THE NEW ZEALAND MEDICAL JOURNAL
Journal of the New Zealand Medical Association

Customised molecular diagnosis of primary immune deficiency disorders in New Zealand: an efficient strategy for a small developed country

Rohan Ameratunga, See-Tarn Woon

Abstract

Introduction Primary Immune Deficiency disorders (PIDs) are uncommon conditions, which necessitate urgent diagnosis in order to prevent disabling complications such as bronchiectasis. Timely diagnosis can be life-saving in children with PIDs such as severe combined immune deficiency.

Methods A customised molecular diagnostic service was established in New Zealand in 2005. Most patients referred to the service have undergone genetic counselling before blood was drawn for testing. Genomic DNA was extracted and polymerase chain reaction (PCR) performed to amplify genes of interest, followed by DNA sequencing. The DNA sequences were then aligned with wild type sequence using computer software to identify possible mutations.

Results Mutational analysis was undertaken in 27 probands with suspected PID. Seven causative mutations were identified in these patients. Family studies have been undertaken after genetic counselling.

Conclusions Customised genetic testing is a cost-effective and efficient method for PID diagnosis in a small developed country.

There have been major advances in the understanding of primary immune deficiency disorders (PID) over the last two decades.[1,2] The genetic basis of many of these conditions has been identified. Most are inherited as single gene defects. Early identification of PIDs can reduce morbidity and mortality, as specific treatment is available for the majority of these conditions.

Early work in New Zealand[3] confirmed the value of molecular analysis of these disorders. The mutation responsible for a family with X-linked hyper-Immunoglobulin M (XHIM) syndrome was identified shortly after the molecular basis for the disorder was discovered.

As a result of this work, the diagnosis was confirmed in the proband and his sister was reassured she was not a carrier. Thus, molecular diagnostic testing can play a vital role in patient management.

In 2003 the Immune Deficiency Foundation of New Zealand (IDFNZ) funded a study to explore the feasibility of establishing a molecular diagnostic service in New Zealand to assist patients and families with PIDs. Following the report presented by the late Dr Karen Snow-Bailey, senior management at LabPlus in Auckland City Hospital made a decision to fund the service. A senior scientist was appointed in 2004 to lead the programme.

Over 120 genes have been implicated in the pathogenesis of PIDs. For most of these conditions, commercial tests are not available. The molecular immunology laboratory offers customised testing on a fee-for-service basis with rapid turnaround times. Turnaround time is usually 1 week for established tests. For customised tests, the turnaround time is approximately 2 to 3 weeks.

Quality assurance is a critical part of laboratory testing. Currently there are no external quality assurance programmes for the genetics of PIDs.[4] As part of the quality assurance programme, blinded samples with previously identified mutations were received from European and North American diagnostic laboratories. These were correctly identified. A sample initially sequenced in Perth for XLP was subsequently sequenced in Auckland and the results were confirmed[5] (see below).

The service has been accredited by IANZ, the laboratory-accrediting agency in New Zealand. The programme has also been discussed with NATA, the Australian laboratory accreditation agency (Andrew Griffin personal communication Sydney, 14.3.09). The service follows the guidelines for molecular diagnostic laboratories issued recently by the Centres for Disease Control.[4]

In this paper, we review the results of patients referred to the service from 2005 to 2008. The analysis of these patients illustrates the power and limitations of molecular diagnosis of PIDs. This programme is working well for New Zealand's small population (pop 4.2 million) and can serve as a model for other PID diagnostic services.

Methods

Mammalian genes are usually coded by exons with intervening introns. In our laboratory, genomic (DNA) sequencing is undertaken. All exons are sequenced with primers designed to anneal to introns, in order to identify potential splice site mutations. Splice site mutations can alter the sequence of mRNA leading to clinical disease as a result of absent or dysfunctional proteins.

Wild type gene sequences are downloaded from public databases such as Genbank™ and Ensembl. Primers flanking the exon regions are designed using Oligo version 6.44 (Molecular Biology Insights, Cascade, CO, USA).

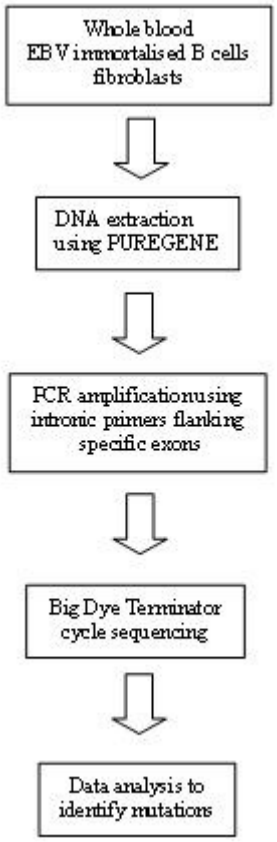
Genomic DNA from blood samples are extracted using PUREGENE DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA). Genes of interest are amplified using polymerase chain reaction (PCR).

