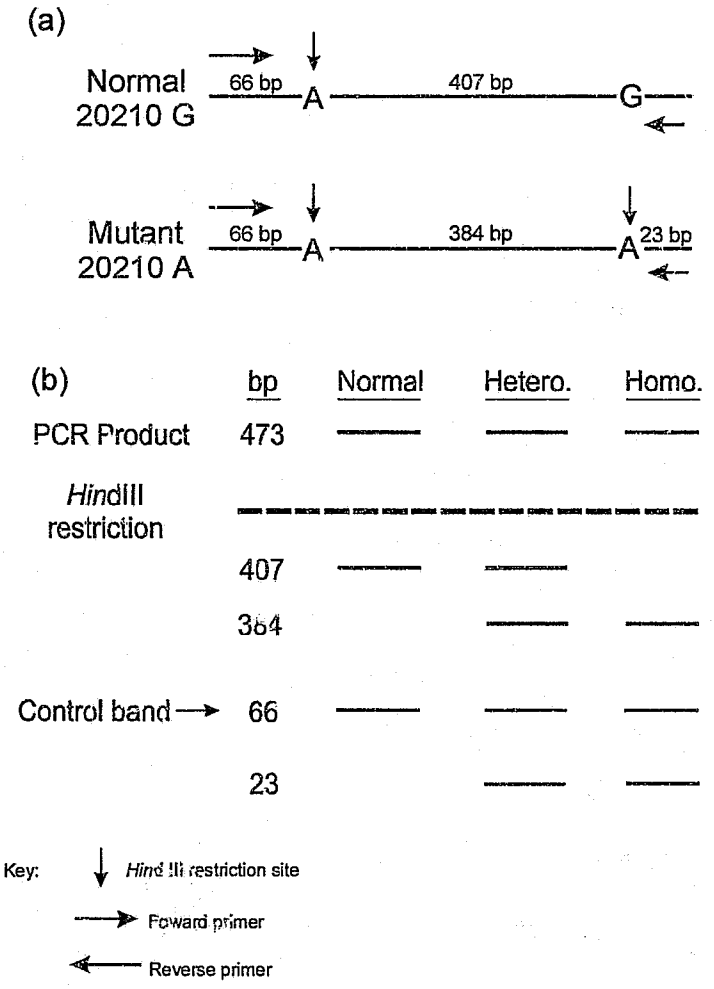

Figure 26: Schematic representation of (a) the prothrombin G20210A mutation and (b) its restriction pattern with *Hin*dIII post PCR.

3.3 The methylenetetrahydrofolate reductase C677T mutation

- Journal article: Development of an Internal Restriction Control in the PCR Detection of the Methylenetetrahydrofolate Reductase (MTHFR) C677T Mutation.

R M Dubreuil Lastrucci, Debbie Dawson, Marion Münster.

Molecular Diagnosis, 1999, 4 (2), 159 - 161.

3.3.1 Journal article

Development of an Internal Restriction Control in the PCR Detection of the Methylenetetrahydrofolate Reductase (MTHFR) C677T Mutation.

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Key Words: Polymerase Chain Reaction, Quality Control

Running Title: A Restriction Control for the C677T MTHFR PCR

Abstract:

Background:

Detection of the presence of the 677C/T allele in the human MTHFR gene is easily achieved by amplification using primers designed to span this region. *Hinf*I digestion, occurring only in the 677T allele, subsequently discriminates between the two alleles. Existing methods, however, do not control for failed restriction endonuclease digestion.

Methods and Results:

A new forward, modified primer was designed and placed further upstream so as to create a *Hinf*I site which, because it is present in both alleles, would serve as an internal restriction control.

Conclusions:

By allowing an additional restriction to take place in both the normal and mutated alleles, the use of the new primer provided for an internal restriction control.

Introduction

Hyperhomocysteinaemia has been identified as a risk factor for coronary artery disease and venous thromboembolic disease. 5,10-Methylenetetrahydrofolate reductase (MTHFR) catalyses the reduction of 5,10- methylenetetrahydrofolate to the circulatory form of folate, 5-methlyltetrahydrofolate, a cofactor involved in the remethylation of homocysteine to methionine. [1, 2, 3] [129 69 118]. A common mutation (C677T) in the MTHFR gene has been described, that causes an amino acid substitution (Ala to Val) and renders the MTHFR protein thermolabile. In the homozygous state this mutation is thought to interact with environmental factors, such as a deficiency of folic acid to predispose to hyperhomocysteinaemia [1,2,3].

Using the primers described by Frosst *et al.* [2] [69] (Table 1) [Table 9], a 198 bp polymerase chain reaction (PCR) product is obtained. Their primers were designed such that when the mutated allele is amplified, a *Hinf* I restriction site is present in the PCR product. The normal allele, therefore, would remain undigested.

In the event of a failed digestion, a patient may be falsely interpreted as normal. The presence of a second identical restriction site, unrelated to the mutation, would serve as a restriction control, as demonstrated by Bertina *et al.* [4] [115] in the detection of the Factor V Leiden mutation using *Mnl* I.

Using the original reverse primer [2] [69] we have developed a new forward primer (Table 1) [Table 9] derived from the MTHFR gene sequence reported by Goyette *et al.* [1] [129], which creates an additional *Hinf*I site starting at nucleotide 625 to act as a restriction control [Figure 28].

The variable nucleotide 'N' in the *Hinf*I restriction site (*viz.* GANTC) is a guanine for both the control and diagnostic restriction sites.

Methods

DNA extraction

Using the method described by Talmud *et al.* [5] [88], DNA was extracted from whole blood collected in EDTA from patients presenting with venous thromboembolic disease. All studies were carried out with ethical clearance from the Committee for Research on Human Subjects (University of the Witwatersrand, South Africa).

Amplification

PCR amplification was carried out using the MTHFR:r and MTHFR:x primers (Table 1) [Table 9] in the Expand High Fidelity PCR system (Boehringer Mannheim) as per the manufacturer's instructions. Each 50 µl reaction contained approximately 0.5 µg genomic DNA, 0.5 µM of each primer, 1.5mM $MgCl_2$ and 0.25 µl Expand enzyme mix. A first denaturation step at 94°C for 3 minutes was followed by 35 cycles of 94°C for 1 minute, 65°C for 1 minute and 72°C for 2 minutes followed finally by 4 minutes at 72°C.

Twenty microlitres of PCR product from each sample was digested with 10 U of *Hin*fI (Boehringer Mannheim) and visualized .

Results

The expected product of 233 bp obtained by PCR prior to digestion is shown, in Fig. 1 [Figure 27], lane v. The disappearance of this original parent band and the subsequent generation of a 17 bp band (lanes ii, iii and iv) indicates that complete digestion has occurred in all these samples. The generation of a 216 bp band only, indicates an individual that is a homozygote for the normal MTHFR gene (lane iv), and the additional generation of 175 bp and 41 bp fragments (lane iii) identifies this patient as a heterozygous carrier of the MTHFR C677T gene mutation.

The absence of the 216 bp band altogether, with the presence of the 175 bp and 41 bp bands, represents the digestion pattern of a homozygote for the MTHFR 677T mutation (lane ii).

Discussion

Rigorous quality control protocols are generally accepted to be an essential component of the daily functioning of routine diagnostic laboratory services. In contrast, although molecular technology has proved not only to be an invaluable tool for basic and applied research, and has helped in the diagnosis and early intervention of human disease, very little emphasis has been placed on its quality control. Although considered to be accurate and precise, in view of

the numerous variables associated with molecular technology, there is an absolute requirement for quality control mechanisms to be in place.

In the detection of the MTHFR C677T allele, the PCR amplification is automatically controlled for as the absence of the 233 bp product implies a failed reaction. In the absence of an internal restriction control site however, the MTHFR genotype based on digestion patterns could be incorrectly interpreted in the event of a partial or failed digestion. By moving the forward primer further upstream we have created an additional *Hinf*I restriction site in the PCR product. The use of this new forward primer, directly controls for the diagnostically relevant *Hinf*I restriction digestion of the mutant allele, by allowing an additional restriction to take place in both the normal and mutated alleles.

Received February 18, 1999

Received in revised form March 25, 1999

Accepted March 30,1999

Table 9: Primers used in the PCR spanning the 677C/T allele of the MTHFR gene

Primer name	Nucleotide Co-ordinates	Primer sequence
MTHFR:f (original forward primer)	644 to 666*	5' tga agg aga agg tgt ctg cgg ga 3'
MTHFR:x (new forward primer)	609 to 634#	5' cga agc agg gag ctt tga gTc tga c3'
MTHFR:r (reverse primer)	*	5' agg acg gtg cgg tga gag tg 3'

'T' - depicts the base changed in the primer to generate the control restriction site.

* Frosst *et al* 1995 [69].

Derived from MTHFR gene sequence published by Goyette *et al* (1994) [129].

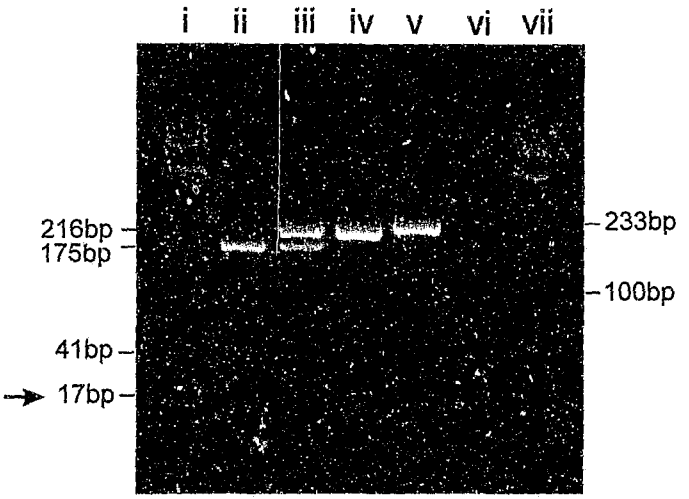

Figure 27: A 3% (w/v) agarose electrophoretic gel depicting the PCR products of the MTHFR gene pre- and post digestion. Lanes i and vii, 100 bp molecular weight ladder (Boehringer Mannheim); Lane ii, is an individual homozygous for the 677T allele; lane iii, is an individual heterozygous for the 677T allele; Lane iv, a normal individual; Lane v, undigested PCR product; and Lane vi is a reagent blank. The arrow depicts the 17 bp fragment, which confirms that digestion has occurred.

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Additional information:

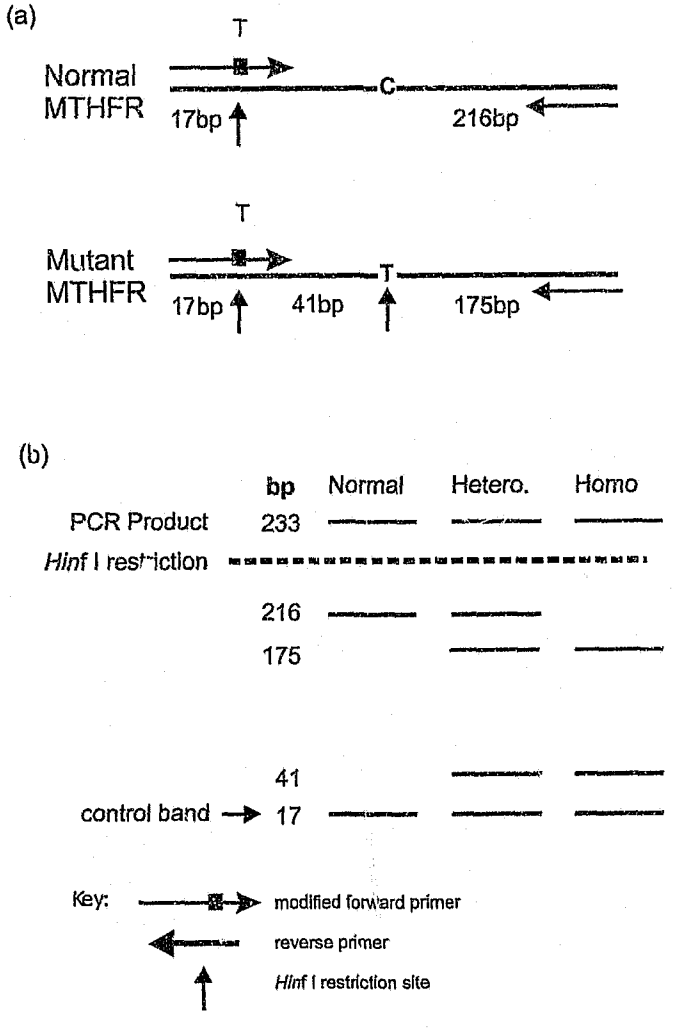

Figure 28: Schematic representation of (a) the MTHFR C677T mutation and (b) its restriction pattern with *Hinf*I post PCR.

