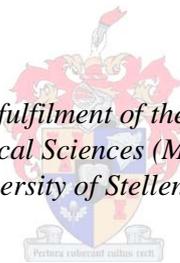


Sequence-based molecular diagnosis of X-linked Agammaglobulinemia in South African individuals

by

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Declaration

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Summary

Background: Primary immunodeficiency disorders (PID) disrupt the proper functioning of the immune system. The prototypic PID is X-linked Agammaglobulinemia (XLA). This disorder is caused by mutations in the Bruton tyrosine kinase (Btk) gene and results in an arrest in B cell development which leads to a profound reduction of all classes of serum immunoglobulins (i.e antibodies). Patients with a lack of antibodies experience recurring bacterial infections during early childhood that can be fatal if not treated. Intravenous gammaglobulin replacement therapy (IVIg) is the standard treatment for XLA. It provides passive immunity thereby reducing the number and severity of infections as well as limiting many of the infectious complications. Early detection and treatment of XLA allows affected individuals to live a relatively normal life.

Objective: The purpose of this study was to determine the molecular basis of XLA in South Africa using a direct sequence-based method to detect abnormalities in the Btk gene to aid clinical diagnosis of the disease.

Methods : Male patients with a clinical diagnosis of XLA were included in this study. Genetic analysis was used to explore the exonic region of the Btk gene of 5 unrelated male patients and compared to 10 healthy controls. Family members were followed up to determine carrier status, where possible.

Results: Mutational analysis revealed Btk abnormalities in 4 of the 5 patients leading to a definitive diagnosis of XLA. Two of the three mutations found in this study have been previously described while one mutation appears to be novel. The novel mutation is a one base pair deletion in exon 16 which leads to the truncation of the Btk protein. Despite the clinical findings suggestive of XLA, no mutation was identified in the exonic region of the Btk gene of the remaining patient, indicating that this patient might have a different form of PID. Maternal follow-up confirmed the maternal inheritance pattern as all mothers screened were carriers of the Btk mutation present in the affected individual.

Discussion :

Using a direct sequence-based method abnormalities were identified in the Btk gene of three patients. Molecular diagnosis coupled to clinical history of the patient provides a definitive XLA diagnosis. This study supports the use of molecular techniques in the diagnosis of PID and underlines the synergy that could be possible in a clinical setting.

Opsomming

Agtergrond: Primêre immuungebrek siektes (PIGS) word gekenmerk aan 'n gebrek aan teenliggame in die immuunsisteem wat lei tot herhaalde infeksies in jong kinders wat fataal kan wees indien dit nie vroegtydig behandel word nie. Die prototype van die bekende PIGS is X-gekoppelde Agammaglobulinemia (XGA). Die siekte word veroorsaak deur mutasies in die Bruton Tiroisien kinase (Btk) geen en lei tot 'n stilstand in B sel ontwikkeling en gevolglik 'n vermindering van alle klasse van serum immuunoglobulins (teenliggaam). Intraveneuse gammaglobulien vervangingsterapie(IVIg) is die standaard behandeling vir XGA. Dit voorsien passiewe immunitet en gevolglik verminder dit die getal en erns van infeksies en beperk baie van die aansteeklike komplikasies. Vroeë diagnose en behandeling van XGA laat toe dat geaffekteerde individue 'n relatiewe normale lewe ly.

Doel: Die doel van hierdie studie is om die molekulêre basis van XGA in Suid Afrika te ondersoek, deur gebruik te maak van direkte volgorde bepaling van die Btk geen in die hoop om die kliniese diagnose van die siekte aan te help.

Metode : Manlike pasiente met 'n kliniese diagnose van XGA was by die studie ingesluit. Genetiese analise was gebruik om die "exonic" omgewing van die Btk geen te ondersoek van 5 onverwante manlike pasiente en vergelyk teenoor 10 gesonde kontrole. Waar moontlik was familie lede ogevolg om draers te bepaal.

Resultaat: Mutasies in die Btk geen is geïdentifiseer in 3 van die 4 pasiente, klinies gediagnoseer met XGA. Die mutasies sluit 2 reeds beskryfde variante in en een nuwe mutasie, 'n een basis paar delesie in ekson 16 van die Btk geen. Ten spyte van die kliniese profiel suggestief van XGA in die 5de pasient, was geen mutasies geïdentifiseer in die "exonic" omgewing van die Btk geen nie, dit kan moontlik toegeskryf word aan die teenwoordigheid van 'n ander vorm van PIGS in hierdie pasient. Opvolg analise op die DNA van die moeders van die pasiente het die moederlike oorerwings patroon van die siekte bevestig aangesien al die moeders draers van die geïdentifiseerde mutasie in die Btk geen van die geaffekteerde individu was.

Gevolgtrekking: Genetiese analise van die Btk geen blyk 'n sensitiewe en spesifieke metode te wees om individue met XGA te diagnoseer. Hierdie studie ondersteun die gebruik van molekulêre metodes in die diagnose van PIGS en beklemtoon die moontlike sinergie wat kan bestaan tussen hierdie tipe benadering in die kliniese omgewing.

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Chapter 1 Introduction

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1. Background

1.1 The immune system

Throughout history, infections are known to be the most frequent cause of death [1]. The immune system functions to protect against these infectious complications by establishing 'barriers' or defence mechanisms. These defence mechanisms can be divided into two groups, namely innate and acquired (or adaptive) immunity (Table 1.1). Innate immunity can be defined as the host's first line of defence and is present before the body is exposed to any infectious agents (antigens). Upon exposure, the innate system recognises a foreign organism and initiates a response by using macrophages to migrate towards the antigen, engulf and destroy it in a process called phagocytosis. Cells used in the initial response depend on where the antigen is detected. For example, if the antigen was detected in the skin, the Langerhans' cells would be the first to migrate towards it, similarly, Kupffer cells in the liver, osteoclasts in bone and peritoneal macrophages in the intestine mediate the phagocytic response in other tissues. Besides their primary function of destroying foreign organisms, macrophages are responsible for the synthesis and release of chemokines and cytokines. These are chemical messengers that recruit other cells to the affected area. Some of the recruited cells include polymorphonuclear neutrophils (or monocytes), basophils, eosinophils and natural killer cells which all function in the successful eradication of the foreign organisms. Other molecules known as Complement are a collection of proteins in the blood that fight against bacterial infection. As its name suggests it complements and forms part of the innate immune system. [2].

It is important to note that innate immunity is, however, not antigen-specific and has no memory cells which respond quicker to antigens that have previously entered the host. Acquired immunity however, is both antigen-specific and it has memory cells. It incorporates the use of specialised cells called B (bone marrow) and T (Thymus) lymphocytes. These cells only recognise specific antigens via B cell receptors (BCR) and T cell receptors (TCR) which are exposed on the surface of the B and T cells respectively. B cell response to pathogens is triggered when innate immunity fails to contain the invasion of infectious agent. i.e. some evolved microorganisms are able to inactivate phagocytic cells and thus would require the help of the host adaptive immunity for fighting the infection. T cells, respond to intracellular infections and are subdivided into T Helper (T_H) cells and Cytotoxic T (T_c) cells. T_H help B cells to make antibodies and also activate macrophages to eliminate intracellular parasites. T_c cells eradicate the invasion by viral infections. Thus, acquired immunity is more specific and effective in its eradication of pathogens [2]. It is important to note that the immune system is not always successful in protecting the host from infectious complications and this can be attributed to a defect known as immunodeficiency.

Table 1.1 Components of the immune system

Immunity	Innate	Acquired
Cells	Phagocytes Monocytes Basophils Eosinophils Natural Killer (NK)	B cells T cells
Molecules	Cytokines Chemokines Complement	Cytokines Antibodies

1.1.1 Immunodeficiency

Immunodeficiency (or immune deficiency) affects the hosts' ability to fight against infection. An individual with a defective immune system is said to be immunocompromised. These individuals are categorized into two groups depending on how the immunodeficiency is obtained. Most immunodeficiency states are due to acquired (or secondary) causes e.g. Acquired Immune Deficiency Syndrome (AIDS), tuberculosis, leukemia or more commonly to malnutrition, aging and sometimes specific medication [2]. A smaller number of immunocompromised individuals are grouped under Primary immunodeficiency disorders (PID). This type of immunodeficiency is congenital, usually due to genetic defect in the host's defense mechanism.

PID disrupts the proper functioning of the immune system [3]. This disruption is caused by mutation in genes, which are involved in the expression of components required for proper immune functioning [4, 5]. Research within the PID field has led to the classification of over 150 PIDs and more than 120 have been characterised genetically [6,7]. Most PIDs are characterized by the individual's susceptibility to opportunistic organisms. These infections are usually persistent or recurrent regardless of aggressive treatment and affected individuals often have poor growth and fail to thrive. [3]. Onset of symptoms is usually in early infancy with 70% younger than 20 years old and 60% occurring in males due to the X-linked manner in which many syndromes are inherited [8]. Classification of PIDs are based on the component of the immune system that is disrupted.

For example, humoral immunity disorders affect B cells and antibody production which predisposes individuals to increased susceptibility for bacterial infections, whilst patients with cellular immunity disorders, due to T cell defects, are more prone to fungal, viral and parasitic infections [4, 5]. Similarly, phagocytic defects characteristically cause oral and skin infections and granuloma formations [5]. The most frequently diagnosed and treated PID is the primary humoral immunodeficiencies, more specifically, the antibody deficiency syndromes. These syndromes result from genetic abnormalities in genes involved in B cell development and are characterized by the individual's inability to produce adequate amounts of

circulating antibodies (Table 1.2), increasing their susceptibility for bacterial infections [9-11]. The clinical characteristics of antibody deficiency syndromes are similar with a marked reduction in all serum Ig isotypes, however the underlying genetic mechanisms differ. The prototypic congenitally inherited antibody deficiency syndrome is X-linked Agammaglobulinemia [10].