The amplicons then undergo BigDye® Terminator sequencing cycle sequencing and the products are subjected to electrophoresis in an Applied Biosystems (ABI PRISM®) 3100 Genetic Analyzer. The DNA sequence files are compared to wild type sequence using SeqMan v5.01 software (DNASTAR, Madison, WI, USA).

The laboratory creates immortalised Epstein-Barr virus (EBV) transformed B cell lines, which can be the source of DNA for genetic studies. Fibroblast cell lines are useful after bone marrow transplantation, as B cells may be of donor origin. These cell lines obviate the need for multiple blood tests. Creation and storage of the cell lines are undertaken with the consent of the patient or family in the case of children.

The laboratory also offers analysis of T cell receptor excision circles (TRECs), which is a marker of T cell production by the thymus.[6] This assay has a variety of uses including confirmation of a Severe Combined Immune Deficiency (SCID) phenotype, when the mutation is not obvious. It can also be used to follow T cell maturation following bone marrow transplantation.

Figure 1. Laboratory workflow of genetic testing



Results

The number of samples received by the laboratory is such that a rapid turnaround time can be achieved. If necessary, a repeat sample can be rapidly tested for confirmation. A list of patients referred to the service is outlined in Table 1.

Table 1. Genetic testing results of the patients referred to the molecular immunology diagnostic service

Proband / family number	Suspected diagnosis	Gene tested	Number of family members tested		Results	Comments
			male	female		
1†	XLP	SH2D1A	2	7	all males are normal; 2 females are carriers	One female carrier status is determined from lymphoma tissue block [5]
2		SH2D1A		1	normal	Family has extensive history of lymphoma; awaiting X-linked inhibitor of apoptosis (XIAP) gene analysis
3		SH2D1A	1		normal	
4		SH2D1A	1		normal	
5		SH2D1A	1		normal	

6		SH2D1A	1		normal	
7		SH2D1A/ XIAP	1		normal SH2D1A, base change in XIAP	XIAP: c.1268A>C, Q423P; 50% Africans have the SNP‡
8		SH2D1A/ XIAP		1	normal SH2D1A, base change in XIAP	XIAP: heterozygous c.1268A>C, p.Q423P; 5-8% Asians have the SNP
9	XLA	BTK	1		splice site point mutation in intron 8	first nucleotide G of intron 8 was substituted with an A (IVS8+1G>A)
10		BTK	1		splice site point mutation in intron 15	second to last nucleotide A of intron 15 was substituted with a C (IVS15-2A>C)
11		BTK	1		deletion of 4 nucleotides (TTTG) in exon 16	4 base pair deletion (g.66795_66798delTTTG, c.1581_1584delTTTG)
12		BTK	1		normal	
13		BTK	1		normal	
14	XHIM	CD40L	1	2	point mutation detected	proband: c.475G>A, p.W140X, leads to premature stop codon; mother is a carrier
15			1		normal	
16			1		normal	
17			1		normal	
18				1	normal	
19	AR-HIM	UNG/ AICDA		1	normal	
20	WAS	WASP	2	4	Point mutation detected	WASP: c.431G>A, p.E133K, known pathogenic mutation, mother, grandmother and grand aunt are carriers
21	X-SCID	IL2RG	1		normal	
22	SCID	RAG1/ RAG2/ Artemis	1	3	normal sequences of RAG1 and RAG 2; point mutation in Artemis	Single copy of c.728A>G, p.H243R; present in healthy older female sibling; inconclusive result
23		JAK3	2	1	2 point mutations detected in proband, both parents are carriers	Proband is a compound heterozygote (c.1351C>T, p.R451X; c.2148G>A, p.W716X)
24		JAK3		1	normal	
25		DNA ligase 4/ Cernunnos factor		1	normal	
26*	Type III HAE	Factor XII		5	normal	*DNA sent to Sonic Laboratories in Sydney (after discussion with patients)
27	ALPS	FAS	1		normal	

Abbreviations: ALPS: autoimmune lymphoproliferative syndrome, AR-HIM: autosomal recessive hyper immunoglobulin M syndrome, HAE: hereditary angioedema, SCID: severe combined immune deficiency, WAS: Wiskott-Aldrich syndrome, XLA: X-linked agammaglobulinemia, XHIM: X-linked hyper immunoglobulin M syndrome, XLP: X-linked lymphoproliferative syndrome, X-SCID: X-linked severe combined immune deficiency.