3.4 Multiplex PCR: factor V Leiden and the prothrombin 20210A

- Journal article: Development of a Simple Multiplex PCR for the Simultaneous Detection of the Factor V Leiden and the Prothrombin 20210A Mutations.

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Molecular Diagnosis, 1999, 4, (3), 247-250.

3.4.1 Journal article

Molecular Diagnostics Vol. 4 No 3 September 1999

Development of a Simple Multiplex PCR for the Simultaneous Detection of the Factor V Leiden and the Prothrombin 20210A Mutations.

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Johannesburg, South Africa, Charlottesville, Virginia.

Key Words: Multiplex Polymerase Chain Reaction, Restriction control, *Hind* III.

Running Title: Multiplex PCR for Prothrombin and Factor V mutations.

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Abstract

Background:

The demand for thrombophilia testing at the molecular level is increasing, and consequently so is the work load of the routine molecular laboratory. Efforts to lighten the work load, economize on time, and strive for reduced costs while still maintaining quality assurance are thus necessary.

Methods and Results:

A multiplex polymerase chain reaction (PCR) for the detection of the factor V Leiden and the prothrombin 20210A mutations was designed that enables the use of the same inexpensive restriction enzyme, controls for the digestion, and produces easily interpretable results.

Conclusions:

The use of this new multiplex PCR and digestion analysis enabled us to simultaneously perform a routine screen for the factor V Leiden and prothrombin 20210A mutations.

Introduction

Venous thrombotic disease is a common health problem contributing considerably to morbidity and mortality in the population. The factor V Leiden and the prothrombin 20210A mutations are currently described as the most common known genetic risk factors for venous thrombosis in Caucasians [1,2] [114 117]. The factor V Leiden mutation has been the prototype for the use of the Polymerase Chain reaction (PCR) in the detection of the mutations related to thromboembolic disease [3] [115]. The use of the *Mnl* I restriction enzyme site, abolished by the factor V Leiden mutation, is often the method of choice for this analysis [3] [115]. Other investigators [4] [133] relied on the creation of a *Hin*d III site by the same mutation for their analysis of the factor V gene.

Detection of the presence of the 20210A/G allele in the human prothrombin gene is also easily achieved by amplification using primers designed to span this region. Discrimination between these two alleles is achieved because the downstream primer creates a *Hin*d III restriction site should the 20210A variant be present [5,6] [68 48]. The upstream primer incorporates a naturally occurring *Hin*d III site that, because it is present in both alleles, serves as an internal digestion control [6] [48]. If the occurrence of digestion failure is not recognised, erroneous genotyping will occur. To combine both the factor V Leiden and the prothrombin 20210A PCR techniques into a multiplex PCR and digestion would obviously be prudent in a busy routine laboratory. Whereas multiplex techniques involving these

prothrombotic mutations have been reported [7-9] [130 131 132], these did not include a control site for failed digestion.

Using both these PCR techniques [4,6] [133 48] we designed a cost effective, multiplex PCR for the factor V Leiden and the prothrombin 20210A mutations that exclusively requires the use of only one restriction enzyme (*Hind* III) in a single PCR reaction, to generate products pre and post digestion that are easily distinguishable from one another. In addition, this multiplex PCR technique uses the *Hind* III control digestion site from the prothrombin PCR to control for the factor V digestion, as well.

Using this multiplex PCR we analysed the DNA of 60 unselected patients with venous thromboembolic disease.

Methods

DNA extraction

Using the method described by Talmud *et al* [10] [88], DNA was extracted from whole blood collected in ethylenediaminetetraacetic acid from 60 patients presenting with venous thromboembolic disease. The specimens were frozen and then thawed to facilitate lysis of contaminating red cells before DNA extraction. Four hundred microlitres of freshly prepared ammonium chloride, 0.17M, was added to 100 µl of thawed buffy coat specimen. This was mixed well by inversion and then left at room temperature for 20 minutes before being spun down in a microcentrifuge for 30 seconds. The supernatant was then discarded, the pellet resuspended in a 0.9% solution of sodium chloride by vortexing and then centrifuged again. This washing process was repeated three times. After the third wash, the pellet was resuspended in 200 µl 0.05 M sodium hydroxide. This was boiled for 10 minutes and neutralized by the addition of 25 µl Tris hydrochloric acid, 1 M (pH 8.0). The extracted DNA was approximated by spectrophotometric means and stored at -20 °C until further analysis. All studies were carried out with ethical clearance from the Committee for Research on Human Subjects (University of the Witwatersrand, Johannesburg, South Africa).

Amplification

PCR amplification was carried out using the forward and reverse primers for both the factor V Leiden and the prothrombin 20210A mutations (Table 1) [Table 10]. Each 50 µl reaction contained approximately 0.5 µg genomic DNA, 0.5 µM of each primer, 1.5mM $MgCl_2$, 200 µM of each dNTP and 1.25 U Taq DNA polymerase (PCR Core Kit, Roche). A first denaturation step at 94°C for 3 minutes was followed by 35 cycles of 94°C for 1 minute, 62°C for 1 minute and 72°C for 2 minutes followed finally by 5 minutes at 72°C. Thirty microlitres of PCR product from each sample was digested with 15U *Hind* III (Roche) and visualized. In order to aid the interpretation of the results, the gel could be visualized more than once during the fragment separation, and restained with ethidium bromide if necessary.

Results

Clear, interpretable bands were obtained from the multiplex PCR and subsequent digestion (Table 2) [Table 11]. As shown in fig. 1(lane iii) [Figure 29], the expected products of 473 bp and 241 bp were obtained after PCR but before digestion. Subsequent disappearance of the 473 band (lanes iv – viii) indicated that digestion had been successful, and the generation of the 407 bp band indicated that the patient had a normal prothrombin allele(s) (lanes iv – viii). The appearance of a 334 bp band indicated that the patient had an allele with the 20210A prothrombin mutation (lanes v and vii). An undigested 241 bp factor V band indicated that the factor V Leiden mutation was absent in at least 1 allele (lanes iv – vii), whereas the

generation of a 209 bp band was evidence of the factor V Leiden mutation being present in an allele (lanes vi – viii). Of the 60 patients investigated for thromboembolic disease, 7 patients were heterozygous for the factor V Leiden mutation, 1 was homozygous for the factor V Leiden mutation, and 5 were heterozygous for the prothrombin 20210A mutation. These findings concurred with the genotypes obtained when all these samples were previously screened for factor V Leiden and the prothrombin 20210A mutation independently. No patient was found to have both mutations.

Discussion

The demand for thrombophilia testing at the molecular level is increasing and consequently, the work load of the routine molecular laboratory is also increasing. Efforts to lighten the work load, economize on time and strive for reduced costs are being made globally. Also important is the need at all times to attain and maintain interpretable results and rigorous quality control.

By combining the factor V Leiden PCR and the prothrombin 20210A PCR into 1 conventional PCR reaction, we have saved on time and cost of the analysis. The PCR and digestion products are grouped and easily distinguishable from one another. In addition, only 1 restriction enzyme is now required instead of 2, and we have done away with the relatively expensive *Mnl* I.

Finally, without the presence of an additional *Hin*d III restriction site serving as a digestion control, the factor V Leiden analysis on its own is flawed. However, by the

incorporation of the prothrombin PCR and its digestion control, we feel that a failed or partial ' stion in the factor V Leiden analysis, is better controlled for. Should only one mutation analysis be requested this can be performed using the individual primer sets. Practically however it would be easier to perform the tests as a multiplex analysis. Should the unrequested analysis reveal a mutation, we would advise that the attending clinician be informed.

In summary, we feel that both the factor V Leiden and the prothrombin 20210A mutations are important risk factors for thrombophilia and need to form part of routine patient screening. Their inclusion into a multiplex PCR and digestion in the laboratory investigation is thus considered prudent and expedient.

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Table 10: Primers Used in the Multiplex PCR of the Factor V Leiden and the Prothrombin 20210A/G Alleles

Primer name	**Primer sequence**
Factor V – 10A (forward primer) †	5' tca ggc agg aac aac acc at 3'
Factor V – 506 (reverse primer) †	5' ggt tac ttc aag gac aaa ata cct gta aag ct 3'
Proth:x (forward primer) #	5' cct gat gaa ggg aaa cga ggg 3'
Proth:r (reverse primer) * #	5' ata gca ctg gga gca ttg aag c 3'

† Gandrille *et al* 1995*[4] [133]

Lastrucci *et al* 1999 [6] [50]

* Poort *et al* 1997 [5] [68]

Table 11: Diagnostically Relevant Fragment Sizes Predigestion and Postdigestion of the Factor V and Prothrombin PCR Products

GENE	FACTOR V	PROTHROMBIN
Fragment size, undigested	241	473
Normal allele post digestion	241	407
Mutated allele post digestion	209	384

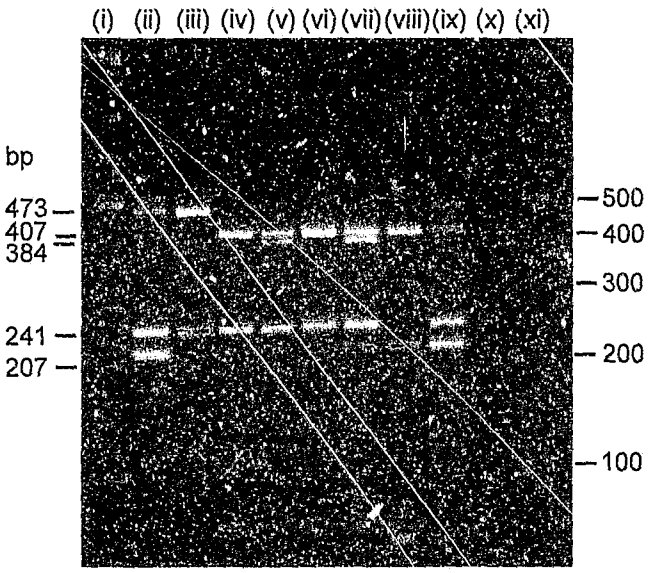

Figure 29: A 2% (w/v) agarose electrophoretic gel depicting the multiplex PCR products of the factor V and prothrombin genes (listed in Table 2) [Table 11]. Lanes i and xi, 100 bp molecular weight ladder (Roche); lane ii and ix, an in-house molecular weight marker made by the authors specifically to depict the expected fragments. Lane iii, undigested PCR product; lane iv, a normal individual; lane v, an individual heterozygous for the prothrombin 20210A allele; lane vi, an individual heterozygous for the factor V Leiden allele; lane vii, a sample obtained from an international quality control scheme, heterozygous for both the prothrombin 20210A and factor V Leiden alleles; lane viii, an individual homozygous for the factor V Leiden allele; lane x, a reagent blank.

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4 QUALITY ASSURANCE

4.1 Introduction

The aim of this chapter is not to be a comprehensive treatise on quality control and quality assurance in the molecular laboratory but to focus on aspects of molecular analysis in the developing world.

Molecular medicine has been notoriously tardy in defining reference methods, which are as yet not as well developed as those for the traditional chemical and haematological pathology laboratories. However, the development of this field is a rapidly evolving process and definitive guidelines will become readily available with time, as they have in other medical fields.

Molecular technology is a powerful tool for obtaining data on a wide range of cells. Its use has been transferred from the research situation into the clinical arena, where molecular diagnosis has become indispensable as a resource for the qualitative detection and identification of genotype, for the analysis of mutations, for population discrimination and for the quantitation of gene transcripts. The clinical utility of molecular technology is evident for both qualitative and quantitative analyses. Procedures that identify malignant populations or disease-associated mutations in haematopoietic cells may be considered qualitative in nature. For diagnostic, as opposed to prognostic or

monitoring purposes, it is usually important only to know whether or not the abnormal genotype is present. However, evaluations that require the quantitation of diseased populations in a background of normal cells are becoming more prevalent in routine clinical laboratories. Both types of molecular analysis require that laboratories establish comprehensive quality assurance (QA) programmes and maintain rigorous documentation of their analyses.