Table 1.2 Antibody deficiency syndromes

Disease	Inheritance	Affected gene	Affected Serum Ig
B cell linker protein (BLNK) deficiency	Autosomal Recessive	BLNK	All isotypes ↓
X-linked Agammaglobulinemia (XLA)	X-linked	Bruton Tyrosine Kinase (Btk)	All isotypes ↓
Igα deficiency	Autosomal Recessive	Igα	All isotypes ↓
Igβ deficiency	Autosomal Recessive	Igβ	All isotypes ↓
μ heavy chain deficiency	Autosomal Recessive	μ heavy chain	All isotypes ↓
λ5 deficiency	Autosomal Recessive	IGLL1 (λ5)	All isotypes ↓

BLNK: B cell linker protein; α : alpha; β : beta; μ: mu ; λ: lambda; (↓) decrease

1.2 X-linked agammaglobulinemia

X-linked Agammaglobulinemia (XLA), also known as Bruton's disease, is the prototypic PID and was first reported and defined by O.C Bruton in 1952. XLA is inherited in an X-linked recessive manner and affects approximately 1 in 200 000 individuals [12]. Males are almost exclusively affected, whereas carrier females, having both the normal and mutant allele appear immunologically normal. Transmission of the mutant allele to the progeny however, will result in the development of XLA in males and a heterozygous genotype in females [13,14]. During the first 9-12 months after birth the condition goes undetected as the male infant is protected by transplacentally acquired maternal immunoglobulin (Ig) G. As these levels decrease physiologically without compensatory production of the infant's own immunoglobulin, the infant becomes extremely vulnerable to recurrent infections (mainly caused by encapsulated bacteria). Most infections are due to pyogenic bacteria and the most common sites of infections include the lower respiratory tract, middle ear and sinuses [3, 15]. The greatest threat to agammaglobulinemia patients are enteroviral infections which can result in chronic meningoencephalitis [16, 17]. Common organisms found in enteric infection are *Giardia lamblia*, *Campylobacter jejuni* and *Salmonella spp.* Common respiratory pathogens that lead to infection, are *Streptococcus pyogenes*, *Haemophilus influenzae* and *Bordetella pertussis* ([3], Table 1.3). Lack of antibody supplementation and immunoglobulin (Ig) replacement treatment leads to chronic obstructive pulmonary disease and premature death. T cell function appears

normal and therefore normal resistance is present against viruses and fungi, with the exception of enteroviruses and hepatitis [18, 19].

Table 1.3 Infection and common pathogens in X-linked Agammaglobulinemia

Recurrent Infections	Upper and Lower Respiratory tract infection Sinusitis Otitis Skin lesions
Major Infections	Pneumonia Septicaemia Meningitis
Common Pathogens	<i>Giardia lamblia</i> <i>Campylobacter jejuni</i> <i>Salmonella spp</i> <i>Streptococcus pyogenes</i> <i>Haemophilus influenzae</i> <i>Bordetella pertussis</i>

1.3 Treatment

1.3.1 Intravenous Immunoglobulin (IVIg) treatment

For XLA patients to live a near normal life, appropriate treatment must be given. Intravenous gammaglobulin replacement therapy (IVIg) is the standard of care for XLA, which provides passive immunity reducing the number and severity of infections as well as limiting many of the infective complications. Thus, early detection and appropriate intervention of XLA, increases the patient's lifespan and can allow the XLA-affected individual to live a relatively healthy life.

IVIg, is a cold alcohol fractionation, pooled from thousands of human blood donations [12]. IVIg is infused at 3-4 week intervals, for the duration of life. The dose is dependent on the trough levels of immunoglobulin and clinical symptoms of the patient. Thus an increase of infective problems may need more frequent infusions. Infusion side-effects include nausea, headaches, flushing, myalgia and vomiting [12, 20]. Home administration is possible in the case of older patients on a regular regime for convenience.

1.3.2 Alternative immunoglobulin treatment

Subcutaneous treatment literally means 'under the skin' treatment. Subcutaneous immunoglobulin (SCIg) is an alternative approach that involves use of intramuscular preparations of gammaglobulins and is usually recommended to patients who have adverse reactions to the IVIg treatment and for more even trough levels. This approach of administration is also sometimes preferred in the pediatric population as venous access in infants is exceptionally difficult [21]. The disadvantage, however, is that there is an increase in the frequency of infusion, as only a limited volume of Ig can be administered per infusion [12]. A study done by Quartier *et al.* revealed that the initiation of immunoglobulin replacement therapy early in life (within 3 months of diagnosis) significantly improved the patients wellbeing. It resulted in a drastic reduction in infectious problems and prevented severe, life-threatening bacterial infection and pulmonary complications [22].

It is important to note that none of these treatments are the cure for XLA, as currently available treatment only provides passive immunity, reducing the number and severity of infections as well as limiting many of the infective complications. It is therefore important that patients are monitored on a regular basis (every 3 months) regardless of the excellent clinical response of most patients, to ensure that optimal levels (recommended replacement dosage : ~0.4 – 0.6gm/kg every 3-4 weeks) are being maintained.

1.4 Bruton tyrosine kinase (Btk) gene

In 1993, two different research groups discovered that the disorder known as XLA, is caused by a mutation in the Bruton tyrosine kinase (Btk) gene [23, 24]. Mutation in the Btk gene results in an arrest in B cell development and almost an entire absence of serum immunoglobulin [10, 25].

1.4.1 Structural organization of the Btk gene

The structural organization of the Btk gene was determined using “positional cloning” strategies as well as a reverse genetic approach [23, 24]. It was mapped to the long arm of the X chromosome in the region of Xq21.3-Xq22 [26] and consists of 19 exons that extend over 37kb of genomic DNA [26]. The protein product is 77kD in weight and comprises of 659 amino acids [23,24].

This gene encodes for the Btk protein which consists of five structural domains (Figure 1.1). Proximal to the N-terminus is the Pleckstrin homology (PH) domain. This domain is the activation site of the Btk gene, and is followed by the proline-rich Tec homology (TH) domain [27], the Src homology 3 (SH3) domain, Src homology 2 (SH2) domain, and the catalytic kinase (also known as Src homology 1) domain. All are involved in protein-protein interaction except for the kinase domain [19]. Even with the vast amount of information, gained over the past few years, the exact mechanism(s) that results in Btk activation and the principal phosphorylation is yet to be established. Understanding the crystal structure of the Btk protein contributes to the structural comprehension of how mutations disturb the proper functioning of the Btk gene and subsequent XLA disorder. Crystal structures provide a more comprehensive view of interactions than other models. And thus understanding the protein at a structural level will provide better insight into how mutations within the Btk gene (genotype) leads to different ‘*physical manifestations*’ of the XLA disorder (phenotype) [28]. Mutation distribution within the 5 domains is approximately proportional to the length of the domain, with the exception of the TH domain [29].

These (variable) domains are frequently found in signalling proteins [30], other than the Btk gene and (the proteins) are involved in a multitude of cellular interactions with cell surface receptors, transcription factors, signal transduction and more [19]. In order to better understand properties and functions of the Btk gene and these other proteins, focus has been shifted to the domains involved as knowledge of the function of one domain in one protein may reveal the functioning in many other proteins carrying the same domain [30,31].

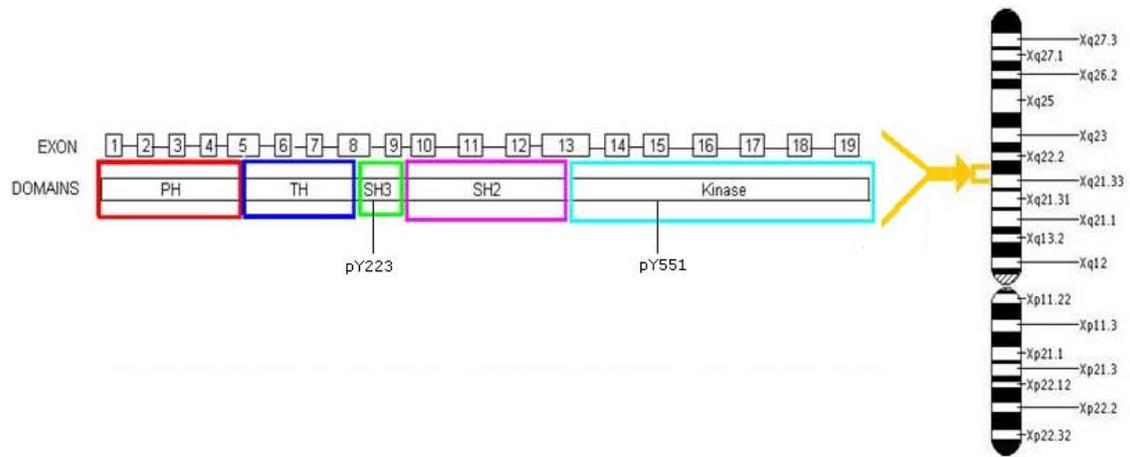


Figure 1.1 The alignment of exon 1 - 19 in the various domains [left] (showing the N-terminal PH domain (red), TH domain (blue), SH3 domain (green), SH2 (pink) and the kinase domain (light blue). Intronic regions are represented by the solid straight lines between exons) and the location of the Btk gene in the X-chromosome [left]. Phosphorylation regions (pY223 and pY551) are also indicated on the figure, and play an important role in the activation of Btk. Adapted from : [32] and Genetic Home Reference. (1993). (Accessed : September 27, 2010 at : <http://ghr.nlm.nih.gov/gene/BTK>)

1.4.2 Btk and B cell development

The Btk gene is mainly expressed in cells of the lymphoid and myelomonocytic lineages and is crucial for B cell development, growth regulation and differentiation [16,23,24]. Btk expression allows for normal B cell development which leads to the expression of functional light and heavy chain genes and thus normal B cell lineage. Antigenic stimulation of the B cells ultimately leads to the making of plasma cells which produce specific antibodies, with the assistance from T cells [16].

Most individuals with the XLA disorder have normal amounts of progenitor (pro-) and precursor (pre-) B cells in their bone marrow, but few or no mature B cells in their peripheral circulation [16, 34]. Whilst pre-B cells express Ig heavy chains they do not express the light chains [16, 35]. Due to the Btk-deficiency B lymphocytes functional light genes are not expressed in XLA patients, and thus mature B cells do not develop (Figure 1.3). This block results in B cell immunodeficiency which predominantly affects antibody production. Low or absent mature B lymphocytes in the periphery (peripheral circulation) and lymph node germinal centers leads to a severe reduction of serum immunoglobulins (Ig) levels of all isotypes [19, 33]. These individuals are therefore more susceptible to recurrent bacterial infection because of their lack of immunoglobulin production. The defect appears to be specific to the B-cell lineage as other lymphocyte populations (i.e. T cells) are not affected [16,17].