‡ Single nucleotide polymorphism (SNP) is a small genetic change, or variation, that can occur within the DNA sequence of an individual; * Currently DNA from patients with suspected type 3 HAE are sent to Sonic laboratories in Sydney, which offers a quick and cost effective service. Patients are made aware of the need to send samples overseas; † The original mutation analysis of the proband was undertaken in Perth, during the time the service was being established. The mutation was subsequently confirmed in New Zealand.

Discussion

Funding and the role of genetic services—Genetics services in New Zealand are centrally funded. Testing is thus free to New Zealand citizens and permanent residents. Diagnostic studies are undertaken after patients undergo genetic counselling. The benefits and limitations of testing are explained to patients.

The laboratory offers a fee-for-service testing programme. The referring clinical service is invoiced for the testing. The cost depends on the size of the gene. The recombination-activating gene 2 (RAG2) gene, implicated in some cases of SCID, attracts a higher fee due to its larger size in comparison with genes such as the CD40 ligand. Once the mutation is identified, only the abnormal exon is sequenced in other family members and therefore the cost is proportionately less. Government funding is available for testing family members.

The exact cost of testing also fluctuates based on the value of the NZ dollar, as reagents have to be imported. In general the cost is considerably less than overseas diagnostic laboratories based in Europe or the United States. Furthermore, many overseas diagnostic laboratories do not sequence the full gene. Mutations in less commonly affected areas of the gene may thus be missed.

The programme employs one full time molecular biologist (S.-T.W.) and a part time immunopathologist (R.A.). The use of shared molecular diagnostic resources at Lab Plus has minimised costs. Currently the service at Auckland City Hospital is financially self-sufficient with revenue covering costs. Samples have been received from around New Zealand and also Australia.

Advantages of genetic testing—The ability to identify a disorder at the genetic level in most cases eliminates any uncertainty about the underlying diagnosis. Genetic diagnosis may allow treatment decisions to be made with more confidence. The identification of a PID has profound implications for other family members. This is illustrated by family 1 with X-linked lymphoproliferative syndrome (XLP). Currently no males in the immediate family are at risk of disease.[5]

The two brothers (family 20) identified with Wiskott-Aldrich syndrome (WAS) have undergone bone marrow transplantation. The decision to undertake bone marrow transplantation in these children was based on the results of mutation analysis by the molecular immunology diagnostic service. In WAS, the phenotypic severity of the disorder can be predicted in many instances, based on the nature of the mutation.[7] These two patients were predicted to have severe disease based on their mutation (E133K).[8]

If the situation is urgent, such as a baby with a Severe Combined Immune Deficiency (SCID) phenotype needing bone marrow transplantation, testing can be undertaken immediately to confirm the genotype. Confirmation of the diagnosis may assist with the decision to undertake bone marrow transplantation. The type of SCID may influence treatment decisions such as whether to offer conditioning prior to bone marrow transplantation.[9,10] A detailed analysis of advantages of PID genetic testing, based on our experience, will be the subject of a future review. (Ameratunga R, Woon S-T and Neas KN submitted)

Limitations of genetic testing—Despite the advantages of genetic testing, the limitations of the technology must be made clear to the patients. This underscores the importance of genetic counselling. The testing strategy described here has some disadvantages. Promoter mutations and complex DNA rearrangements for example, may not be identified by DNA sequencing of the coding region of a gene. Some mutated genes such the C1 inhibitor have a higher probability of complex mutations.[11] Identification may require Southern blotting and/or analysis of cDNA. These additional tests are available through the laboratory.

Mutation analysis can be problematic. The significance of an identified mutation may be uncertain (e.g. patient 7). A mutation may be non-pathogenic and therefore does not alter cellular function. Several mutations (e.g. patients 7 & 8) may represent single nucleotide polymorphisms (SNPs). Furthermore, some patients may be compound heterozygotes, where the second mutation has not been identified. This was seen in one of the SCID patients, who may have had Artemis deficiency (patient 22). The second mutation has not been identified.

Another baby with SCID was identified as having JAK3 deficiency as a result of compound heterozygosity. The second mutation was not obvious in the patient and required careful evaluation of both parents. Both babies had very low numbers of T Cell Receptor Excision Circles (TRECs), confirming the SCID phenotype. A protein or functional assay may be able to determine deleterious effects of the mutation but could not be performed as the two patients are deceased. Creating cell lines may allow functional assays, such as cell signalling and phosphorylation studies, with greater ease.