The objective of QA is to achieve accuracy in generating a correct result and to ensure its reproducibility and precision. Ensuring that the sample handling, instrumentation, reagents and techniques employed are checked and controlled for, will go a long way to generate accurate and precise results. The precision of a result is measured by replicate testing, by comparison to previously tested samples and the statistical analysis of these comparisons. Accuracy can be measured by comparison to reference or standard materials of which the outcome is known. Dacie and Lewis [134] recognises four components of QA: internal quality control (IQC), external quality assessment (EQA), proficiency surveillance and standardisation. These issues are addressed with special reference to a molecular diagnostic laboratory in a country with limited resources. Issues dealt with include sources of uncertainty, the need for procedural standardisation, the need for reference measurement procedures and current methods of IQC and EQA

4.2 Sources of uncertainty and their quantitation

4.2.1 Technical

The QA programmes for molecular diagnostic techniques should be designed to control for all of the sources of error and variability in procedures. One way to consider these sources of variability is to view the process critically, identifying the assumptions that are made in order to generate results.

In general, a laboratory's QA programmes are established to ensure that correct results are obtained. The QA programme monitors and evaluates the effectiveness of laboratory policies and procedures for the pre-analytical, analytical and post-analytical testing phases [135].

- Pre-analytical testing
    - Specimen collection, transport and storage - the maintenance of specimen integrity.
    - Specimen processing, to ensure consistency and that minimal variability is introduced during sample handling and processing.
- Analytical testing
    - Instrument sensitivity and performance.
    - Reagent specificity and reagent reproducibility.
    - Considerations particular to molecular technology - the detection of contamination and inhibition, illegitimate product generation, restriction

failure, DNA contamination of RT PCR and tube to tube variation in quantitative PCR.

- Post-analytical testing
    - Result analysis, to ensure sample analytical reproducibility.
    - Data handling, its acquisition, analysis, storage and reporting.

Good quality control programmes in existing routine haematology laboratories test the validity of the assumptions on a regular basis, which could be hourly, daily or monthly. In the routine haematology laboratory, the analytical instruments are checked for reproducibility first, using replicate samples of fresh whole blood. The coefficients of variation are calculated and these should not exceed the acceptable limits that are defined for the different parameters. Every ½ hour, or every 20th sample, aliquots of the same material source are checked and using the cumulative sum method (CUSUM), drift away from the original mean is monitored [134]. It thus implies that all the variables that can be controlled in the analytical process have been monitored and found to be satisfactory. These methods are less applicable to qualitative data, but have relevance to the quantitative measurement of RNA levels, as an indication of gene expression. For each assumption quality control assessments must be built into the testing protocol in order to assure accurate results [135].

4.2.2 Biological

There are a number of factors that could result in biological uncertainty. It needs to be established exactly which biological entity is being measured and what the interpretation

of the result is going to mean. The measurement of RNA versus DNA often can have two different meanings. RNA variation is indicative of gene expression and viability whereas DNA is an easily detected but fixed entity. This can have biological significance. Not dealt with in this thesis is the issue of the viability of certain infectious agents as determined by the presence of their RNA (dictating active gene transcription) as apposed to DNA.

Cancer is a multi-step evolutionary process and often its diagnosis at the molecular level is subject to debate. When certain entities related to tumourigenesis e.g. t(14;18), are found in normal tissue, questions are raised as to the significance, if any, of these findings [55 54]. In the case of minimal residual disease (MRD) there is uncertainty as to what a negative/positive result using a molecular technique such as PCR really means in the clinical setting. Some studies have shown that in patients that have residual disease, the presence of 1 malignant cell in 10^2 to 10^3 normal cells is predictive of relapse. At even lower levels (1 in 10^4) there is a greater chance of continuous clinical remission [136 137]. Single positive results soon after therapy are not indicators of relapse, but negative results post therapy appear to predict good long term disease-free survival [137]. Molecular assumptions made in order to carry out a molecular technique can be flawed. For example, the success of RFLP-based analyses is well known and often quoted as the 'gold standard' for PCR based diagnosis. However, a note of caution was issued recently by the Association for Molecular Pathology (AMP) regarding the over estimation of the number of patients homozygous for the C282Y haemochromatosis mutation. This was due to the

presence of an unknown, frequently occurring polymorphism in one of the primer binding sites [138].

4.2.3 Nosological

Initially, in the classification of tumours, lineage was interpreted by morphology. Immunophenotyping then introduced an aspect that went some way to identify the molecular nature of the tumour. With the advent of molecular technology it was found that homogeneous morphology often concealed a molecular heterogeneity. This was illustrated in a paper in Nature recently [58], where it was shown that the molecular classification of tumours on the basis of gene expression could identify clinically significant and previously undetected subtypes of cancer. Two molecularly distinct forms of diffuse large B-cell lymphoma (DLBCL) were identified in patients with DLBCL correlating with two different overall survival rates [58]. On the other hand, molecular technology identified molecular homogeneity amongst what initially appeared to be morphologically heterogeneous populations. Thus, according to the French, American and British (FAB) classification of acute myeloblastic leukaemia (AML), AML is classified into 8 morphological subgroups (M0 – M7 inclusive) [139 140 141 142], yet Golub [143] using molecular characterisation showed recently that there were functionally only 3 broad genotypic groups in AML. Using microarray technology, molecular self-organisation mapping, class discovery and class prediction, Golub and colleagues were also able to predict leukaemia subtypes with 100% accuracy [143 144]. In this study it was shown that patients with either AML, T-cell ALL or B-cell ALL could be distinguished from one another, entirely on the basis of gene expression pattern recognition.

4.3 Need for procedural standardisation - what can and cannot be standardised?

4.3.1 Specimen collection, transport, and storage

It is often assumed, erroneously, that the end result is not biased by specimen collection, shipping, or storage, yet with reference to most analytes (DNA is something of an exception), once the cells/tissue leave the body and are collected, the specimen begins to deteriorate [11]. The rate of deterioration depends on the anticoagulant used to collect the specimen, the temperature at which the specimen is stored, and the length of time elapsed before it is processed. For this reason, it is essential to standardise the shipping and storing procedures and to select the proper anticoagulant/media for specimen collection. Cut-off times for each type of specimen/test need to be determined and implemented.

One reason for complexity of QA in the pathology laboratory is the type of sample received. Some specimens are fresh, some are fixed and others arrive days after dispatch from outlying rural facilities. Laboratories need to know how these variables might influence the testing procedures and if necessary, have the appropriate control samples or data should these be required. Depending on the test, the molecular procedure may require either RNA or DNA to obtain a result. In the event of RNA the sample must be received fresh. DNA testing, however, has a significant advantage in that it is extremely stable under many environmental conditions, and testing can generally still be carried out on

destroyed, degraded, fixed, old and even ancient tissue. (The paper presented in appendix 2 illustrates this phenomenon) [a].

4.3.2 Specimen processing

Different methods of processing can affect results in different ways, depending on the sample type (blood/tissue) and how it is stored (fresh/frozen/formalin-fixed). The above determinants result in qualitatively different molecular analytes, and this has to be considered in conjunction with the assay to be performed. For example, different DNA/RNA extraction methods are employed for different types of specimen and/or storage method used. It is important to know how the specimen processing affects the success of the downstream analysis and the final results. This also means that it is important that there be consistency in the method used to process the specimen so that the *results can be compared with other results obtained at a different time.* Standard guidelines of IQC and EQA programmes are ideal ways of dealing with result variation due to processing differences and inconsistencies.

4.3.3 Instrumentation

The purpose of instrument quality control is to ensure that the instruments used, operate identically from day to day. These would not only include PCR based equipment [e.g.: conventional PCR thermal-cyclers, Light Cycler (Roche), COBAS AMPLICOR (Roche) and the ABI sequence detection systems (PE Biosystems)], but also pipettes, heating and

[a] Urbani C, **Dubreuil Lastrucci R**, Kramer B. The effect of temperature on sex determination using DNA-PCR analysis of dental pulp. Journal of Forensic Odonto-Stomatology, 1999; 17 (2): 5-39.

cooling devices, and centrifuges. Instrument quality control must test all of the components with respect to established specifications. Some of the instrument checks should be performed at the beginning of each run to show that the instrument meets minimal requirements, and many of the thermal-cyclers for PCR have inbuilt checks that are automatically carried out and recorded on start-up. Others, such as the pipettes, heating blocks, water baths, fridges and freezers need checking and/or calibration on a monthly or quarterly basis.

4.3.4 Reagents

Reagents can vary from manufacturer to manufacturer, lot to lot and under different environmental conditions. With regard to sensitive molecular techniques these variations can cause an analysis to fail, and any changes need to be controlled. Optimisation for local conditions should also be considered essential. When a test is implemented and routinely run in the laboratory, it needs to be evaluated according to either the manufacturer's specifications or the literature. This is usually done by means of relevant positive and negative controls. If the desired results are not obtained, the test will need to be optimised and this is dealt with in section 4.3.5.1.

4.3.5 Considerations particular to molecular technology

Molecular techniques as a rule are exquisitely sensitive. The power of PCR as a diagnostic tool lies in its sensitivity and the ease with which it can be carried out. Inherent in this, however, lie many problems associated with the use of PCR in the

clinical laboratory. The extreme sensitivity of PCR means that there is a tendency to produce false positives. On the other hand, the potential fastidiousness of the enzymes can result in a failed reaction and thus false negative results. No diagnostic test is of any value when the possibility of incorrect results is high. Correct and precise control is however, achievable in most cases with the use of the correct controls and procedures (section 4.5), a little innovation and some modification of existing protocols. The publications emanating from this thesis (sections 2.2, 2.5, 3.2, and 3.3), are examples of what can be achieved in respect of these aspects of molecular quality control.

4.3.5.1 PCR optimisation

Optimisation of any molecular protocol is essential to its success in both research and routine analyses. PCR optimisation is no exception and the following examples all play a major part in reducing the occurrence of failed PCR (false negatives) and spurious products (false positives), and obtaining the best, most reliable result [145 146]:

- Variations in temperatures
- Cycle number and times
- Nucleic acid contamination
- Reagent concentrations.

To complicate matters, these variables also act in concert with each other and thus need to be optimised in relation to one other in a matrix type analysis to establish the best set of conditions for any particular assay.

4.3.5.2 Contamination and false positives

A standard 30 cycle PCR reaction produces approx. 10^9 amplicons per natural molecule. Their relative abundance as contaminants in the laboratory thus presents a major source of contamination and therefore false positives, should they infect a sample [146]. It is essential that the possibility of contamination is minimised and contaminants, if present, destroyed using uracil-N-glycosylase (section 4.5.5.1).

4.3.5.3 Inhibition and false negatives

PCR is an easily inhibited reaction, and a common problem in routine PCR for diagnostic purposes is the presence of inhibitors in clinical samples which would cause false negative results. There is a need for simple procedures that permit isolation of good quality DNA and RNA but exclude inhibitors of the PCR reaction. Each PCR system is different in its sensitivity to inhibitors. While one system may be robust enough to tolerate the inhibitors present in a crude DNA extraction, another may be extremely sensitive to their presence. It is essential to monitor for the occurrence of inhibition in each sample [146] (section 4.5.5.2). With regard to RFLP PCR, the failure of the PCR reaction is automatically controlled for by the absence of a product. The failure of digestion however, is often overlooked due to the lack of adequate restriction controls. The objective in publications 3.2 and 3.3 (emanating from this thesis) was to design restriction controls for the prothrombin G20210A and methylenetetrahydrofolate reductase (MTHFR) C677T PCR mutation analysis.

4.3.6 Result analysis and data handling

Consistency from sample to sample is an essential prerequisite for an efficient laboratory. Depending on the test, data can be generated in many different forms e.g. automated printouts, ELISA readings, electrophoretic gels and/or membrane detections. Automated instruments often generate clear cut results and analyses, leaving very little to be interpreted by the user. However, systems such as ELISA, electrophoretic gels and membrane based assays still need interpretation by the user, and stringent guidelines need to be put in place for their analysis. Computer data programs that allow the logical, accessible storage of data are already in place in the routine laboratory and the downstream reporting procedure will already be well established. These tried and tested systems can be utilised by the molecular laboratory to store and issue results.