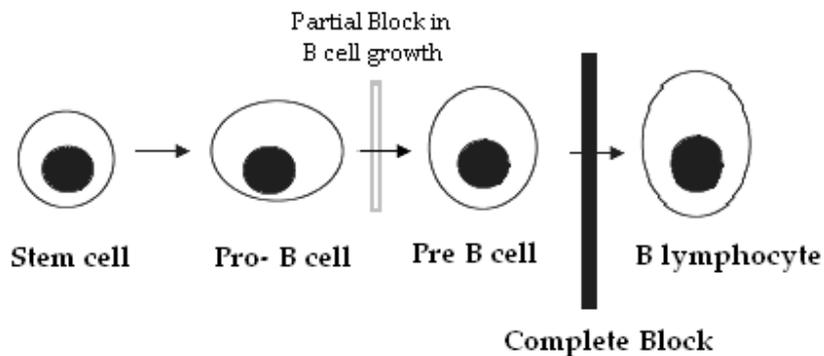


Figure 1.2 Blockage in the B cell development. B cell production is arrested at stages between pro-B and pre-B cells which results in B cell apoptosis and the subsequent XLA phenotype.[Adapted from Ref. 30].

1.4.3 Btk activation and function

Btk is dependent on its association with various ‘partners’ for its activation and regulation.

Btk activation is initiated by antigen stimulation of BCR on mature B cells, which induces tyrosine phosphorylation of Btk [36]. This partially activates Btk and places it in the BCR signal transduction pathway which reiterates the essential role of Btk in driving B cell differentiation. BCR-crosslinking ultimately leads to PH domain-mediated translocation of Btk to the plasma membrane [37], where it is further phosphorylated at Y551 by Src family kinase, either lyn or fyn. This phosphorylation increases the proteins activation considerably [36] and also leads to the subsequent autophosphorylation event involving the Y223 of the SH3 domain which results in the full activation of Btk [36, 38]. Btk is primarily found in the cytosol with small amounts in the plasma membrane fraction. This suggests that most of Btk function is exercised in the cytosol. [39]. Activated Btk recruits B cell linker (BLNK) protein and phospholipase C-gamma-2 (PLC- γ 2) to the plasma membrane. Btk and BLNK interaction contributes to the complete tyrosine phosphorylation of PLC- γ 2 [40] and the succeeding PLC- γ 2 phosphorylation ignites the downstream signal pathway which provides the platform for a variety of signal proteins [39].

Several studies have shown that a defective Btk gene results in an ‘autonomous B cell lineage defect’ [12,16, 34, 41]. This affects pro-B cells ability to thrive, reduces the pre-B cell proliferation [42] and also results in low to no numbers of B cell in the peripheral blood. Apart from Btk’s crucial role in B cell development [43] Btk also has a role in intracellular calcium concentration in response to antigen receptor stimulation as it sustains low levels of intracellular Ca^{2+} , required for proliferative responses of B cells. In this way it provides protection from bacterial infection. Btk has also been implicated in regulation of transcriptional factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B). NF- κ B plays a role in regulation of immune responses to infections and is involved in a cellular response to stimuli such as cytokines as well as bacterial infection [44, 45]. This implicates the kinase in a role that involves the functioning of immune cells of innate as well as adaptive immunity [46, 47].

1.4.4 Types of mutations found in the Btk gene

Mutations are distributed along all five functional domains, almost evenly throughout the gene, however, studies show that they tend to group at the tyrosine kinase domain (Table 1.4, [48, 49]). There are several types of mutations that leads to disease-causing XLA. Namely, single base pair substitutions that result in amino acid substitution, splice defects, premature stop codons as a result of nonsense mutations as well as small insertions and deletions that result in frameshift mutations. These mutations are found in both the coding (exonic) and non-coding (intronic) region of the Btk gene however most disease-causing mutations are found in the exonic region. Having effects on the protein expression, stability, activity as well as other properties of the protein [13]. Several DNA analysis studies done by several groups have found that approximately one-third of human Btk mutation causes amino acid substitution [13,17, 50-52] which results in unstable proteins, but normal amounts of Btk mRNA [18, 51]. Analysis of Btk expression in XLA patients revealed that regardless of the mutation type, most of these individuals did not express the protein, others led to a truncated protein [13, 53]. Mutations are mostly found in the coding region and over 12% of mutations have been found to interrupt on splice site recognition sequences [13]. Mutations usually affect conserved residues and functionally significant regions, causing alterations to the protein [13, 54]. The effect of some genetic variations are however better tolerated than others and clinical severity can usually be ascribed to the type and location of the mutations [55].

It is no surprise that drastic changes in protein properties are likely to have a drastic effect on its functioning. A database for XLA, was founded in 1994, as the first immunodeficiency mutation database (IDbase). The BTKbase Mutation registry for XLA Version 8.5 (last updated 21 August 2008) has a large collection of comprehensively, described Btk mutations that result in the XLA disorder and also contains clinical information of patients [29] This database can be accessed in the XLA mutation registry at: <http://bioinf.uta.fi/btkbase/> [59].

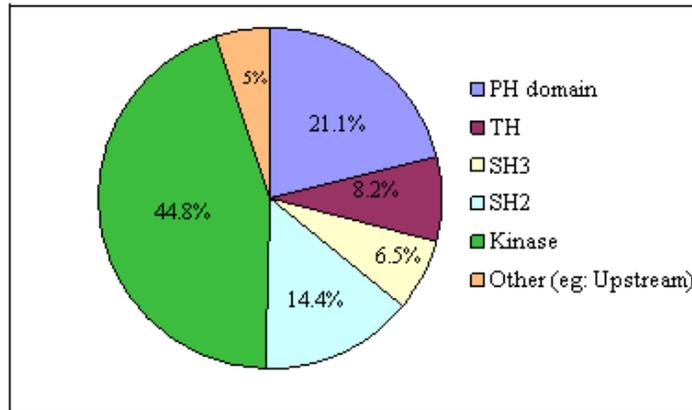


Figure 1.3 Mutation distributions in the 5 domains

- Mutations appear to be evenly spread throughout all structural domains except SH3 domain.
 - Mutation distribution also correlates to the length of the domain.
- Pleckstrin homology (PH) domain; Tec homology (TH) domain ; Src homology 3 (SH3) domain; Src homology 2 (SH2) domain

1.5 XLA diagnosis in South Africa

There is still a considerable amount of diagnostic delay despite available treatment. This is mainly due to a lack of awareness of PID (in the less established or under privileged communities) and due to lack of appropriate diagnosis.

1.5.1 Diagnostic testing

At present, XLA in South Africa is mainly diagnosed using clinical and immunological parameters such as recurrent (severe) infection, low to no serum immunoglobulin levels, no CD19⁺ B cells and a family history [3,10, 11, 43]. One third of XLA cases occur sporadically [17] and in these cases a clinical and immunological diagnosis is not sufficient. The seemingly low prevalence of XLA in S.A could be due to the fact that the clinical parameters used to diagnose XLA is similar to that of the Common Variable Immunodeficiency disorder (CVID) [60], resulting in XLA patients being misdiagnosed. Another possibility is that the severely affected infants may not survive long enough for diagnosis or intervention.

This study does not dismiss the value of clinical and immunological features as a useful method of diagnosing XLA but rather emphasises the importance of adjunct genetic confirmation. An extensive genetic Btk analysis on Agammaglobulinemia patients will allow for the identification of mutations within the Btk gene which would confirm XLA status within patients. Sequence-based diagnosis provides a definitive, convenient method that can be used in routine XLA genetic diagnostics, to support clinical diagnosis of patients without family history of XLA. Majority of patients have significantly reduced B cells and Ig levels. Thus another form of diagnosis is by monitoring the number of CD19⁺ and CD20⁺ B cells,

IgG, IgM and IgA levels which are usually reduced in an XLA patient. Table 1.4 shows the clinical, immunological and genetic diagnosis required for an accurate diagnosis of XLA.

Table 1.4 Diagnostic Testing of XLA

Clinical Diagnosis	<ul style="list-style-type: none"> a. Male patient b. Recurrent bacterial infections in the first two years of life. c. Btk mutation presumed
Immunological Diagnosis	<ul style="list-style-type: none"> a. < 2% of CD19 positive B cells in the blood b. Low Serum concentration of IgA, IgG and IgM (more than 2SD below the mean for age)
Genetic Diagnosis	<ul style="list-style-type: none"> a. Mutation in the Btk gene

1.5.2 Diagnostic test benefits

The implementation of an XLA diagnostic screen test will allow physicians to promptly provide adequate clinical treatment plans and interventions. It will also allow for patient and family screening with diagnosis of XLA. Early recognition will lead to early treatment, appropriate medical follow-up and hopefully prevention of complications of the affected individual.

The clinical data from the affected XLA patients of this study, are recorded in our Primary Immunodeficiency South Africa (PISA) Registry, which is a Registry for PIDs in South Africa. The registry and our study are part of an effort to improve awareness of PID in S.A and for early diagnosis and better patient care.

1.6 Research design and methodology

This research was conducted to identify abnormalities in the Btk gene of patients, clinically diagnosed with XLA. Molecular diagnosis coupled to clinical history of the patient provides a definitive XLA diagnosis. The research focused on five unrelated individuals and their available family members. They were selected primarily from the Primary Immunodeficiency South Africa Registry. In an attempt to investigate XLA in South Africa, a call was sent out to doctors throughout S.A (Appendix A) but the response was poor and thus the study was limited to 5 male patients. Figure 1 presents a flow chart of the process undertaken in this study.

The inclusion criteria for this study were a clinical diagnosis of XLA compared to a group of healthy 'negative' controls. Each participant provided informed consent and a blood sample. DNA is subsequently extracted from the blood and all 19 exons of the Btk gene are analyzed using PCR techniques. After amplifying all 19 exons individually for each patient, the PCR product is purified using a Wizard® SV Gel and PCR Clean-up System kit standard procedures (Promega, Maddison USA), sequence reactions are carried out using a BigDye Terminator Cycle Sequence Kit (Applied Biosystems, Foster City USA) and assembled to the Btk reference sequence (Accession: U78027) using BioEdit. Once the mutation was identified, mothers of the patients were tested using a mutation-specific test to identify maternal carrier status. Mutations are also compared to those found on the online Btk database in order to further classify mutations as known or novel.