As illustrated by several patients in this series, a causative mutation may not be identified in spite of the patient having the classical phenotype. This is seen in several patients with suspected XLP (patients 3–6) and suspected XLA (patients 12 & 13). One possibility is genocopy, where mutations in unrelated genes can cause a similar phenotype. A good example of genocopy is a defect of BLNK, where the phenotype produced is very similar to X-linked agammaglobulinaemia. [12]

The results of testing are only meaningful if they are interpreted in the appropriate clinical context. The laboratory offers an extensive panel of tests for PIDs including flow cytometry, lymphocyte proliferation and vaccine antigen responses. Weekly meetings are held to discuss results and progress with clinical staff. The results of other tests including flow cytometry are also discussed at the same time.

Diagnostic tests in research laboratories—Some research laboratories offer free testing as part of their research programme. These laboratories are often run by leading authorities in the field with extensive clinical knowledge of these disorders. While the main advantage is free testing, potential disadvantages need to be carefully evaluated. The cost of the DNA extraction and sample transportation need to be considered. Some overseas research programmes require patients to travel to their institutions before being offered free testing. The cost of travel needs to be balanced with a free service.

Another concern is a long turnaround time. Samples may be batched in research laboratories until a sufficient number have been received. Sometimes results may not be available for months, which could impact on the ability to offer a family prenatal

diagnosis, where time is of the essence. Genetics services in many centres including Auckland require the results from research laboratories be confirmed in a diagnostic laboratory before being used for prenatal diagnosis.

Molecular studies are expensive and labour intensive. Because of cost constraints, testing may be performed by a junior staff member or a student in a research laboratory. Testing could be discontinued abruptly if a research grant is not renewed or the senior investigator moves to another institution. We also had the experience of free testing for a limited time after the discovery of a novel disease-causing gene. Free testing is not offered after some time, presumably because new mutations cannot be published in high-impact journals.

Research laboratories may not be obliged to participate in external quality assurance programmes, which can be an expensive process. Sample mix ups and PCR contamination can occur in any laboratory. Without close clinical communication, there may not be an easy way to identify an error in a remote laboratory. Repeating a test may also take a considerable amount of time, especially in a distant country.

Cultural issues need to be considered. *Tikanga* is the traditional system of beliefs, values and spirituality of Māori. For Māori there is concern about sending tissue and DNA samples abroad. For some Māori, there may be added concerns about long-term storage of DNA samples in overseas laboratories. Culturally appropriate disposal of tissue and DNA samples is important. The Auckland City Hospital follows *Tikanga*-recommended best practice policy. If safe to do so, there is the option of returning specimens to patients if requested. Samples are stored long term only with the consent of the patient.

If a mutation cannot be identified in a diagnostic laboratory, the condition being investigated may not have been previously described. In such cases, a candidate gene approach may need to be considered. Genes that are likely to be mutated, based on the known physiology of the suspected gene can be analysed with ethics approval and patient consent. Alternatively samples could be sent to a research laboratory once ethics and cultural issues have been addressed. In this specific situation, research laboratories can play a complementary role to diagnostic laboratories.

Conclusions

The model presented here is an efficient and cost-effective solution for a small developed country. It allows self-sufficiency in PID diagnosis. We have shown here that a dedicated PID programme allows rapid diagnosis, leading to early treatment and improved patient outcomes with reduced mortality and morbidity. A similar model may be feasible for other specialties in New Zealand, where genetic diagnosis plays a critical role. More importantly, close involvement of referring clinicians is likely to improve the quality of the results.

Competing interests: None declared.

Author information: Rohan Ameratunga, Adult and Paediatric Immunologist; See-Tarn Woon, Senior Scientist; LabPlus, Auckland City Hospital, Auckland

Acknowledgements: We are very grateful to IDFNZ for its assistance in creating and supporting this programme. We would like to thank the late Dr Karen Snow-Bailey

for her support in the early stages of this programme, Dr Kitty Croxson and senior management at Auckland City Hospital for their ongoing support, and Dr Maia Brewerton for advice on cultural issues relating to Maori. We thank colleagues Drs Don Love, Miriam Hurst, Salim Aftimos, and Lochie Teague for helpful suggestions. The authors can be contacted for more information on specific tests.

Correspondence: Associate Professor Rohan Ameratunga. LabPlus, Auckland Hospital, Park Rd, Grafton, Auckland, New Zealand. Fax: + 64 (0)9 3072826; email: rohana@adhb.govt.nz

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