4.4 The need for reference measurement procedures

Reference measurement procedures could conceivably take the form of either reference material or a reference method. As most of the tests done in a molecular laboratory are still qualitative in nature (i.e. an entity is either present or absent), conventional reference measures are not necessarily applicable. With molecular diagnostics the definitive test is ultimately the actual nucleic acid sequence of the sample or PCR product. With the availability of the entire human genetic sequence recently becoming a reality, and with sequencing techniques becoming standardised and automated, it is possible for any result to be sequenced to check for its validity. In fact, the chances of an incorrect sequence that

would give the same result is as little as 1 in 1.6×10^{60}, and thus makes sequencing an ideal reference method for validating molecular methodology.

For qualitative molecular assays, independently authenticated positive and negative controls largely replace reference material as a means of ascertaining the validity of the test being performed, and these were employed routinely in the present study. Reference material could be sequenced material as discussed above or, more practically, positive and negative controls obtained independently. For example, an ideal reference material for use in the t(14;18) PCR analysis, would be a sample that has been cytogenetically shown to contain this translocation.

Thus for qualitative determinations the sequence definitive of the disease would serve as a reference measurement, whereas with quantitative determinations, the situation is more difficult and these will have to evolve with time, following principles established in the traditional haematology laboratory.

4.5 Current methods of IQC - can these be adapted to a developing country?

Many of the IQC procedures that are required in a molecular diagnostics laboratory equate with similar procedures in other pathology laboratories, and all of them are potentially implementable in a developing country. It is however important not to lose sight of the need to contain costs in a developing country; if unnecessary, elaborate quality control will increase these costs, the cost saving objective could be defeated. On the other hand

QA in a developing country must continually be mindful of these principles. It is worse to produce an unreliable result, than no result at all. Many of the simpler procedures (similar to some of those discussed in this thesis) as well as the more automated ones, in fact require minimal quality control maintenance, provided the inherent and appropriate checks and controls are in place.

The following are the IQC procedures carried out by the SAIMR Molecular Diagnostics Laboratory, Department of Molecular Medicine and Haematology, University of the Witwatersrand.

4.5.1 Specimen collection, transport, and storage

Due to the sensitivity of some of the specimens and tests, directives regarding the handling of samples for each test are issued to clinicians and phlebotomists. Records of the time of collection, time of receipt and all other relevant information is kept on all specimens. Ideally, specimens not complying with the directives should be discarded, however these specimens are generally still processed and the results subsequently obtained are viewed in light of this fact.

4.5.2 Specimen processing

Extensive standard operating procedures (SOPs) as well as working procedures have been laid down for all samples and tests done, and are reviewed annually. These are strictly adhered to, with no deviation. Stringent sample identification and documentation is provided for each sample. Bar coding is used to identify the sample before, during and

after analysis. An audit sheet is kept for each specimen recording every procedure that is carried out, the reagents and methods used, the results and their interpretation.

4.5.3 Instrumentation

The implementation of instrument quality control procedures can control for a number of potential sources of variability regarding instrumentation. Initial start up, calibration and periodic re-calibration of thermal-cyclers, thermometers, biohazard hoods, heating blocks, fridges, freezers, balances and pipettes need to be performed regularly. Instruments such as the PCR-based thermal-cyclers and biohazard hoods are placed on maintenance programmes where the suppliers carry out maintenance and calibration at 6 to 12 monthly intervals. In addition to this there are daily start up procedures self-initiated by the PCR thermal-cyclers, and on a monthly basis, user initiated checks are carried out. Thermometers are checked against SABS (South African Bureau of Standards) approved thermometers, and these in turn used to test and calibrate other heating and cooling equipment. The temperature of fridges and freezers is checked daily and charted. They are mapped inside for consistency of temperature and are all fitted with alarms. Pipettes are calibrated quarterly. Biohazard hoods, heating blocks and centrifuges are swabbed down with ethanol and distilled water on a daily basis, to maintain sterility and to prevent contamination. In order to validate plugged tips, it is important to check the performance, effectiveness and the integrity of the aerosol barrier pipette tips.

4.5.4 Reagents

There is stringent stock control to monitor lot, usage and expiry dates of all reagents used. Expiring reagents are marked and either discarded or used in practice research and development sessions. When new/fresh reagents are brought into use, there is a period of reagent overlap where the incoming reagent is checked against the older one.

4.5.5 Considerations particular to molecular technology

4.5.5.1 Contamination and false positives

When many of the commercially available kits are used, contaminating amplicons are destroyed using uracil-N-glycosylase. Prevention of contamination is accomplished by physical separation of the different stages of PCR: reagent and template preparation, PCR set up and post-PCR analyses. The use of dedicated pipettes, coats, gloves, pens and workbooks at each step is necessary [146 147]. Laboratory techniques such as sterile procedures, glove changing and careful pipetting to prevent aerosol formation are exercised. The aliquoting of reagents and constant surveillance for contamination are essential steps to control for false positives. Low copy number for positive controls, a number of negative controls and reagent controls are incorporated into every run. False positives may also arise when primers are able to amplify DNA other than their designated target. An example of this is where primers designed to span and amplify the t(14;18) major breakpoint, have also been shown to amplify Epstein-Barr viral DNA [56]. Additional methodology was thus incorporated to confirm a positive result (section 2.5).

4.5.5.2 Inhibition and false negatives

It is essential to monitor for the presence of inhibition in each sample. This is achieved by 'spiking' the sample with a known control, which although it represents the 'target', can still be distinguished from it [148]. There are various types of internal controls ranging from unrelated genes such as β-globin in the immunoglobulin gene rearrangement PCR (section 2.4), to true 'competitors' using the same primers as the target, but which are slightly different in size or sequence. The latter two can be designed with various levels of ease. Although designed specifically for quantitative RT-PCR, the principle of xeno-competitive PCR developed and published as an original contribution from this thesis (section 2.2), could be used as an internal control for inhibition.

4.5.5.3 Standards and controls

Controls and checkpoints are designed to alert the worker to problematic results. Over and above this, controls need to be employed to detect contamination and inhibition, illegitimate product generation, restriction failure, DNA contamination of RT PCR and tube to tube variation in quantitative PCR (sections 2.2, 2.4, 2.5, 3.2 and 3.3).

Every PCR run should include positive and/or sensitivity controls, negative controls and a reagent blank. The negative or blank control should always be replicated within a run and should yield only background signal. The positive control, if possible, should generate a moderate signal. If the positive control yields a negative or unusually weak signal, the entire PCR run should be repeated. A positive result with the negative or blank control

necessitates that all reagents be checked for amplicon or target DNA contamination or both. When nested PCR is performed, every test sample should be alternated with a negative control to check for carryover during transfer of first-round PCR products into new tubes [149]. Controls can be RNA/DNA extracted from cell lines, validated clinical specimens, cloned DNA or purchased positive/negative controls.

Workers in the field will attest to the fact that fragment size is not sufficient to establish that the correct product is present. In order to do this, the fragment generated is checked by some other means at least once, preferably periodically. Possible methods of validation include sequencing, enzyme restriction, secondary PCR and probe hybridisation. An original contribution from this thesis presented as a published manuscript in section 2.5, is valuable in this regard.

Whilst in the detection of the factor V Leiden mutation, failed digestion was controlled for by the inclusion of a published control digestion site in both the normal and mutant Alleles [65], such published controls were lacking for prothrombin G20210A and the MTHFR C677T mutation analysis [69 130]. This vacuum was the justification for the published manuscripts presented in sections 3.2 and 3.3. Should these digestions fail without the presence of a restriction control, patients could have been incorrectly genotyped. The addition of control digestion sites in both these tests, alerts the user to its occurrence.

RT-PCR necessitates additional steps to control for DNA contamination of RNA, failed reverse transcription and RNA degradation. DNA contamination of RNA may not necessarily yield false positive results. However, should this be a concern, primers need to be designed to span an intron, thus signalling the presence of DNA by the generation of a larger band or no band all. Positive controls are exclusively RNA molecules, and are placed in the run at the outset. Both degradation and failed reverse transcription are thus controlled.

Quantitative, 'real time' PCR is becoming the PCR of choice for routine diagnostic work. The newer, automated equipment such as the Light Cycler (Roche) and the ABI PRISM sequence detection systems (PE Biosystems) all make use of technology which allow cycle by cycle amplicon monitoring. Quantitative PCR is one of the most complex PCRs to analyse, due to tube to tube variation disallowing the direct quantitative comparison of two results from two different tubes. The placement of a 'competitor' or a control in all the tubes is essential to monitor and then compare the PCR performance between two or more tubes (section 2.2). This, although in practice at the SAIMR Haematology Department, probably falls outside of the developing country context, at least for the time being.

4.5.5.4 Primer design

Primer design is an essential part of PCR optimisation. Inefficient primers may contribute to failed PCR reactions as well as incorrect product generation. The primer design is the core principle of the PCR diagnostic protocol. Some of the PCR primer design principles are illustrated by the following:

(a) Chromosomal translocations bring two normally distant primers into proximity of one another to generate a product.

(b) Mutations are spanned and often modified to allow subsequent restriction digestion or sequencing.

(c) IgH gene rearrangement PCR relies on the primers spanning the area where these rearrangements occur and so doing could detect the presence of a single monoclonal band.

(d) The presence or absence of genetic entities (e.g. infectious agents) relies on specific primer design. Consequently, diagnosis is based on the presence or absence of a PCR product.

Primers should be chosen to be as robust as possible, without promiscuous binding to any other sequences. Primers should span and incorporate any controls necessary for the accurate and confident analysis of results; such as restriction controls, internal controls, consensus regions and introns in the case of RT-PCR. Examples of such restriction controls are extensively demonstrated in chapter 3 and the consequence of failed restriction discussed. RT-PCR and internal controls are presented in publication 2.2.

4.5.5.5 Hot starts

Hot starts prevent the spurious association of the polymerase, primers and target DNA before the correct, stringent temperatures are reached. False products and artefacts can be obtained if these associations are allowed to occur. There are numerous ways of accomplishing a hot start, some involve physical separation of the reagent components

and others modify the polymerase so that it is inactive until sufficiently high temperatures are reached, however, many of these techniques are expensive. A simple, inexpensive method to prevent the polymerase from extending spurious nucleic acid associations at low temperatures is to set up the PCR reaction on ice. Once the reaction mixture is prepared, the tube should be transferred directly onto a hot PCR block (94°C), ensuring that the tube is at no point at ambient temperatures where promiscuous associations can occur.

4.5.5.6 Systems of quality assurance relating to research and development

All projects requiring molecular investigations have approved protocols by the relevant controlling bodies which clearly stipulate the methods of analysis and quality control programmes required. These guidelines are strictly adhered to by laboratory staff and the involved researchers are supplied with the relevant documentation. Wherever possible the same instrument is used for all analysis of a particular project to avoid the problem of instrument variability. All analytical kit evaluations are run concurrently with the approved methods and are usually run in conjunction with trials in other laboratories. Only the results obtained from the approved method of analysis are made available to clinicians. For new test validation, clinical sensitivity is established on a number of known positive and negative samples that have been previously evaluated using validated methodology. The two methods are then executed in parallel for a number of runs to check the validity of the new method.

4.5.6 Result analysis and data handling

Well constructed qualitative tests with the appropriate controls should leave little room for error when analysing the test results. Providing all the correct maintenance checks have been carried out, results generated from automated procedures should pose no problem. However, each result obtained is checked by at least two experienced, laboratory personnel before being issued. All the parameters surrounding the test are rechecked, such as instrument reliability and controls. Once a result is confirmed, it is entered into the laboratory computer against the patient information and then made available to the clinicians/pathologists.

4.6 Current methods of EQA - can these be adapted to a developing country?

EQA is essential in a molecular diagnostics laboratory and is arguably more important in a developing country, where reliance on the result may be greater than in a developed country, specifically so that the cost savings potential can be realised. Belonging to a sample exchange programme is a simple and inexpensive exercise, and it would become immediately apparent if any laboratory is unable to produce and assure reliable results. One advantage of DNA molecular technology, is that DNA is very stable and readily lends itself to EQA sample exchange programmes, without undue concern for its degradation during transportation.