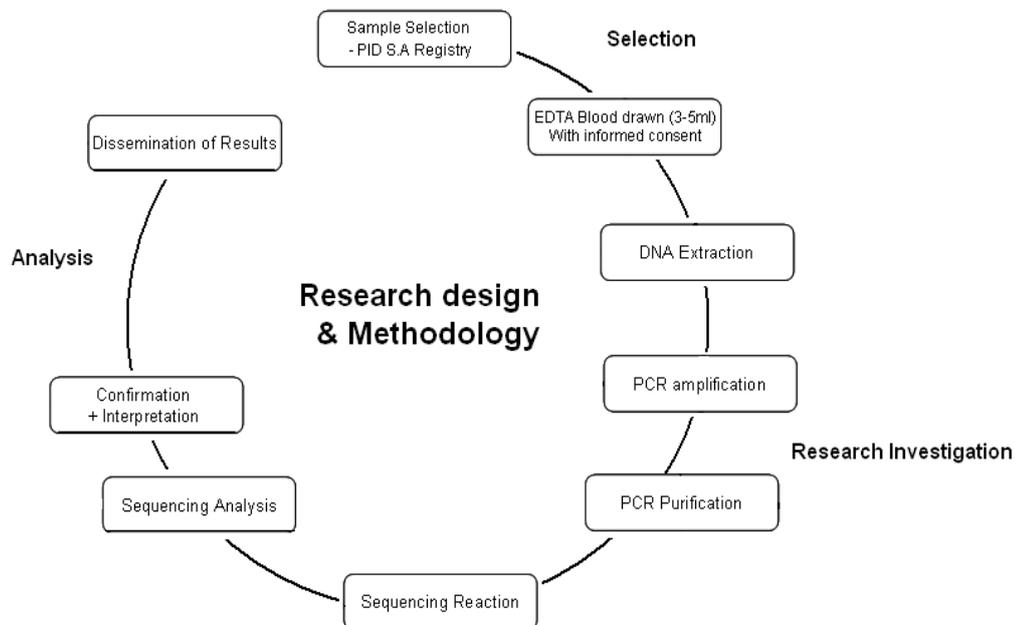


Figure 1.4 Research plan of action

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Chapter 2

Sequence-based diagnosis of X-linked Agammaglobulinemia in South Africa

Sequence-based diagnosis of X-linked Agammaglobulinemia in South Africa

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Abstract :

X-linked agammaglobulinemia (XLA) also known as Bruton's Disease, the commonest primary immunodeficiency syndrome, results from Bruton tyrosine kinase (Btk) gene mutations. Affected patients fail to generate mature B cells and are unable to produce antibodies. Diagnosis is frequently delayed until onset of severe and recurrent infections. Early diagnosis by genetic studies can prevent morbidity and mortality. With DNA based PCR we investigated unrelated male patients, with a clinical diagnosis of XLA. We recorded three different mutations, one of which was novel. This method proved to be efficient for the detection of Btk mutations providing a definitive diagnosis for patients and carriers in South Africa.

Keywords : Primary Immunodeficiency (PID), X-linked agammaglobulinemia (XLA), Bruton tyrosine kinase (Btk), Polymerase chain reaction (PCR).

Introduction

Primary immunodeficiency disorders (PID) disrupt the proper functioning of the immune system [1]. This is caused by mutation in genes, required for proper immune functioning [2,3]. The prototype PID is X-linked agammaglobulinemia (XLA), which was reported in 1952, by O.C Bruton [4]. The patients are usually male, have less than 2% CD19⁺ B cells in their peripheral blood and subsequently lack humoral immune responses. Deficient antibody production of all immunoglobulin isotypes results in an excessive susceptibility to infections. It was later discovered, that this disorder is caused by a mutation in the cytoplasmic tyrosine kinase gene known as Bruton's tyrosine kinase (Btk) [5,6]. The Btk gene is mapped to the long arm of the X chromosome in the region of Xq21.3-Xq22 [7] and consists of 19 exons that extend over 37.5 kb of genomic DNA [8]. The protein product is 76kD in weight and consists of 659 amino acids [5,6]. The Btk gene has five functional domains. Proximal to the N-terminus is the Pleckstrin homology (PH) domain, followed by the Tec homology (TH) domain, Src homology 3 (SH3) domain, Src homology 2 (SH2) domain and the catalytic kinase domain (Figure 1). Mutation distribution within the 5 domains is approximately proportional to the length of the domain with the exception of the TH domain [9].



Figure 1. The domain structure of the Bruton's Tyrosine kinase protein.

The Btk gene is mainly expressed in cells of the lymphoid and myelomonocytic lineages and is crucial for B cell development, growth, regulation and differentiation [5,6,10]. Mutations result in an arrest in B cell development due to complete block in B cell development (Figure 2). This predominantly affects antibody production with low or absent immunoglobulin levels and lack of lymph node germinal centers, [11,12]. The defect appears to be specific to the B-cell lineage as other lymphocyte populations (i.e. T cells) are not affected [13].

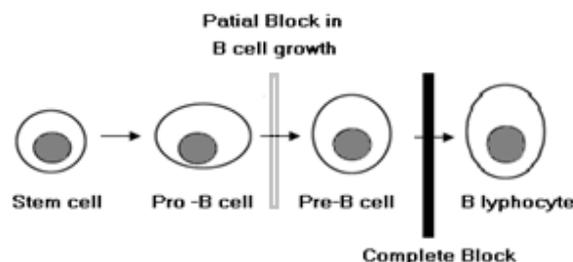


Figure 2. Blockage in the B cell development. B cell production is arrested at stages between Pre-B and B lymphocyte which results in B cell apoptosis and the subsequent XLA phenotype

The severity of XLA varies with each individual, even relatives that share the same mutation differ in their clinical phenotype [14,15]. The most common clinical and immunological features include recurrent infections especially with bacteria (Table I) and very low B cells and immunoglobulins. The standard treatment for XLA is intravenous gammaglobulin replacement therapy (IVIg), which provides passive immunity thereby, reducing the number and severity of infections as well as many of subsequent complications. Diagnostic delay is still common however and this may be due to a lack of availability of screening tests and lack of awareness of PID in especially the less privileged communities. Nevertheless, the XLA is suspected in males with early onset, recurrent bacterial infection, a marked reduction in B cell numbers ($< 2\%$ CD 19⁺ B cells) with low to no detectable levels of serum immunoglobulin upon clinical and /or immunological investigation [1,4,11,16]. A definitive diagnosis is confirmed only in individuals who have genetic analysis and who are identified with a mutation in their Btk gene [17]. Genetic testing is also the most reliable way of identifying female carriers of XLA. Carrier testing should be offered to females who have a family history of XLA.

Table I Clinical Presentation of XLA

Recurrent Infections
<ul style="list-style-type: none"> • Otitis • Sinusitis • Conjunctivitis • Rhinitis • Pneumonia • Arthritis
Most common infectious agents
<ul style="list-style-type: none"> • <i>Streptococcus</i> • <i>Pneumococcus</i> • <i>Staphylococcus</i> • <i>Giardia</i> • <i>Echovirus</i>

A registry containing mutations and phenotypes for XLA was founded in 1994, and was the first immunodeficiency mutation database (IDbase) (BTKbase : <http://bioinf.uta.fi/btkbase/>). It contains mutation entries for over 1000 patients from more than 900 unrelated families, representing more than 600 unique molecular events [9,18]. In this study we performed genetic analysis on a group of 5 male unrelated patients with a clinical diagnosis of XLA. Polymerase chain reaction (PCR)-based method was used for detection of mutation in the Btk gene and subsequent XLA diagnosis.

Method

The screening approach used to characterize the mutations identified in the Btk gene of the XLA patients was DNA-based PCR method, using 38 primers (Appendix C, [19]) to amplify all 19 exons of the Btk gene, followed by direct sequencing and subsequent sequence analysis of the Btk gene. Mutational analysis was performed on five male patients from five unrelated families, four of the patients had an XLA compatible phenotype. The remaining patient presented with recurrent infection and a phenotype similar to XLA, however did not fit the strict criteria for a clinical diagnosis of XLA. His participation in the study served as a negative-diseased control with hypogammaglobulinemia. Two patients included in this study receive care at our clinics at Tygerberg hospital. The remaining three were referred to by physicians from different institutions. Table II shows the laboratory data of the 5 unrelated patients.

Table II Relevant Immunological data at diagnosis

Patient No.	001	002	003	004	005
Ethnicity	White	White	Coloured	White	Black
Age at diagnosis	4yr	1yr 3months	3months	7yr 3months	7yr 9months
Serum Ig Levels (g/L) *					
IgG	1.09↓	0.1 ↓	0.33 ↓	0.33 ↓	<1.41 ↓
IgM	0.2↓	0.02 ↓	0.1↓	0.08 ↓	<0.25 ↓
IgA	0.07↓	0.06 ↓	0.07 ↓	0.07↓	1.09 N
B cell enumerations (%)					
CD19 ⁺	<2%	0%	0%	1%	1%

* Serum Immunoglobulin level and B cell number reported at the time of diagnosis.

Reference ranges from Ref 16.

↓ Low

N Normal

Patient 003 had a family history of XLA and was previously reported on by Pienaar *et al.* [20], he was included as a positive control. Female carrier detection was done when possible, this included the mother and sibling of Patient 001 and the mother of Patient 002. Ten healthy individuals with various ethnicities were included as negative controls. The initial step involved the preparation of DNA from (EDTA-lined) blood followed by the amplification of the 19 exons in the Btk gene using PCR method. PCR products were then directly sequenced and in a final step mutation analysis was performed in the 5 unrelated patients. Ethical clearance was obtained from the Ethics Committee for Human Research at Stellenbosch University and written consent was obtained for each participating individual.

Results

PCR analysis followed by direct sequencing analysis revealed three different mutations (Figure 3). Two mutations were previously described [20-22] and one novel mutation was identified. Mutations were observed in the PH, SH2 and Kinase domain. Two XLA causing mutations identified within the Btk gene were located in the Pleckstrin-homology (PH) domain. The Btk mutational analysis of Patient 001, revealed a mutation at the amino acid position 28 (R28H) resulting in a missense mutation in exon 2 which is consistent with the classical diagnosis of XLA. This amino acid change was first identified by de Weer *et al.* [21] and the BTKbase contains 17 patients with the R28H mutation (<http://bioinf.uta.fi/BTKbase/index.php?content=index/IDbases>). The second Btk mutation (in Patient 003) found in the PH domain was identified by Pienaar *et al.* (2000), a Δ 60 mutation of the Btk gene as a AAG deletion found at amino acid positions 308-310, which results in the loss of a single amino acid, lysine at codon 60. The BTKbase contains 7 patients with the K60del mutation, six of the patients were collected from the study done by Pienaar *et al.* the 7th patient was reported in 2003 by a study done in Argentina [23]. Patient 002 had a point mutation in the SH2 domain of the Btk gene which results in a C-to-T change at position 862 in exon 10 of the Btk gene. This mutation has been reported in 29 other unrelated families [21] and can also be found in the Btkbase.