Variations in procedures within a laboratory and between laboratories can lead to inadequate and faulty results. The ultimate standardisation of molecular techniques is

essential to eliminate problems arising from variations, such as reagent concentrations, reaction temperatures and different equipment suppliers. Initially, quality control programmes in molecular diagnostics were difficult to establish and direct inter-laboratory comparisons were a practical problem. Today, various externally based testing programmes have become available to provide an indication of a laboratory's performance in nucleic acid typing.

A multi-centre study to assess the need for standardisation of the IgH and T cell receptor (TCR) PCR protocols, was initiated and set up by WS Stevens of the SAIMR Department of Haematology between six centres – five in the UK and our routine PCR laboratory. Again, there was considerable variation in the results obtained depending on the centre, the test and the protocol, highlighting the need for standardisation of these analyses. Although the sample size was small, there appeared to be a significant difference in results between laboratories for B cell clonality assays with the immunoglobulin heavy chain gene rearrangement using primers directed at framework region 3. In addition, there also appeared to be significant differences between laboratories for T cell receptor β chain gene rearrangement studies. The problem is however not unique to this programme.

At the Association for Molecular Pathology (AMP) meetings in 1998 and 1999, multi-centre studies set up over the USA for Ig and TCR gene rearrangements were presented. The 1999 studies showed a fairly large range in the malignancies detected:

- B-cell: 57 – 94 % (average 77 %)

[11 – 80 % (mean 36 %) false negative, and

0 – 20 % (mean 5%) false positive results]

- T-cell: 75 – 95 % (average 86%)
  - Jh/bcl2: 35 – 75 %

The conclusion was that there was substantial variability in the methodology employed by the different laboratories, ranging from the DNA extraction and enzymes used, through to the final analysis and detection.

The SAIMR Molecular Diagnostics Laboratory has recently joined an AMP initiated sample exchange programme with respect to the t(14;18) and t(9;22) translocation, the latter specifically for use on the Light Cycler (Roche).

Our Haemostasis and Thrombosis laboratory has joined three international thrombophilia external quality assessment sample exchange schemes. These are ECAT (European Concerted Action on Thrombosis) involving 85 laboratories in 14 different countries (March 1998), UKNEQAS (United Kingdom External Quality Assessment Schemes) and the RCPA (Royal College of Pathologists of Australia) molecular diagnostics QA programme. To date samples received and processed by our laboratory were in keeping with the expected results.

4.7 What are the overall quality assurance recommendations - which are achievable and which are idealistic?

There are many intricate quality control and QA programmes in a routine haematology laboratory that monitor all procedures involved in generating an accurate result. Many are already in place in the routine molecular diagnostic laboratory, but there are still some that require implementation, or an equivalent found. Instrument checks are a basic requirement by both types of laboratory, and as discussed are practicable. Specimen and data handling as well as reagent control and monitoring is a necessity and has been implemented, and in many instances these methods have been borrowed from the conventional laboratory. For sample processing and analysis, there are already distinct guidelines and more are rapidly becoming available.

Daily statistical analyses (e.g. standard deviations, control charts and CUSUMS) of the routine haematological laboratory's performance are carried out daily; these are not yet implemented in the molecular diagnostic laboratory, and equivalent measures need to be implemented for the quantitative determinations.

With automation and kit manufacture (not yet available for many of the molecular tests), the incorporation of fluorescent dyes into PCR products, the automatic sizing of bands, quantitation by means of 'real time' PCR and actual sequence determination, the burden of QA will gradually be eased [11]. As automation becomes cheaper and more readily available, with it will come inbuilt standardisation and quality control [11].

As the molecular laboratory and molecular techniques are different from the conventional haematology laboratory in so many ways, latitude is needed and unique solutions have to be found for the distinctiveness of the tests carried out. In time, there is no doubt that all the relevant QA procedures will be implemented.

4.8 Conclusions

In order to develop effective QA, an integrated QA programme must be designed to validate all of the variables inherent in obtaining results. An awareness of which variables can be monitored, is important in understanding how the QA programme should be structured. Once the programme is established, it is essential to follow quality control criteria in order to minimise bias in the results and to provide consistent data that can be compared over time.

Molecular techniques such as PCR are feasible, cost effective and important for the diagnosis and therapy of diseases in the developing world. The ever-increasing number of samples received for molecular diagnosis dictates the need for QA. Standardised procedures and the use of common algorithms are essential for ensuring reliable results, and these are certainly attainable in the routine diagnostic laboratory situated in a developing country.

- Abstract: RMD Lastrucci, DJ Clifford, BV Mendelow. *Quality control in molecular diagnostics with particular reference to the polymerase reaction (PCR).*
Federation of South African Societies of Pathology Congress, Bloemfontein, July 1995.
(Appendix 1.11)

- Abstract: D Clifford, W Stevens, R Lastrucci, BV Mendelow. Quality control in the routine molecular haematology laboratory – nightmare or reality?
Outreach into Africa, Federation of South African Societies of Pathology Congress, Cape, July 1997.
(Appendix 1.12)

- Abstract: Stevens W, Stevens G, Sherman G, Du Breuil R, Mendelow B
The feasibility of a routine molecular diagnostic laboratory : A third world experience.
ISH-EHA, Amsterdam, Netherlands, July 1998.

Published abstract: British Journal of Haematology, 1998, 102,1, 293
(Appendix 1.13)

5 CONCLUSIONS

The Human Genome Project is likely to produce technological developments which will have far-reaching effects both within and outside of medicine. Automation is another development which has progressed very rapidly indeed as can be seen by instruments such as the LightCycler (Roche), COBAS AMPLICOR (Roche) and the ABI sequence detection systems (PE Biosystems), not to mention all the current automated technology for the forensic laboratories.

The ultimate aim is of course to provide even cheaper and simpler techniques that could be used in a greater number of laboratories. In the long term, it would not be surprising to find DNA sequencing based analysis as the method of choice for the diagnosis of genetic defects. In the meantime, screening for mutations should become less laborious as more effective strategies are described. Important future developments will also include the increasing availability of DNA diagnostic kits which have non-radiolabelled DNA or RNA probes. These will be user-friendly and very applicable to many clinical laboratories. The development of newer methods to amplify DNA or the removal of the contamination problem with the present techniques will see greater use of DNA amplification across many areas including clinical practice, research and industry. It is not inconceivable that DNA amplification will become so user-friendly that testing at the bedside or the local family physicians office will become commonplace.

History has taught us that technology cannot and should not be halted or kept from reaching the developing world. If we could harness the technology of molecular diagnostics sooner rather than later, it would be of huge benefit to a country (South Africa) who's scientific infrastructure is relatively sound. The aim would be to implement this technology where it is needed most: to diagnose the sick in the absence of the numbers of heath care professionals that are available to other countries. In the face of reduced resources, both financial and expert in nature, molecular diagnostics can fill a niche in providing the diagnosis, prognosis and monitoring of disease in a clear, objective manner, in order to aid the few professionals trying to carry out this task single-handedly.

Molecular diagnostics can be used to definitively diagnose diseases that up until now have relied on less objective methods. Inherited genetic disorders are 'tailor-made' for methods such as Southern blotting and PCR. Neoplastic disease has come 'into its own' diagnostically with the advent of molecular diagnostics. The ability to recognise a cancer by virtue of it genotype and the identification of markers of clonality have revolutionised and universalised the diagnosis of cancer. Provided molecular quality controls are attained and maintained both in the first and the developing world, diagnosis as we know it and the disciplines based on anatomical structure are an entity of this past century.

Molecular diagnostics is:

- Definitive and digital
- Cost effective
- Simple

- Quality controllable
- Multi-disciplinary

Suffice to say that molecular medicine will take the first and the developing world **together** (our global village) into the next millennium.

Ethical Clearance

All studies were carried out with ethical clearance from the Committee for Research on Human Subjects and the Animal Ethics Committee (University of the Witwatersrand, South Africa) for all experimentation done on the relative subjects.

The following clearance numbers were allocated:

M961015

M940210

M960418

91/160/4

APPENDIX

1

1.1 Abstract

QUANTITATION OF SPECIFIC mRNA USING XENO-COMPETITIVE PCR.

R.M. du Breuil and B.V. Mendelow.

Department of Haematology, SAIMR and the University of the Witwatersrand, York Road, Parktown, Johannesburg.

- South African Biochemistry Congress, June, 1992.

Quantitation of mRNA transcripts in monolayer cultures may present problems of imprecision caused by the low yield of total RNA generally extracted from such cultures. One solution to this problem is the application of competitive PCR, in which a quantified competitive sequence is co-amplified with target mRNA. Relative abundance of target and competitor are then analysed using a predefined and exploitable difference between the target sequence and that of the competitor. Differences which have been reported previously involve restriction site variations induced by site-directed mutagenesis and the incorporation of an intron within the competitor.

In this recent study we report a simplified competitive PCR system based on interspecies sequence differences. By comparing the β-actin gene nucleotide sequence of the rat with that of the human we were able to choose an appropriate set of primers that was identical

in both species, as well as a unique *Pvu*II site present only in the rat sequence. This allows the amplification of a fragment that can be identified as either competitor or target in this competitive PCR technique.

1.2 Abstract

SERUM MODULATION OF ACTIN EXPRESSION IN PRIMATE MACROPHAGES R.M. Du Breuil, J.N. Patel and B.V. Mendelow.
Department of Haematology, SAIMR and University of the Witwatersrand Medical School.

- South African Biochemistry Congress, 1991.

Actin is a constitutive cellular protein which forms an important part of the cytoskeleton. The actin genes are highly conserved and as they constitute a substantial proportion of eukaryotic cell protein, actin mRNA expression is often used as a reference standard relative to which other mRNA levels can be measured. However, recent findings have suggested that β-Actin is in fact a regulated early-response gene.

We analysed β-Actin mRNA levels in human HL-60 and primate macrophages. The HL-60 line was grown in medium supplemented with 10% horse serum, whereas the primate macrophages are unique in that they were maintained in culture in the total absence of exogenous protein. Differences were recorded in the actin mRNA expressed in the two situations, as follows:

For equivalent amounts of total RNA there was far less actin message in the primate macrophages, compared to HL-60 cells. In view of the conservation of β-Actin, this

difference is unlikely to be artefactual. More importantly, the amount of β-Actin mRNA and total RNA in the macrophages was strikingly increased after 1½ hour's exposure to serum.

Phenotypically, the macrophages under serum-deprived conditions have a distinctive, stellate morphology which is dramatically altered upon exposure to serum (Patel *et al*, 1989) [150]. In view of actin's defined role as an important component of the cytoskeleton, it is feasible that the morphological changes were a consequence of the induction of actin mRNA by serum. These findings reinforce the notion that β-Actin transcription is subject to modulation by serum factors.

1.3 Abstract

MICRO-ARRAY TECHNOLOGY: THE ANALYSIS OF GENE EXPRESSION PATTERNS USING A CHEMILUMINESCENT DETECTION PROTOCOL

- International Society of Haematology. South Africa, September 1999.
- Presented again by request at BIOY2K Combined Millennium Meeting, Grahamstown, South Africa, January 2000.

R Lastrucci, G Stevens, and W Stevens, Elaine Wick Poplin*, Kel Locklar*, Barry Mendelow. Department of Molecular Medicine and Haematology, School of Pathology SAIMR and University of the Witwatersrand, South Africa. *Research Genetics Inc, Huntsville, Alabama, USA.

Objectives: The rapidly advancing technology of high density cDNA arrays offer the first opportunity of providing a systematic approach to surveying RNA and DNA variation or RNA expression profiles in disease. Several recent reviews have suggested that they may become standard tools in molecular research and clinical diagnostics. Many currently available commercial arrays monitoring RNA expression levels of 5000-10000 genes/EST clusters are fixed onto nylon membranes and hybridised to a radioactively labelled cDNA 'probe', and the resulting data is recorded with a phosphor-imager. The use of

radioactivity is not practical in the routine diagnostic laboratory and the growing trend in research is to move away from the use of radioactive isotopes. We describe a method using a non-radioactive label combined with a chemiluminescent step, as an alternative.

Design and Methods: mRNA extracted from normal and leukaemic individuals was labelled with DIG-dUTP and hybridised to GENEFILTERS Research Genetics, Alabama. Using anti-DIG-AP and CDPstar, the resulting chemiluminescent signal was detected using a Lumi-Imager, and recorded in a 16bit-tif format. Further processing of the data was then carried out with software allowing single and multiple filter analysis.

Results: The images obtained using chemiluminescence were more than satisfactory and allowed complete filter analysis to the full extent of the software.

Conclusions: Using a non-radioactive labelling and detection method for the GENEFILTERS has enabled the cost-effective use of micro-array technology in our laboratory. Without this innovation, use of this modern technology to its fullest potential would have been out of our reach.

1.4 Abstract

MICRO-ARRAY TECHNOLOGY: COMPARISON OF GENE EXPRESSION IN B-CHRONIC LYMPHOCYTIC LEUKAEMIA AND NORMAL PERIPHERAL BLOOD.

RMD Lastrucci, WS Stevens, C Crowther*, D Brittain, and G Stevens

Department of Molecular Medicine and Haematology, School of Pathology SAIMR and University of the Witwatersrand, South Africa

*Department of Medicine, University of the Witwatersrand, Johannesburg, South Africa

- BIOY2K Combined Millennium Meeting, Grahamstown, South Africa, January 2000.

Aims: Micro-array technology is poised to become a standard tool of both molecular biology research and clinical diagnostics. The focus of most commercially available arrays is the monitoring of RNA expression levels. The overall gene expression profile of Chronic B cell Lymphocytic Leukaemia (B-CLL) is yet to be characterised.

Methods: Using micro-array technology we compared the RNA expression pattern of normal B-lymphocytes from healthy donors to that of B-lymphocytes from B-CLL patients.

Patients, all of whom were CD19 and CD5 positive with light chain restriction, were analysed. Control CD19 positive B cells were purified from peripheral blood lymphocytes using CD19 microbeads. RNA was extracted from patient and control lymphocytes and Dig- labelled cDNA was generated by reverse transcription. These labelled 'probes' were then hybridised to commercially available gene arrays and analysed using computer software. These expression arrays and software are available from Research Genetics, Alabama.

Results: A number of differential gene expression patterns were observed between the normal control and the patients.

Conclusions: It is evident that micro-array technology will play an increasingly important role in the identification and quantitation of genes integral to the pathogenesis of disease. Elucidation of the molecular basis of disease will aid in the implementation of appropriate therapies.

1.5 Poster

MICROARRAY TECHNOLOGY: COMPARISON OF GENE EXPRESSION IN B-CHRONIC LYMPHOCYTIC LEUKAEMIA (B-CLL) AND NORMAL PERIPHERAL BLOOD

RMD Lastrucci, WS Stevens, C Crowther*, D Brittain, and G Stevens
Department of Molecular Medicine and Haematology, School of Pathology SAIMR and University of the Witwatersrand, South Africa
*Department of Medicine, University of the Witwatersrand, Johannesburg, South Africa

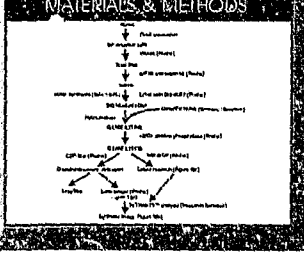
ABSTRACT

Aims: Microarray technology is poised to become a standard tool of both molecular biology research and clinical diagnostics. The focus of most currenercially available arrays is the monitoring of RNA expression levels. The overall gene expression profile of Chronic B cell lymphocytic Leukaemia (B-CLL) is yet to be characterized.
Methods: Using microarray technology we compared the RNA expression pattern of normal B-lymphocytes from healthy donors to that of B-lymphocytes from B-CLL patients.
Patients, all of whom were CD19 and CD5 positive with light chain restriction, were analysed.
Control CD19 positive B cells were purified from peripheral blood lymphocytes using CD19 microbeads. RNA was extracted from patient and control lymphocytes and Dig-labelled cDNA was generated by reverse transcription. These labelled 'probes' were then hybridised to commercially available gene arrays and analysed using computer software. These expression arrays and software are available from Research Genetics, Alabama.
Results: A number of differential gene expression patterns were observed between the normal control and the patients.
Conclusions: It is evident that microarray technology will play an increasingly important role in the identification and quantitation of genes integral to the pathogenesis of disease. Elucidation of the molecular basis of disease will aid in the implementation of appropriate therapies.

INTRODUCTION

The rapidly advancing field of microarray technology offers the first opportunity of providing a systematic approach to surveying RNA or DNA variation or RNA expression profiles in a parallel fashion on a large scale. Several recent review articles have suggested that they may become standard tools in molecular research as well as in the molecular diagnostics arena (Duggan *et al.* 1999, Lander *et al.* 1999). Many currently available commercial arrays monitoring RNA expression levels of 4,000-10,000 genes/EST clusters are fixed onto nylon membranes and hybridized to a radioactively labeled cDNA probe, and the resulting data is recorded with a phosphor imaging system. The microarray system (GENEFILTER ®) available from Research Genetics, Inc., Alabama, is a 7cm x 5cm nylon membrane containing approx. 5000 known genes and ESTs arrayed only 750 µm apart. Recently we described a method using a nonradioactive label combined with a chemiluminescent and/or color precipitate to differentially screen mRNA on these highdensity membrane arrays (GF211, GENEFILTERS, Research Genetics).
An individual with CLL was selected and control CD19 positive B cells were purified from peripheral blood lymphocytes using CD19 microbeads. RNA extracted from the patient and control lymphocytes was reverse transcribed and labeled with DIG-dUTP (Roche) and hybridized to two GENEFILTERS (Research Genetics). Using anti-DIG alkaline phosphatase (Roche) and CDP-Star (Roche), the resulting chemiluminescent signal was detected using a Lumi-Imager (Roche) available to us (Fig. 1a), and recorded in a 16 bit Tiff format. Further processing of the data was then carried out with the Pathways™ (Research Genetics) software allowing single and multiple filter analysis (Fig. 2&3). In addition, the filters were then directly detected with NBT/BCIP (Roche), and neat, contained, coloured spots were obtained (Fig. 1b). This allowed the immediate analysis of the membrane without the use of any instrumentation and further direct visual verification of the results obtained with chemiluminescence. The images obtained with the chemiluminescence or NBT/BCIP were more than satisfactory and allowed complete filter analysis to the full extent of the software

MATERIALS & METHODS



RESULTS

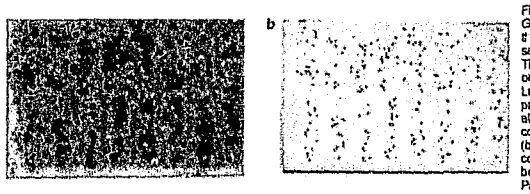


Figure 1: The figures show a GENEFILTER (GF 211) depicting the mRNA expression profile of a sample labeled with Digoxigenin. The CDPStar chemiluminescent output was detected on a Lumiimager™ and image (a) was produced. The GENEFILTER was also processed with NBT/BCIP colour reaction to generate image (b), which produced neat, contained dots that could either be analysed visually or with Pathways.

The results are depicted in the following synthetic filters (Fig. 2 and Fig. 3), each showing genes either over expressed in the normal control or the CLL patient. These are based either on the ratio or the difference between the normal control and the patient.

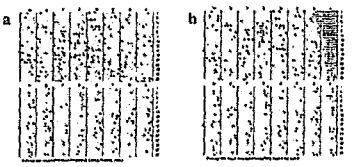


Figure 2: These images were then processed using Pathways software to produce a computer-generated synthetic image. (a) Normal control. (b) CLL

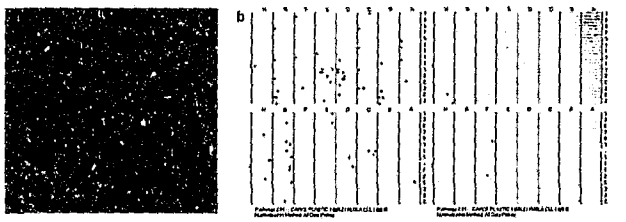


Figure 3: The stored data on the patient and control Genefilters was then compared in a variety of ways to generate the following images:
(a) Comparative ratios of signal intensities. Red = normal control, green = CLL.
(b) Comparative differences of signal intensities. Red = normal control, blue = CLL

As expected, certain of the genes, such as CD20 and the immunoglobulin related genes were over expressed in the normal. Potentially relevant genes are listed in Table1.

Table 1: Potentially relevant genes, based on findings in the literature, were selected from the Pathways generated lists of the differences and ratios between the patient and normal control filters.

Gene	Up/down regulated in patient	Association
RAP 1A	Up	Ras oncogene family - deregulated expression in hu
TGF R	Down	Known to down-regulate B-cell growth
		Down regulated in CLL
MTCP (p13) (oncoprotein)	Up	Previously shown to be increased in malignant T-cell lymphoproliferature
Glutathione Transferase (P)	Down	Previously shown to be decreased in CLL
MDM	Up	Up regulated at more advanced stages of disease in
Homeobox genes		
Hox A9	Up	Deregulation of homeobox gene expression in leu
Hox B5	Up	
TPM 1(α- tropomycin gene)	Up	Important in cell growth and differentiation
Myc binding protein	Down	
Multidrug resistance associated protein (MRP)	Down	Previously low levels shown in CLL and other chronic leukaemias.
CD58 antigen	Down	
SAK (Serine threonine kinase	Down	? expression defined more advanced disease
Pak 1 (Human protein kinase	Down	Plays a role in cell proliferation.
Bd-1 (Human bcl2 related)	Down	Important role in tumour growth and survival. Bcl 2 related protein. CD40 inducible gene.
IL 13 RA receptor complex	Down	Cell proliferation activities.
Mef 2 (Myocyte enhancer factor	Down	Important in B-cell differentiation
		Found in nuclear extract of λ producing B-cells

CONCLUSION

Although cancer classification has improved over the last 30 years, there has been no general approach to identifying new cancers or assigning tumours to known classes. Definitive diagnoses of patients would be greatly facilitated by a set of gene expression parameters defined for a specific malignancy. In this exploratory work, the gene expression in a CLL patient is analysed w.r.t. a control sample. Genes up or down regulated, as identified by a microarray system, show some distinct features that have previously been associated, described and applied to a CLL.

Research Genetics, Alabama
Roche Diagnostics, South Africa

1.6 Abstract

IMMUNOGLOBULIN GENE PCR: DIAGNOSTIC APPLICATION AND INTERPRETATION. N. Sioutos, A. Bagg, R du Breuil-Lastrucci, W.C. Pugh, J Locker, J. Cossman. Georgetown University, Washington D.C., University of the Witwatersrand, Johannesburg, SA, M.D. Anderson Cancer Center, Houston, TX, University of Pittsburgh, PA.

- International Academy of Pathology, San Francisco, March 1994

We have developed a simple PCR assay for the detection of clonal immunoglobulin heavy chain (IgH) gene rearrangements which requires only a single V_H/J_H primer pair. Here, we have used only a single V_H primer homologous to the 3' end of FR3. When applied to a large series of B cell lymphoproliferative processes (9 non-Hodgkin's lymphomas, 5 chronic lymphocytic leukaemias, 18 acute lymphoblasic leukaemias and 37 clonal post-transplant lymphoproliferative disorders), 80% (55/69) cases were found to have IgH gene rearrangements by PCR. Based upon our experience, several principles underlying the interpretation of this assay have emerged:

1. One or 2 bands (size range 100-160 bp) are seen in clonal B cell processes.

2. Although the PCR product can often be visualized in 2% agarose gels, we found that 6% polyacrylamide is superior in terms of sensitivity and specificity (detection of faint, minor clonal B cells) and the 6% polyacrylamide gel can be used alone.
3. The technique works equally well in fresh tissues, formalin and B5 fixed tissues.
4. The present PCR assay is preferred because of its simplicity: it does not require multiple primers, nested priming, blot hybridization or sequencing.
5. Negative PCR results should be interpreted with caution: the use of a positive control (e.g. erb-B2) PCR is necessary to exclude cases without amplifiable DNA (in this study 8 such cases were excluded).
6. The probable explanation for the false negative results in cases with amplifiable DNA is that the V_H primer we used recognized most, but not all V_H genes. We conclude that the single pair V_H/J_H PCR assay designed here when carefully interpreted, and with the use of appropriate controls, can be of significant value in the detection of the majority of clonal B cell processes.