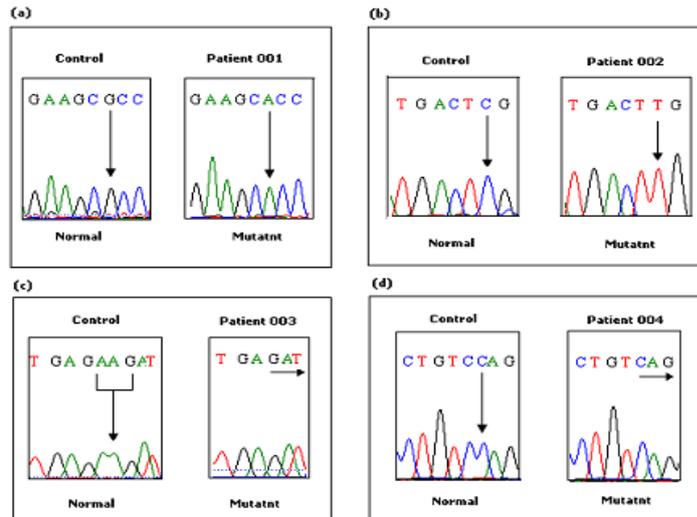


Figure 3. Schematic view of the sequence analysis of patient 001 (a), Patient 002 (b), Patient 003 – the positive control (c) and Patient 004 (d). Mutated Nucleotide is indicated with an arrow

The mutation found in Patient 004 in the kinase domain appears to be a novel mutation as no published literature was found. This mutation has been submitted, to the website. Once the mutation has been validated the publication of the entry will appear in the next database released. Although Patient 005 did not conform to the strict criteria for clinical diagnosis of XLA with normal serum IgM levels, he was included in this study on the basis of his low levels of B cells, recurrent infections and similar phenotype to that of XLA patients. His inclusion served as a negative-disease control and was thus not expected to have a detectable Btk gene mutation, this was confirmed on mutation analysis of his Btk gene. Table III summarizes the mutations identified and also indicates whether or not relatives were affected. Two mothers and one other family member were available for evaluation in this study. Screening the sequence of Patient 001's mother indicated that she was a carrier (heterozygous) of the mutation. Although the mother of Patient 001 had no desire of having any more children and therefore declined the offer to speak to a genetic counsellor she did however wish for her daughter, the twin of Patient 001 to be tested as well. She too was a carrier of the mutation. Patient 002's mother was tested in the same manner and her results indicated that she was also a carrier affected by XLA mutation found in Patient 002. As for the mother of Patient 003 she was previously documented to have the Btk mutation identified in Patient 003 and her participation in this study was useful as a positive carrier control. Detected mutations were described according to den Dunnen and Antonarakis [24] using the Btk cDNA sequence as reference sequence (GeneBank accession number NM_000061.1) in which the A of the ATG translation 'initiates' the start site representing nucleotide +1[9, 24].

Table III. Characterisation of the mutations found in the Btk gene of unrelated XLA patients

Patient No.	Exon	Btk domain	Type of mutation	Nucleotide position ^{1,2}	Amino Acid Change ²	Affected relatives
001	2	PH	1bp substitution	c.83G>A	p.R28H	+
002	10	SH2	1bp substitution	c.862C>T	p.R288W	+
003	3	PH	3bp deletion	c.308-310delAAG	p.K60del	+
004	16	Kinase	1bp deletion	c.1629delC	p.S543del	N.D
005	N.D	N.D	N.D	N.D	N.D	N.D

¹Numbering of the nucleotide and amino acid positions refers to cDNA sequences (GeneBank accession number NM_000061.1). A of the ATG translation initiates the start site representing nucleotide +1 [9,23]. Patients who had an affected relative are indicated with a (+). The novel mutation is indicated in **bold**. Patient 005 had no mutation in his Btk gene. (ND : Not determined).

Del : deletion; **In Bold** : Novel mutation

Discussion

This study explored the molecular diagnosis of unrelated XLA patients in South Africa. The method used was a DNA-based, to detect abnormalities in the Btk gene. Data represent three male patients identified with a Btk mutation. Two of the patients (P001, P002) had mutations previously described in the Btk mutation database (BTKbase) and one novel mutation was identified in Patient 4 (P004). Patient 005 illustrates an XLA-like phenotype with a defect possibly in another gene required for proper B cell development for example μ -heavy chain or the λ 5/14.1 genes. One study of three patients indicated that patients with mutation in μ -heavy chain, the λ 5/14.1 and Btk gene all resulted in having impairment in differentiation from pro-B cell to pre-B cell [11]. Thus demonstrating that defects in any of these genes can result in similar clinical features.

In South Africa, agammaglobulinaemia is currently diagnosed only according to clinical and basic immunological features. A definitive diagnosis of XLA without genetic analysis is possible in a patient with a family history of XLA. However one-third of XLA cases occur sporadically [25] and in these cases a clinical and immunological diagnosis is insufficient and commonly delayed. These patients especially require the addition of molecular diagnostics involving a specific Btk mutational analysis to provide a clear diagnosis. Though this study involved a limited number of patients, investigators believe that the results obtained will be of value and direct beneficial use for family members of the five patients. The study also aimed to increase awareness as well as providing accurate diagnosis of XLA. Using Btk mutational analysis the above mentioned knowledge can be applied to ensure early detection of Btk gene abnormalities of affected males and also carrier females. This will allow physicians to provide appropriate clinical treatment plans and early interventions for best prognosis and outcome. The clinical data from all patients are recorded in our Primary Immunodeficiency (PISA) Registry for PIDs in South Africa. The registry and studies such as this one aim to identify the prevalence of these genetic diseases in South Africa for appropriate diagnostics and treatment algorithms to improve patient care.

Summary

Mutational analysis led to a definitive diagnosis of XLA in 3 of the patients each were identified with a different Btk mutation. Direct DNA sequencing of the Btk gene revealed mutations on exon 2 of Patient 001, exon 10 of Patient 002, Patient 003 had previously been diagnosed with XLA and served as a positive control. And a novel mutation in exon 16 of Patient 004. Patient 005 was not definitively diagnosed with XLA even though he demonstrated clinical features similar to that of patients with the XLA status. This study promotes awareness of PID and may encourage more specific genetic mutational analyses of other Primary Immune disorders in South Africa.

Declaration of conflict of interest

The authors declare no conflict of interest.

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Chapter 3

A Novel mutation : XLA in South Africa

A Novel mutation : XLA in South Africa

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Abstract

X-linked agammaglobulinemia (XLA) is a result of mutations in the Bruton tyrosine kinase (Btk) gene. Genetic analysis of the BTK gene was performed on a group of 4 unrelated male patients with a clinical diagnosis of XLA using a direct sequencing approach. Analysis revealed 3 different mutations in 3 of the 4 patients, namely c.83G>A (p.R28H), c.862C>T (p.R288W), and a novel mutation, c.1629delC (p.S543del). Evaluation of 2 available mothers revealed x-linked carrier status in both of the mothers. This study supports the usage of genetic analysis as a specific and sensitive method for the possible detection of Btk mutation and definitive diagnoses of XLA in South Africa.

Key Words: X-linked agammaglobulinemia (XLA), Bruton tyrosine kinase (Btk), Pleckstrin homology (PH) domain, Tec homology (TH) domain, Src homology 3 (SH3) domain, Src homology SH2 domain, kinase domain.

Introduction

X-linked Agammaglobulinemia (XLA, MIM # 300300) is a primary immunodeficiency disorder (PID), first defined and reported by O.C Bruton in 1952 [1]. The underlying genetic basis of the disease was later discovered to be caused by mutations in the Bruton tyrosine kinase (Btk) gene [2,3] mapped to the long arm of the X chromosome in the region of Xq21.3-Xq22 [4] and consists of 19 exons that extends over 37kb of genomic DNA [5]. The protein product is 77kD in weight and comprises of 659 amino acids [2,3]. The Btk gene consists of five structural domains, proximal to the N-terminus is the Pleckstrin homology (PH) domain, followed by the Tec homology (TH) domain, Src homology 3 (SH3) domain, SH2 domain, and the catalytic kinase (SH1) domain. All are involved in protein-protein interaction except for the kinase domain. [6]. Mutations are almost evenly distributed along all five functional domains; however, studies have shown that most mutations can be found in the tyrosine kinase domain [7, 8]. The severity of the disease is associated with the type and location of the mutations [9]. A database for XLA was founded in 1994, as the first immunodeficiency mutation database (IDbase). The BTKbase Mutation registry for XLA Version 8.5 (last updated 21 August 2008) contains more than 900 different Btk mutations combined with clinical information associated with the mutations (<http://bioinf.uta.fi/btkbase>) [10, 11]. The aim of this study is to determine the molecular basis of XLA in South Africa using a direct sequence-based method to detect abnormalities in the Btk gene. Molecular diagnosis coupled to clinical history of the patient provides a definitive XLA diagnosis.

Materials and Method

Sampling of patients

Blood was obtained from four unrelated, clinically diagnosed XLA male patients, each with an XLA compatible phenotype, namely <2% circulating CD19⁺B cells, very low levels of all serum immunoglobulin isotypes and recurrent infection, together with ten healthy individuals as study controls and a fifth patient who served as a negative diseased-control. After the initial screening on the index cases, family members were followed up to determine carrier status, where possible. Ethical clearance was provided from the Ethics Committee for Human Research at Stellenbosch University and a consent form was obtained from each participating individual.

Genomic DNA extraction, Primers and PCR assay

Total genomic DNA from Ethylenediaminetetraacetic acid (EDTA) blood samples was extracted by using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany), following the recommendations of the manufacturer. All 19 exons, of the Btk gene, were screened using exon-specific primers as previously described [12]. PCR amplification reactions consisted of: 1.00 mM MgCl₂, 0.20 mM dNTP mix, 1x Taq Colourless PCR buffer, 0.02 U/μl Go Taq Polymerase (Promega, Maddison USA), 0.20 μM of each primer [12] and 100 ng template DNA. The total PCR amplification reactions were prepared to 25 μL for each sample. The amplification conditions used for exons 1, 2, 4-7 and 17-19 was as follow: initial denaturation at 95°C for 5 min; 15 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec, followed by 30 cycles of denaturation at 95°C for 30sec, annealing at 55°C for 30sec and extension at 72°C for 30 sec; followed by a hold cycle at 4°C. The parameters for exons 3, 15 and 16 were similar to the above with the exception of an touch-down annealing temperature of 55° for 15 cycles and a further 30 cycles at 50°C. Amplification products were then visualized on 2.0% TBE agarose gels and the PCR amplicons varied in size from approximately 143-315bp. Product sizes were confirmed by running a 100bp ladder on either end of the gel.