1.7 Abstract

EXTENSION OF A COLD LABELLED OLIGOPROBE TO ANALYSE POLYMERASE CHAIN REACTION PRODUCTS

RMD Lastucci, W Stevens, BV Mendelow.

Department of Haematology, School of Pathology, University of the Witwatersrand and SAIMR.

- Outreach into Africa, Federation of South African Societies of Pathology Congress, Cape, July 1997.

Introduction: DNA fragment size is often not sufficient to confirm the legitimacy of a PCR product, especially when there is no specific size but rather a size range. Further manipulation of the PCR product, such as nuclease restriction or secondary PCR, can be used to confirm that the observed PCR product is real. One approach to definitive analysis of PCR products is the traditional 'Hot Blot' described by Parker *et al.* In the present study we describe a new method which achieves the same objective, but without the use of radioactive probes.

Methods and Results: DNA was extracted from patients with Follicular Lymphoma, as an example, and PCR was performed using primers designed to amplify the t(14;18)

translocation. An oligonucleotide probe for the Major Breakpoint Cluster Region was end-labelled with biotin and used to probe the PCR product as follows: A cold labelled, internally nested oligoprobe was added to an aliquot of the PCR product, and one PCR cycle performed. The product was run on an electrophoretic gel, electroblotted onto a nylon membrane and detected using streptavidin alkaline phosphatase. A positive result was interpreted if the oligoprobe was able to bind internally to the PCR product and was extended by the Taq polymerase, thus incorporating the cold label into the PCR product.

Conclusion: It was found that this method was able to successfully distinguish between legitimate and illegitimate product bands.

1.8 Abstract

IDENTIFICATION OF CANCER ASSOCIATED CHROMOSOMAL TRANSLOCATIONS USING DIFFERENTIALLY LABELLED CHROMOSOMES AND BOOLEAN LOGIC.

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- American Society of Haematology, 39th Annual Meeting & Exposition, December 1997, San Diego CA, USA Logic.
- Blood, November, 1997, 90 (10) supplement 1, 217b.

Rapid progress in the understanding of neoplasia at the molecular level is exposing the inadequacy of leukaemia/lymphoma classification based on phenotype alone. While a growing number of molecular lesions definitive of disease entities have been described, these generally are directed investigations which confirm or reject a specific diagnosis. Cytogenetic analysis has the potential to identify disease-associated chromosome rearrangements, but lacks the speed and efficiency required for clinical application. This study was designed to test the hypothesis that molecular techniques applied with Boolean

logic could be used to identify specific DNA lesions without prior knowledge of the rearrangement. Chromosomes 14 and 18 were used as models of the system. Labelled chromosome 14 and 18 specific material was obtained by amplification of flow sorted chromosomes using degenerate oligonucleotide primed (DOP) PCR, and simultaneous labelling with biotin and Digoxigenin (DIG) respectively. Individual chromosomal material was purified by selectively eliminating all sequences homologous to Cot I DNA and sequences common to both chromosomes. The resultant specific chromosomal DNA was used as PCR primers to amplify DNA from negative controls and patients with Follicular Lymphoma bearing the t(14;18) translocation. DNA amplification occurring across the translocational breakpoint incorporated primers from both chromosomes 14 and 18 and thus also their respective tags viz. biotin and DIG. Molecules containing both the biotin and DIG moieties were selected using a solid support of streptavidin and then detecting with an anti-DIG alkaline phosphatase colour reaction, or vice versa. A positive reaction, identifying exclusively hybrid molecules (t(14;18)), was found in 3/3 patients and 0/3 controls. This study has demonstrated the feasibility of this novel molecular technique to identify specific translocations where the only prior knowledge available was the chromosomes involved. The same approach could be extended to a 2 dimensional matrix of paired chromosome-specific material covering all possible translocation partners allowing analysis of any patient sample. It should also be possible to purify the DNA spanning such breakpoints using the unique combination of tags to permit molecular characterisation of unknown breakpoints.

1.9 Abstract

DEVELOPMENT OF AN INTERNAL RESTRICTION CONTROL IN THE PCR DETECTION OF THE PROTHROMBIN 20210A MUTATION.

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Department of Haematology, School of Pathology, University of the Witwatersrand and the South African Institute for Medical Research.

- Association for Molecular Pathology Annual Meeting, Arlington, VA, USA, November 1998.
- American J Pathology, 1998. 153 (5), 1648, G2

Detection of the presence of the 20210A/G allele in the human prothrombin gene is easily achieved by amplification using primers designed to span this region. Discrimination between these two alleles is achieved because the downstream primer creates a *Hin*dIII restriction site should the 20210A variant be present. A new forward primer was designed, placing it further upstream so as to incorporate a naturally occurring *Hin*dIII site that, as it is present in both alleles, serves as an internal restriction control. Using the above technique we have analysed the DNA of 132 patients who were being investigated for thromboembolic disease. Of the 59 Caucasians, 5 (8.5%) were heterozygous for this mutation but none of the 73 African Black patients were positive. The results are in keeping with global findings.

1.10 Abstract

PROTHROMBIN 20210A MUTATION IS ABSENT IN THE SOUTH AFRICAN BLACK POPULATION.

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- International Society of Haematology, Durban South Africa, September 1999.

Objectives: To determine the frequency of the Prothrombin 20210A mutation in patients with thromboembolic disorders in South Africa.

Design and Methods: Patients presenting with thromboembolic disorders who were investigated for hypercoagulability by the coagulation laboratory between June 1998 and March 1999 were included in the study. PCR amplification was performed on DNA extracted from peripheral blood (EDTA) followed by *Hin*dIII digestion to assess Prothrombin gene genotype.

Results: Of 272 patients, 148 were White, 110 were Black and 14 were Asian. The site of thromboembolism was venous in 145, arterial in 72 and unspecified in 55 (all had

detectable D-Dimers). The Prothrombin 20210A mutation was present, in the heterozygous state, in 7 of the Whites all of whom had a venous site of disease (7/85 = 8.2%) The mutation was not found in individuals with arterial disease or in any of the Black or Asian patients.

Conclusions: The Prothrombin 20210A mutation appears to be absent in Blacks whilst the prevalence in White patients with venous thromboembolic disease in South Africa is in accordance with the published literature.

1.11 Abstract

QUALITY CONTROL IN MOLECULAR DIAGNOSTICS WITH PARTICULAR REFERENCE TO THE POLYMERASE CHAIN REACTION (PCR).

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• Federation of South African Societies of Pathology Congress, Bloemfontein, July 1995.

Molecular technology has proved not only to be an invaluable tool for basic and applied research, but has helped in the diagnosis and early intervention of human disease. However, numerous variables are associated with these techniques (e.g. in PCR) and although easily performed in the laboratory, variables such as reagent concentrations, reaction temperatures, different equipment and suppliers are involved. Thus, techniques are modified from laboratory to laboratory for any one type of technique and variation in results among different laboratories can easily occur when these techniques are used for diagnosis. For example, in PCR the possibility of false positive results due to contamination and false negative results caused by enzyme inhibition are potential problems for PCR use in clinical diagnostic laboratories. Therefore reliable PCR

procedures are needed to ensure reliable results. Standardised procedures can help to avoid confusion, mistakes and complications and they encourage efficiency, reproducibility and quality control. Some standards are only a set of recommendations that offer guidance; others are definitive procedures to produce acceptable results. Quality control of and laboratory accreditation for molecular diagnostics are in their infancy, but at a conceptual level, it is important to recognise the digital nature of a molecular diagnosis, as opposed to the analogue nature of many conventional laboratory tests. This concept has major implications for good laboratory practice in molecular diagnostic routines.

1.12 Abstract

QUALITY CONTROL IN THE ROUTINE MOLECULAR HAEMATOLOGY LABORATORY - NIGHTMARE OR REALITY?

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Department of Haematology, School of Pathology, University of the Witwatersrand and S.A.I.M.R.

- Outreach into Africa, Federation of South African Societies of Pathology Congress, Cape, July 1997.

Introduction: Molecular technology using techniques like PCR have proved to be important in the setting of basic and applied research *as* well *as* invaluable *in* the diagnosis, early intervention and monitoring of patients' response to numerous diseases. Quality control and laboratory accreditation in the setting of molecular diagnostics is sadly lacking and the literature is full of errors made in the interpretation of results. Much of our focus has been on lymphoma diagnostics using various primer sets to categorise Non-Hodgkin's lymphomas into distinct biologic entities. The awareness that the use of non standardised assays at numerous sites result in variable sensitivity, specificity and reproducibility of these assays has led us to initiate an external quality assurance programme involving two other laboratories, in the United Kingdom, for evaluation of our

B and T-cell receptor gene rearrangement studies. The idea being to expand both the numbers of laboratories involved as well as the repertoire of investigations analysed. *Conclusion*: Standardised reagents, procedures and the use of common algorithms are essential for ensuring reliable results. For each investigation the possibility of false positive results caused by contamination and false negative results caused by enzyme inhibition are all potential problems that need to be addressed. Equally important is the collection and processing of specimens, the physical separation of parts of the reaction, and the inclusion of positive and negative controls. Further steps may need to be taken to establish the validity of the products which may include techniques like sequencing, post PCR restriction enzyme analysis, cold labelled internally nested oligoprobe analysis and Southern blots using internally placed sequence specific probes.

1.13 Abstract

The Feasibility of Molecular Diagnostics in the Routine Laboratory : A Third World Experience.

W Stevens, G Stevens, G Sherman, R du Breuil, B Mendelow.

- ISH-EHA, Amsterdam, Netherlands, July 1998
- British Journal of Haematology, 1998, 102,1, 293

Introduction: Molecuar technology using techniques like PCR have proved to be important in the setting of basic and applied research as well as invaluable in the diagnosis, early intervention and monitoring of a patient's response to numerous diseases. We initiated a routine molecular diagnostic facility at the Chris Hani Baragwanath hospital in Soweto. Much of our initial focus has been on lymphoma diagnostics using various techniques to categorise non-Hodgkin's lymphoma into distinct biologic entities. Our emphasis has however changed to address the needs of our population in particular the AIDS epidemic which is substantial and worsening. We are in the process of developing further tests that are rapid, cost effective and relevant. Assays of both a quantitative and qualitative nature are currently performed. The awareness that the use of non-standardised assays at numerous sites results in variable sensitivity specificity and reproducibility of these assays has led us to initiate an external quality assurance programme.

Conclusion: Despite limited resources we feel that molecular techniques like PCR are feasible, cost effective and important for the rationalisation of therapy in the third world. Standardised procedures and the use of common algorithms are however, essential for ensuring reliable results. The ever-increasing number of samples received bears witness to the usefulness of this service.

APPENDIX

2

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THE EFFECT OF TEMPERATURE ON SEX DETERMINATION USING DNA-PCR ANALYSIS OF DENTAL PULP

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ABSTRACT

Forensic applications often necessitate the identification of human remains. This is made more difficult when the tissues have been exposed to high temperatures. Previously, metrical and non-metrical assessments of skeletal remains have been used to assess gender. Recent advances in molecular biology allow amplification of DNA from human blood, dental pulp and other tissues using the polymerase chain reaction (PCR), thus facilitating gender identification. The aim of this study was to investigate the efficacy of utilising DNA retrieved from the pulp of human teeth that had been exposed to different

temperatures for different lengths of time, in order to assess gender. DNA was obtained from 94 teeth, 88 of which were isolated (44 male and 44 female), and six male teeth embedded in bone and soft tissue. A 106 base pair fragment from the X chromosome and a 112 base pair fragment from the Y chromosome was amplified from the amelogenin gene. PCR was shown to be 100% reliable when used to assess the gender of teeth which had been heated at 100°C for 15 minutes but less reliable when the teeth were heated at higher temperatures for longer periods of time. Teeth encased in bone and soft tissue yielded better results when subjected to higher temperatures than did the isolated teeth.
(J Forensic Odontostomatology 1999;17:35-9)

Key words: Sex identification, temperature, dental pulp, amelogenin gene, PCR.