Mutational Analysis

After all 19 exons were amplified, amplicons were sequenced with the Big Dye Terminator cycle Sequencing kit (Biosystems, Foster City, USA) using the same primers as that in the PCR method. Samples were finally processed on an ABI3700 capillary Sequencer (Foster City, USA) and sequence variations were confirmed by sequencing in the opposite direction. These mutations were annotated using recommended nomenclature described by den Dunnen and Antonarakis [13] using the Btk cDNA sequence as reference sequence (GeneBank accession number NM_000061.1) in which the A of the ATG translation 'initiates' the start site representing nucleotide +1 [10, 13].

Results and discussion

This study used molecular techniques to definitively diagnose patients with XLA. Individuals were selected based on clinical and immunological parameters compatible with an XLA phenotype (Table 1).

Table 1 Relevant Immunological data at diagnosis

Patient No.	001	002	003	004	005
Age at diagnosis	4yr	1yr 3months	3months	7yr 3months	7yr 9months
Serum Ig Levels (g/L) *					
IgG	1.09↓	0.1 ↓	0.33 ↓	0.33 ↓	<1.41 ↓
IgM	0.2↓	0.02 ↓	0.1↓	0.08 ↓	<0.25 ↓
IgA	0.07↓	0.06 ↓	0.07 ↓	0.07↓	1.09 N
B cell enumerations (%)					
CD19 ⁺	<2%	0%	0%	1%	1%

* Serum Immunoglobulin level and B cell number reported at the time of diagnosis.

Reference ranges from Ref 14.

↓ Low

N Normal

After extensive Btk Analysis, mutations were identified in three of the four unrelated patients. The presence of the mutations detected was confirmed using bi-directional sequencing. Mutations were observed in the PH, SH2 and the Kinase domains. Table 2 provides a summary of the mutations identified, its affected domain and whether the mutation was found in any of the relatives screened.

Table 2. Characterisation of the mutation found in the Btk gene of unrelated XLA patients

Patient No.	Exon	Btk domain	Type of mutation	Nucleotide position ^{1,2}	Amino Acid Change ²	Affected relatives
001	2	PH	1bp substitution	c.83G>A	p.R28H	+
002	10	SH2	1bp substitution	c.862C>T	p.R288W	+
004	16	Kinase	1bp deletion	c.1629delC	p.S543del	N.D
005	N.D	N.D	N.D	N.D	N.D	N.D

¹Numbering of the nucleotide and amino acid positions refers to cDNA sequences (GeneBank accession number NM_000061.1). A of the ATG translation initiates the start site representing nucleotide +1 [10,13] Patients who had an affected relative is indicated with a (+). The novel mutation is indicated in **bold**. Patient 005 had no noticeable mutation in his Btk gene. (ND : Not determined).

Del : deletion

Two patients (Patient 001 and Patient 002) had Btk mutations in conserved residues (R28H and R288W). The R28H substitution identified in Patient 001 (Figure 1a) results in a G→A transition. The R288W missense mutation identified in Patient 002 (Figure 1b) presented with a C→T transition. Both of these mutations are well defined mutations and are known to lead to a classical XLA - causing phenotype [15,16] evident from the way in which the mutation disrupts the functionally important conserved residues of the

Btk gene which leads to an arrest in B cell development and ultimately a deficiency in the immune system [17,18], evident in both index cases. On review of available history and laboratory data, Patient 001 and Patient 002 presented with low CD 19⁺ B cell numbers and various infectious complications, consistent with the criteria for XLA diagnosis. Their infections raised suspicions of a primary immunodeficiency disorder, which was later confirmed when laboratory results of their serum B cells and serum immunoglobulins were also seen to be exceptionally low. The mutations identified in Patient 001 and 002 are both been reported in 17 and 29 other unrelated families respectively, described in the BTKbase [11, (<http://bioinf.uta.fi/BTKbase/index.php?content=index/IDbases>)].

The novel mutation identified in Patient 004 (Figure 1c) leads to a frameshift in the gene. Frameshift mutations are predicted to result in protein truncation that usually prevents the production of normal transcripts [19] which is evident in Patient 004. This single base pair deletion detected in exon 16 encodes for the kinase activity of Btk [18] domain, is found in the β 9 strand [20] and is a phosphorylation site in the kinase domain. It also plays a role in regulation of the kinase [21]. This mutation has been directly submitted, using the electronic submission form on the website. Once the mutation has been validated the publication of the entry will appear in the next database released. Identification of a Btk mutation in three of the patients allowed for a specific-mutation screen test that could be performed on mothers and siblings available for evaluation in this study. The specific mutations were detected in two mothers and a female sibling available for screening, which confirmed their carrier status and the X-linked inheritance pattern of the disease. Patient 005's inclusion served as a negative-diseased control and was thus not expected to have a detectable Btk gene mutation. Even though Patient 005 had no visible mutation in the coding region of the Btk gene, this study excluded screening of the non-coding regions (i.e. the promoter or intronic region) thus the possibility exists that the clinical phenotype observed in this patient could be due to variations in these areas or from a defect in another gene resulting in the same clinical features as that found in XLA patients [22,23].

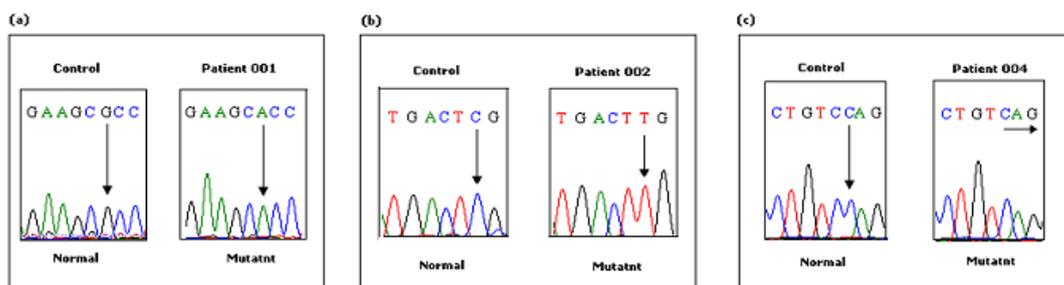


Figure 1. Schematic view of the sequence analysis of normal and variant sequence of Patient 001; c.83G>A (p.R28H) (a), Patient 002; c.862C>T (p.R288W) (b) and Patient 003; c.1629delC (p.S543del) (c). Mutated Nucleotide is indicated with an arrow.

In recent years, publications have focused less on de novo mutations and more emphasis is being placed on structure-function relationships specifically focused on possible genotype-phenotype correlation. The most recent breakthrough by Lee *et al.* [19] describes a correlation between genotype and the severity of the disease in terms of occurrence of severe infectious complications and the age of disease onset. Lee *et al.* classified patients according to their early onset clinical presentation, severity of infections and mutation type. Groups included those with severe mutations presenting with infectious complications at an early age and those who had a less severe mutation, presented later on in life with a less severe form of the disease. Lee *et al.* further notes, that though these findings are not absolute they do suggest that a 'global' genotype-phenotype correlation for XLA does exist [19]. As we are only reporting on a small group of patients consisting of one novel mutation we cannot comment on a correlation between mutation and the severity of clinical phenotype.

In conclusion, to our knowledge this is the first study in South Africa to report different Btk mutations in four unrelated South African patients clinically diagnosed with XLA. Sequence-based diagnosis provided a definitive and accurate method that can be used in routine XLA molecular diagnostics. This would facilitate early diagnosis of patients without a family history of XLA and will allow for the immediate institution of life saving replacement therapy, the detection of carrier status in family members and genetic advice. This study emphasises the importance of genetic analysis as an adjunct to the basic screening of clinical and immunological features in patients investigated for XLA.

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Declaration of conflict of interest

The authors declare no conflict of interest.

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4. Discussion

4.1 Molecular analysis and interpretation

This study, included five male patients from five unrelated families, four of the patients had an XLA compatible phenotype. The remaining patient presented with recurrent infection and a phenotype similar to XLA, however did not fit the strict criteria for a clinical diagnosis of XLA. His participation in the study served as a negative-diseased control with hypogammaglobulinemia. These patients were screened for mutations in the Btk gene using direct sequencing. Mutations were identified in the PH, SH2 and the Kinase domain of the Btk gene. Two of the three mutations were previously described and reported in the BTKbase, whilst the fourth mutation appears to be novel.

Two point mutations were found in conserved residues of the Btk gene [1]. Patient 001 had a missense mutation in the PH domain resulting in a G→A transition in exon 2, which led to an amino acid change of an arginine to a histidine (R28H). The PH domain is a small signal transduction domain [2]. It plays a crucial role in the translocation of Btk from the cytosol to the plasma membrane which is a critical step in the phosphorylation and activation of Btk [3, 4] and is also said to act as a membrane anchor as it binds to phosphoinositides [5, 6]. Most mutations located in the PH domain are located in the positively charged end of the molecule and is presumed to affect the binding site for phosphatidylinositol lipids [7, 8], therefore preventing Btk migration to the cell membrane (via the reduce in its affinity for inositol phosphates).

The genetic defect in Patient 001 was first described by de Weer *et al.* [9] and is located in the conserved Arg-28 residue. This residue plays an important role in binding potential ligands such as phospholipids [7, 8], therefore the genetic defect in Patient 001 leads to drastic reduction in the binding activity of Btk [7]. The mutation found in Patient 002 is in the SH2 domain. This domain plays a role in growth, metabolism, gene transcription and mitogenesis [11]. It also has a role in signal transduction as it mediates the binding of tyrosine-phosphorylated peptide motifs to other molecules in the signal transduction pathway [12, 13]. The R288W mutation detected in Patient 002, substitutes a C → T at codon 288 in exon 10 of the Btk gene. This substitution replaces the large basic arginine (R) with a large sized aromatic tryptophan (W) (R288W) causing a disruption in proper ligand binding [13]. This leads to a 200 fold decrease in stability and peptide binding affinity of the SH2 domain with phosphotyrosine molecules [14].