INTRODUCTION

Disastrous events such as explosions, high impact collisions, crimes and fires complicate the process of human identification, thereby annually increasing the number of unidentified deceased persons. The demand for accurate methods of gender identification is thus on the increase. It is generally easier to establish the identity of an individual from an intact corpse while the degree of difficulty increases in deteriorated, fragmented or mutilated remains.[1 (151)]

Identification of the gender of an individual includes both non-dental and dental parameters [1] and utilises both metrical and non-metrical procedures. The size and shape of the skull and the pelvic girdle along with the length and girth of the long bones are the most common skeletal determinants used.[2 (152)] However, the age of the subject, the degree of fragmentation of the bones and biological variability may influence the accuracy of these methods. The accuracy of gender determination using an intact pelvis is 95%, followed by 85% when using an intact skull and 70 - 75% when using the length of the humerus.[3 (153)]

Skeletal elements subjected to physical trauma such as explosions or fire, require more sophisticated measures for gender determination. Analysis of DNA provides this sophistication, particularly in forensic studies where trace amounts of DNA could yield the necessary information. A rich and reliable source of DNA is the pulp of the tooth. The pulp is cloistered in a hard tissue casing and is well protected from the effects of

heat.[2(152)] Teeth are able to withstand temperatures of between 150°C – 450°C,[4(154)] they are tough, due to their high inorganic content [5(155)] and are easily removed for examination purposes. As teeth are small, it is highly unlikely that all the teeth would be destroyed if a body was crushed or fragmented.

The established effectiveness and refinement of the polymerase chain reaction (PCR) has allowed amplification of degraded samples yielding information about gender [6(156)] amongst other things. PCR is an *in vitro* method for the synthesis and amplification of specific DNA sequences of interest. In a series of cyclic reactions the number of PCR sequence products increases exponentially.[7(157)] It is an extremely fast and sensitive technique and can be tolerant of poor quality DNA. Further, it has a high probability of success and a low assay time [7(157)] and has been applied in the diagnosis of genetic disorders, the detection of pathogenic organisms, the genetic identification of forensic samples and the analysis mutations.[8(158)]

Pillay and Kramer [9(159)] amplified a region of the ZFX and ZFY gene in human pulp by PCR, followed by a restriction digest, to show that the method was an alternative to metrical and non-metrical assessments of skeletal remains for gender determination. A 100% accuracy in determining the gender of human teeth, which were kept at room temperature was demonstrated.

The amplification of the portion of the X - Y homologous amelogenin gene offers a gender typing system that requires only a short DNA sequence, and which is useful when forensic samples contain highly degraded DNA or DNA damaged by fire and explosions. [10 (160)] Large portions of this DNA sequence are highly conserved and the amplification of the X-Y amelogenin gene yields a 106 base pair (bp) fragment from the X chromosome and a 112 bp fragment from the Y chromosome. The use of the amelogenin gene may provide a better result than the primers used by Pillay and Kramer [9 (159)] in the likely event of the DNA being degraded by heat. Use of the amelogenin gene has the added advantage of not requiring additional enzyme restriction. The amelogenin gene has previously been used to sex teeth exposed to high temperatures, but exposure times were relatively short (between 1 and 10 mins). [11 (161)] The aim of the present study was therefore to identify gender from DNA extracted from dental pulp subjected to high temperatures for increased periods of time.

MATERIAL AND METHODS

Eighty-eight extracted carious and non-carious teeth of known gender, consisting largely of impacted third molars, were obtained from the Department of MaxilloFacial Surgery, School of Dentistry, University of the Witwatersrand and from private maxillo-facial surgeons. Six teeth embedded in their bony sockets and surrounding soft tissue were obtained from a male cadaver in the Department of Anatomical Sciences, University of the Witwatersrand.

Preparation of specimens

A calibrated furnace was used to heat the isolated teeth at varying temperatures and for different lengths of time. In each group of isolated teeth, 50% were male and 50% female. The sex of the tooth was not known to the investigators until after the experiment was completed. Teeth were exposed to the following temperatures: 100°C for 15 min (n=14), 100°C for 30 min (n=14), 200°C for 15 min (n=16), 200°C for 30 min (n=16), 300°C for 15 min (n=14) and 300°C for 30 min (n=14). Based on the results obtained with the isolated teeth, six male cadaver teeth, embedded in bone and surrounded by soft tissue, were exposed to slightly higher temperatures of 150°C (n = 2), 250°C (n = 2) and 350°C (n = 2), each for 15min.

Following heating, the teeth were split open and the dental pulp was retrieved using sterile fine forceps. DNA was extracted from the dental pulp by a NaOH and phenol /chloroform method and PCR was applied to determine the gender of the individual.

Extraction of DNA

Blood from normal male and female individuals was used with every run as a reliable, standardised source of control DNA. Amplified control DNA would provide the expected 106 and 112 bp bands for comparison with the male and female pulp samples in every reaction.

DNA was extracted from the buffy layer from 5ml of blood. Three millilitres of a 0.17M NH_4Cl solution was added to the buffy layer, mixed and placed on ice for 20min. The remaining white blood cells were pelleted for 5min at 15 000 rpm, mixed with 8ml of phosphate buffered saline and pelleted at 15 000 rpm for 10min. The pellet was washed three times in a 0.9% NaCl solution. The sample was resuspended in 500 µl of NaOH, boiled at 98°C for 15 min and neutralised by adding 62.5 µl of Tris-HCl (pH 8.0) before being allowed to precipitate at -70°C overnight.

Dental pulp

The teeth obtained were all not of the same size or condition. Some of the isolated teeth showed evidence of caries while others were impacted and showed no evidence of wear. DNA extraction from the pulp was performed by using the above method and further purification of the DNA was carried out using a phenol *I* chloroform extraction method.[8] (158) This method of extraction was chosen as it gave an OD 260: OD 280 ratio of approximately 1.8 indicating the high purity of the DNA preparation.

Primers were synthesised to homologous regions of the human amelogenin gene, spanning the area of the difference between the X and Y chromosomes. The sequences of these primers were:

SULI 5' CCC Tgg gCT CTg TAA AgA ATA gTg 3'

SULII 5' ATC AgA gCT TAA ACT ggg AAg CTg 3,

These were used to amplify the DNA extracted from the heated teeth. PCR was carried out using a PCR Core Kit (Boehringer Mannheim) following the manufacturer's instructions. A 1.5 μM final concentration of $MgCl_2$, 0.25 μg of sample DNA and 2.5 μl of a 10 μM solution of each primer was added per 50 μl reaction.

DNA samples extracted from male and female blood were used as standardised controls, while reagent blanks, which contained no DNA controlled for potential contamination.

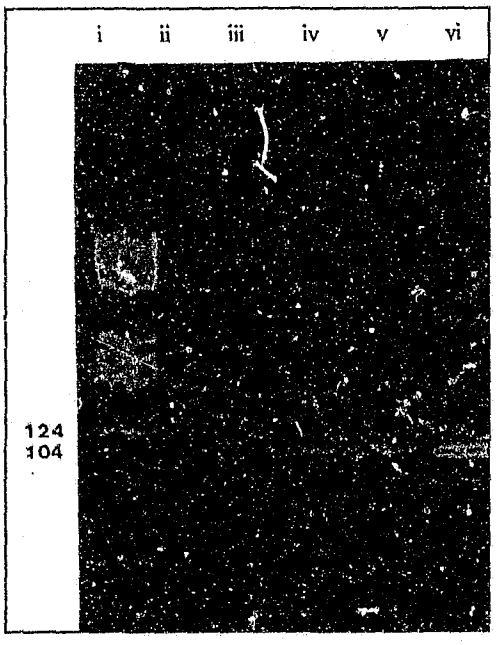
The samples were amplified for 30 cycles in a Perkin Elmer thermocycler (2400). Each cycle consisted of three phases namely: denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec and extension at 73°C for 1min. The first cycle was preceded by a denaturation step at 97°C for 2min.

Twenty microlitres of the PCR product from each tooth was analysed by electrophoresis on a 4% agarose gel.

RESULTS

All control samples gave satisfactory results. Blood and pulp from male subjects were identified as having two bands of 106 and 112 bp, while blood and pulp from female

individuals were identified as having a single band of 106 bp. The reagent blanks showed no product as expected (Fig. 1 [Figure 30]).



[Figure 30] ***Fig1:*** *A 4% agarose gel depicting the amelogenin PCR products from male DNA [lanes iii-v] and female DNA [lane vi]. Lane i - molecular weight marker V (Boehringer Mannheim) and lane ii - reagent blank*

Temperature and time	Male (n)	Percentage correctly sexed	Female (n)	Percentage correctly sexed
100°C for 15min	7	100%	7	100%
100°C for 30min	7	86%	7	71.4%
200°C for 15min	8	62.5%	8	37.5%
200°C for 30min	8	50%	8	25%
300°C for 15min	7	14.3%	7	14.3%
300°C for 30min	7	0%	7	0%

Table 12*: Isolated teeth. The percentage of teeth correctly sexed with increasing temperatures and times.*

At 100°C and with an exposure of 15min, all the isolated teeth were correctly sexed (Table 1) [Table 12]. However, with increasing temperature and length of exposure the ability to sex a sample diminished. The accuracy of sexing varied between the male and female samples. At 300°C and an exposure of 15mins, only 14.3% of both the male and female samples were correctly sexed (Table 1) [Table 12].

The ability to sex a sample following heating improved when the teeth were encased in bone and protected by soft tissue (Table 2) [Table 13]. At 250°C both teeth were correctly sexed. However at 350°C, neither tooth was sexed.

Temperature	Male (n)	Percentage correctly sexed
150°C	2	100%
250°C	2	100%
350°C	2	0%

Table 13: *Teeth embedded in bone and soft tissue. The percentage of teeth correctly sexed following increased temperatures. Incineration time was 15 minutes for all specimens*

DISCUSSION

The term "sexed correctly" simply implies that the gender of the tooth was identified following PCR. No teeth were sexed incorrectly, that is, no male specimens were classified as female and *vice versa.*

Teeth remained unsexed if the PCR failed to produce a result.

In this study, the success of the PCR in sexing individuals decreased as the temperature and exposure time increased. When the teeth were heated for a longer period, that is 30min as compared to 15min, the percentage of sexed teeth decreased even further. Of the 14 teeth heated at 300°C for 30min, no DNA was amplified. Alvarez Garcia *et al.* [11 (161)] obtained an 87% positive identification of sex at 100°C for 10 min (compared with 100% at 15min in this study). When they increased the temperature to 200°C for 10mins, their result was significantly poorer (33% positive). It is of interest that at 200°C (15mins), we obtained 62.5% accuracy in the identification of males and 37.5% for females. At 30mins (200°C), we obtained 50% accuracy for males and 25% for females. Alvarez Garcia *et al.* [11 (161)] were able to obtain positive gender identification at 500°C, but incineration time was for only 2mins.

The discrepancies between the percentage of males and females correctly sexed at 100°C (30min exposure) and 200°C (both 15 and 30min exposure) in this study, may be due to the male teeth being larger and more protective of the pulp during incineration. In addition, the condition of the teeth from different patients varied as some patients had healthy teeth without caries (impacted third molars), while others had carious teeth. Although the number of carious to non-carious teeth was not quantitated, more female than male teeth were carious. The impact of caries on the quality of the DNA extracted from the pulp could not be determined in this study. It is however possible that exposed

dentine would develop cracks at lower temperatures than when protected by enamel (dentine shows multidirectional cracks at temperatures of 400°C [12 (162)]). The fact that more female teeth had caries could account for the higher success in identification of male specimens.

All of the embedded teeth were correctly sexed at both 150°C and 250°C for 15min (Table 2) [Table 13], but no results were obtained for the embedded teeth at 350°C. While Alvarez Garcia *et al.*[11 (161)] obtained positive sexual identification on isolated teeth above temperatures of 300°C, their exposure time did not exceed 2mins. It is possible that longer exposure times at high temperatures leads to incineration of the DNA.

There was a two-fold increase in the success rate of embedded teeth exposed to 250°C (15 min) compared to extracted teeth exposed to 200°C (15 min). This could imply that the mandible and soft tissue have the capacity of absorbing the heat and in so doing, protect the teeth and the pulp.

In summary, using primers designed to amplify 106 and 112 bp from the amelogenin gene of the X and Y chromosome respectively, we were able to accurately sex 100% of isolated teeth exposed to 100°C for up to 15min. Beyond this temperature and length of time, the ability to sex the individuals diminished, but was still possible at 250°C for teeth embedded in bone.

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