The mutations identified in Patient 001 and 002 have been found in 17 and 29 other unrelated families, respectively in the BTKbase (<http://bioinf.uta.fi/BTKbase/index.php?content=index/IDbases>). Both of these mutations are well defined and are known to lead to a classical XLA status. This is evident from the way the mutations disrupt the functionally important conserved residues of the Btk gene, which leads to an arrest in B cell development and ultimately a deficiency in the immune system. On review of available

history and laboratory data, of both Patient 001 and Patient 002, they presented with low CD 19⁺ B cell numbers and various infectious complications, consistent with the diagnostic criteria for XLA status. These infections raised suspicions of a primary immunodeficiency disorder, which was later confirmed when laboratory results of their serum B cells and serum immunoglobulins were also seen to be exceptionally low.

A family history of Agammaglobulinemia prompted Patient 003 to be investigated at the early age of 3 months after birth. Initial laboratory results did not meet the criteria for XLA diagnosis due to the presence of maternal antibodies. He was presumed to have an XLA mutation and genetic analysis confirmed this [15] IVIg treatment was started before the onset of any serious infections. The identification of the family specific mutation described by Pienaar *et al*, in this family was performed previously ([15], data not shown) and this patient served as a positive control during this study. High variability of clinical expression has been reported in case-to-case comparisons of patients even within a family, affected with the same mutation [16,17]. This was also evident within Patient 003's family.

The novel Btk mutation in Patient 004 is found in the kinase domain (also known as the Src Homology (SH1) domain). The mutation identified was a single base pair deletion of a C at position 1629 in exon 16 which leads to a frameshift deletion in the amino acid serine. This novel mutation is found in the β 9 strand [12] in a functionally important site of the kinase domain. Exon 16 encodes for the kinase activity of Btk [11] and S543 is a phosphorylation site which plays a role in regulation of the kinase [18]. Studies thus far, have noted that mutations identified in the kinase domain mostly leads to no detectable protein kinase activity, with different effects on the protein stability [19, 20]. The mutation found in Patient 004 leads to a stop codon (Appendix D) which produces a truncated protein. This leads an alteration, subsequently preventing the production of a normal transcript [21]. Evaluation of Patient 004's clinical history and comparing it to the other patients it would seem to indicate that the mutation identified in Patient 004 is a severe mutation. This discrepancy however could be due to the late age of diagnosis and delay in treatment. Precise characterization of the novel mutation would require Btk expression analysis. This mutation has been directly submitted, using the electronic submission form on the website. Once the mutation has been validated the publication of the entry will appear in the next database released.

It is well known that Btk mutations have been identified in all five domains with XLA-causing disorder in each. In our study, mutations were found in the PH domain which is known to be important for the translocation of Btk to the membrane [7], the SH2 domain which is significant for its interactions with signalling molecules [14] and lastly the Kinase domain which is essential for kinase activity and ATP binding [22]. Thus it is clear that each domain of the Btk protein is in some way functionally important and collectively contributes to the proper B cell development and ultimately a functioning immune system.

Despite the clinical appearance of XLA in our diseased – negative control, Patient 005 had no visible mutation in the coding region of the Btk gene and since this study excluded screening of the non-coding regions (i.e. the promoter or intronic region) the possibility exists that the clinical phenotype observed in this patient could be due to variations in these areas.

Another possibility is that Patient 005 has an XLA-like phenotype which is due to mutations in genes other than the Btk gene, such as the μ -heavy chain or the λ 5/14.1 genes [26]. These genes also play an important role in B cell development and thus mutations in one of these genes could lead to a failure of B cell production and eventually to agammaglobulinemia. One study indicated that studies of patients with mutation in μ -heavy chain, the λ 5/14.1 and Btk gene all resulted in impairment in differentiation from pro-B cell to pre-B cell, in all three patients [25]. Which allows us to conclude that defect in any of these genes will result in similar clinical features.

4.2 Carrier detection and genetic counselling

Identifying a Btk mutation in three of the patients provided a specific mutation screen, which was also used to analyse their relatives. Since XLA has a maternal inheritance pattern, the analysis was offered to the mothers to confirm carrier status.

Females are not usually affected by XLA but those identified with the carrier status carry one diseased copy of the gene and one normal copy. If one of the two X-chromosomes carries a gene defect that is detrimental to proliferation or survival, all the cells of that lineage will demonstrate preferential inactivation of the mutated X-chromosome [27]. Males only have one X-chromosome and therefore show both the clinical and immunological results of abnormality. A carrier mother needs genetic counselling to understand the implications to her unborn offspring. Genetic counselling is a process by which a trained professional provides individuals and /or family with information on genetic disorders such as this and provides detailed explanation of the nature and implications of inheritance of the disorder in laymen's terms to enable the family to make informed medical and personal decisions.

Three mothers and one other female family member were available for evaluation in this study. The mother of Patient 001's was identified as a carrier (heterozygous) of the mutation. Although the mother of Patient 001 declined the offer to a genetic counsellor she did, however, request the testing of her daughter who was confirmed to be a carrier of the mutation. The daughter will be offered genetic counselling when she reaches child bearing age. Patient 002's mother was also confirmed as a carrier of the XLA mutation found in Patient 002. The mother of Patient 003 was previously confirmed to be with the carrier status [15] and her participation in this study was useful as a positive carrier control (data not shown). None of the patients

had male siblings with 'XLA symptoms' and were therefore not included in this study. It is however important to note that any relative, specifically male, that could be at risk, should be evaluated as promptly as possible by using the family specific- mutation screen test (if available).

4.3 Phenotype - genotype correlation

In recent years, publications have focused less on de novo mutations and rather on structure-function relationships and a possible genotype - phenotype correlation relating to the specific site of mutation in the Btk gene and the affiliated XLA phenotype.

Earlier studies attempted to determine a correlation between phenotype and genotype in Agammaglobulinemia patients with mutation in the Btk gene done was unsuccessful [24,28]. However two studies done by two independent research groups brought about significant insight. The study done by Broides *et al.* [29] was a breakthrough in understanding Btk activity, Btk mutation and the subsequent affects. And Lee *et al.* [21] also describes a correlation between genotype and the severity of the disease in terms of occurrence of severe infectious complications and the age of disease onset. Lee *et al.* classified patients according to their early onset clinical presentation, severity of infections and mutation type. Groups included those with severe mutations presented with infectious complications at an early age and those who had a less severe mutation, presented later on in life with a less severe form of the disease. Lee *et al.* further noted, that though these findings are not absolute they do suggest that a 'global' genotype - phenotype correlation for XLA [21].

The only common feature in all patients analysed is the severe reduction in the number of B cells. More precise understanding of the functional consequence of the Btk gene mutation has the potential of defined diagnostic categories that may lead to more specific individual treatment.

Early diagnosis and specific treatment would prevent complications from infections and would sustain a good quality of life. This will be possible through the elucidation of the Btk genomic structure and comparative studies of clinical outcome.

4.4 Value of the methodology

Since the discovery of XLA [30] by O.C Bruton in 1952 a lot of information on XLA and the Btk gene has accumulated. In this study we focused on a molecular technique that proved to be less labour intensive than previous reported methods. We utilized sequence-based diagnosis to support clinical diagnosis of patients with no family history of XLA. This proved to be a definitive method of diagnosis of affected male status as well as carrier determination. Sequence-based diagnosis has clear advantages with respect to speed, reliability and convenience making it suitable for routine XLA genetic diagnostics.

Undiagnosed or late diagnosis of XLA is associated with severe recurrent infections and leads to morbidity and mortality. This is avoided with early detection, which will prompt the institution of life-saving treatment (Intravenous Immunoglobulin replacement therapy) for the management of XLA. Delay in diagnosis or sub-optimal IVIg treatment and a lack of proper follow up can still result in poor outcomes that could lead to chronic infections, organ damage and a failure to thrive. In contrast, early diagnosis before onset of recurrent infections and IVIg treatment including adjustments to the treatment plan results in improved outcomes increasing the patient's lifespan and allows the XLA-affected individual to live a relatively healthy life.

Currently, there is no routine diagnostic facility for patient or carrier detection of XLA, available within the National Health Laboratory Services (NHLS). The first report of XLA in S.A was done by Pienaar *et al.* [15]. No further studies relating to the genetics of the Btk gene or XLA in South African patients have been reported. This study aimed to expand on the genetic knowledge of unrelated XLA patients in S.A using the sequence-based method. The main advantage of this method is that it allows for the detection of specific Btk gene abnormalities that would confirm/diagnose an XLA status within patients. This is a rapid method that can be done as early in life, requires minimal training, can be done by any PCR competent lab technician and can be housed in any laboratory with the 'appropriate' instruments/ apparatus. Results can be obtained within as little as seven working days in the case where the mutations are known and with a high reliability of both the test and instrument. Routine availability of this test would ensure early XLA diagnosis in South Africa and will allow the immediate institution of life saving replacement therapy in affected patients. As mutation detection provides the most definitive method of XLA diagnosis [31].

At present however, XLA in South Africa is still diagnosed according to clinical and immunological features and is suspected in a patient with a positive family history. As most patients lack a positive family history clinical and immunological diagnosis is insufficient for early accurate diagnosis. These patients require molecular diagnostics involving a Btk mutational analysis to provide a clear diagnosis. Patients who present with atypical XLA and one-third of XLA cases that occur sporadically [32] will also benefit from molecular diagnosis. Another advantage of molecular XLA diagnosis is in the differentiation of XLA from Common Variable Immunodeficiency disorder (CVID). Although the clinical features are similar between XLA and CVID these disorders are significantly different at the molecular level [33, 34]. This study aimed to develop a screening test screen test for the NHLS and will be validated, standardized and offered in the Pathology Research Facility (PRF) Laboratory, Tygerberg Campus, Stellenbosch University. Our long term goal is to encourage and motivate efforts for improved management of XLA and other PIDs in South Africa. A secondary aim of this study was to encourage further research in the PID genetic field of South Africa.

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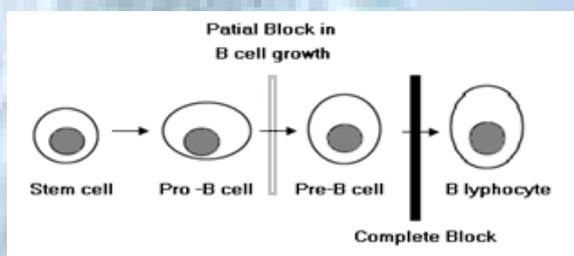


X-linked Agammaglobulinaemia Search

BACKGROUND

X-linked Agammaglobulinaemia (XLA) is a humoral immunodeficiency caused by mutation in the Bruton's tyrosine kinase (Btk) gene. Mutation leads to reduced numbers (<2%) or total absence of B cells which results in low immunoglobulin levels.

Affected males are characterised by a high susceptibility to recurrent bacterial infection with onset in infancy or early childhood that persists for life. Treatment can prevent premature death. Thus the early detection and diagnosis of XLA is crucial.



PREVALENCE: XLA affects approximately 1 in 200 000 individuals.

Approximately 90% of XLA patients have mutation in their Btk gene, & this is seen as the most common type of Agammaglobulinemia.

- Medical and public awareness of Primary Immunodeficiency is scarce and thus not many clinical diagnoses of this sort has been made (known).

HOW CAN YOU HELP ??

We are currently in search of (male) patients presumed to have XLA.

WHAT TO LOOK FOR / CHARACTERISTICS OF TYPICAL XLA :

- low to undetectable serum immunoglobulin levels
- low to no B cells (less than 2% of peripheral lymphocytes)
- increased predisposition to bacterial infection

- ❖ Ethical clearance / permission was obtained for this investigation
- ❖ A consent form will be sent to each participating individual

PROJECT TITLE

Real Time PCR as a diagnostic tool for detecting Btk mutations in Agammaglobulinaemia

AIM

The aim of this project is to determine the spectrum of Btk mutation and to develop a cost effective, reliable diagnostic Real Time PCR detection method for genetic abnormalities associated with the Bruton's tyrosine kinase (Btk), primary humoral immunodeficiency.

ENVISAGED OUTCOMES

To determine the spectrum of Btk mutation in the SA population and to develop a Real Time PCR based diagnostic test for the most common Btk gene mutations. This will be useful for the early diagnosis of X-linked agammaglobulinemia.



Contact: Melanie Leo (MSc. Medical Microbiology Stellenbosch University)
Email: 14393921@sun.ac.za

BRUTON'S TYROSINE KINASE GENE

Accession # U78027 (36721bp)

Indicating introns, exons in **bold** and primer pairs (forward in **blue**, and reverse in **red**).

ORIGIN

1	CTCAGACTGT	CCTTCCTCTC	TGGACTGTAA	GAATATGTCT	CCAGGGCCAG	TGTCTGCTGC	
61	gatcgagtc	caccttccaa	gtcctggcat	ctcaatgcat	ctgggaagct	acctgcatta	Exon1
121	AGTCAGGACT	GAG gtgggtc	tggggtatgg	caggggctgg	gc agcagcag	caatgtacct	
181	tgcttgggac	ccctaaaaac	cagagagaca	gcatggctgg	tgccatttat	cagctagtgg	
241	aggaggctga	cgagggtgg	gagtgtcatc	agcacaaggc	cctggcagtc	ccttctgggtg	
10801	ctaggtagct	aggctgaggg	ggaaccaaga	gggatgagga	ttaatgtcct	gggtcctcag	
10861	gaactttc at	tatcaacagC	ACACAGGTGA	ACTCCAGAAA	GAAGAAGCTA	TGGCCGCAGT	Exon2
10921	GATTCTGGAG	AGCATCTTTC	TGAAAGCGATC	CCAACAGAAA	AAGAAAACAT	CACCTCTAAA	
10981	CTTCAAGAAG	CGCCTGTTTC	TCTTGACCGT	GCACAAACTC	TCCTACTATG	AGTATGACTT	
11041	TGAACGTGGG	gtaagttct	cgactatgaa	aactgagttt	caagatatca	aggacttggc	
11101	cttagatc tt	tcttggggaa	gaggtaaatt	ttcgttggtg	ggaggagggg	agtagaatgg	
11461	cacctcactt	tgttgaatga	ttagataaca	aatctaatc	atctggttg	ttaatccctc	
11521	ttaatccttc	tcattttct	tcctcattct	acttctcagA	GAAGAGGCAG	TAAGAAGGGT	Exon3
11581	TCAATAGATG	TTGAGAAGAT	CACTTGTGTT	GAAACAGTGG	TTCTTGAAA	AAATCCTCCT	
11641	CCAGAAAGAC	AGATCCCG gt	aagaagagac	ca atgtctga	gatggggaac	agcagatttg	
11701	aagaaatttg	caacatttaa	attctctgta	aatagactgg	tgatgctgtg	caacgtggaa	
11761	cacggtcaag	tttccttaa	aaattcttca	ctctaccata	ttggtataa	agaatcttag	
14341	tccttattgg	cctatagaaa	gtaatcacta	agaatcagcc	ttgtctgtg	aggagaaact	
14401	tctgaagcca	cagcaaaa gg	cttctagtac	ctaagg aaat	tcagaaaaga	gcaatgcatc	
14461	aaccaataac	cattttttct	tatttcatta	cag AGAAGAG	GTGAAGAGTC	CAGTAAAATG	Exon4
14521	GAGCAAATTT	CAATCATTGA	AAGGTTCCCT	TATCCCTTCC	AG gtgagtta	ttttctctca	
14581	ccaaatctcg	aaagg cccct	gtaacacaat	tagaaggggt	ggtggggtg	agggcagagg	
14641	gaaggctacg	aggaccagt	gaatagaagt	tgaggtggac	aaggatgctg	aaccaagaaa	
15961	ttcacataac	ctgaacacca	ttgctgactg	aagattctgc	ctttctttgt	tttctacaat	
16021	aaatatacaa	attgtcccct	aagattagta	ctttcaaggt	ctttctcctt	ttttctgaat	
16081	cctaactgct	gaagtCTGTG	TTTTCATCGA	CCCAGGTTGT	ATATGATGAA	GGGCCTCTCT	Exon5
16141	ACGTCTTCTC	CCCAACTGAA	GAACTAAGGA	AGCGGTGGAT	TCACCAGCTC	AAAAAGgtg	
16201	agaattattt	gga aaataaa	tgttccaaa	gaaagaagga	aaggaaggaa	gaggagatag	
16261	acgggaggaa	aaggaaagaa	ggaagagaga	aacgagaaa	aaagagaaa	gagaaacgta	
23341	gtcatgtgag	ccaactggtc	ctgtgatctg	ctctccttca	gggtggccaa	ggagggggca	
23401	gttgcttgaa	gttcaccact	aagctcaaag	aggaaaacat	gcaaatgggtg	gcttct cctc	
23461	catgtcagat	gtgat ctctc	tcttctttcc	tgctacagTA	ATCCGTTACA	ACAGTGATCT	Exon6
23521	GGTTCAGAAA	TATCACCTT	GCTTCTGGAT	CGATGGGCAG	TATCTCTGCT	GCTCTCAGAC	
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23701	cagggtcata	gcataaggtt	gtacactttg	ggtgctaaac	aaaggtcct	gggggagggg	
23821	atgcctagct	actttttcta	acttgcttaa	aggcactcaa	tatggacggc	aaagtccctg	
23881	accaaacctg	tccttggccc	ctttcctaaa	gtgcatt tcca	tatcatcact	ggct tcttgt	Exon7
23941	tttgacag GCT	TAAAACCTGG	GAGTTCTCAC	CGGAAGACAA	AAAAGCCTCT	TCCCCAACG	
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24061	ta gtcaagac	ctaagaacac	actagagtct	tagaattacc	ctgggacttg	gcagtgttaa	
24121	ttggtattct	tgctaagatg	ggggatttgt	tcttctaatt	aggatttct	cctcgatgga	
25261	tgcaaggctg	agaactcctg	cactgggtgct	taaaaatggt	tgttgaatga	ctgctgagta	
25321	atattttagt	atgcctgatt	tgggtgtctt	gagtaagcca	gagagttggg	agagaagaga	
25381	agagtgtctg	gtatcttgac	aaac cctcct	accttttctc	ctaactacat	ag ATCTTGAA	
25441	AAAGCCACTA	CCGCCTGAGC	CAGCAGCAGC	ACCAGTCTCC	ACAAGTGAGC	TGAAAAAGGT	Exon8
25501	TGTGGCCCTT	TATGATTACA	TGCCAATGAA	TGCAAAATGAT	CTACAGTCTG	GGAAGGGTGA	

25561 TGAATATTTT ATCTTGGAGG AAAGCAACTT ACCATGGTGG AGAGCACGAG ATAAAAATGG
 25621 gtgagtcac accagccctt cctgagcctg gtgcctgccc actccctaca cgcaaaaagt

25681 gctgcggcat aattcccgtg atcagcatcc tcatcagaga ctgttcttcc cacaccagac
 25861 cacctcactg gctcactgcc taaccatgca tcagagacat gattgtcttt gaggaggtg
 25921 catgatacat atacctccat ttgagatttg ttctgagctc agggacgtgg gcatatgaat
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 26041 GGAAGGCTAC ATTCCTAGTA ACTATGTCAC TGAAGCAGAA GACTCCATAG AAATGTATGA
 26101 gtaagtatgt ttatgtcagt ccacaatctt ccaggaggaa ctctctctct ctatatatat
 26161 atatatttat ttatttatat atattttata tatattttata tatatatata acacacacaa

Exon9

26701 gctatgcaca tgacagatgc tcagtaaata gggtttgagt gagttgactg aatcactgac
 26761 atggacaagc cctggagggt gctgtaacct ccaa**tctgct** **tatgaccagg** agccactcaa
 26821 gcagcactct cccttcacag GTGGTATTCC AAACACATGA CTCGGAGTCA GGCTGAGCAA
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Exon10

27361 gctgtagctc aagcgtggaa cttggcagta aaatcagggg ttgttctatt ttctctctca
 27421 agtttgggca ggtgggatgc aggtgtgagc acc**cttctc** **cctacagaca** **gCTTCTTTT**
 27481 CGTTGTTTCA GGGGAAAGAA GGAGGTTTCA TTGTCAGAGA CTCCAGCAAA GCTGGCAAAT
 27541 ATACAGTGTCT TGTGTTTGCT AAATCCACAG Ggtgagtgtct actattccaa ggcctgagg
 27601 acaagaaca ggggtac**cct** **cctaatagct** **ccttgatgct** gtgccgtcc cacttctct

Exon11

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 27841 CCCTGAGCTC ATTAACTACC ATCAGCACAA CTCTGCAGgt gactaccagg **ggcaactgag**
 27901 **aagggagtta** cagacaccag gataagcaat gcaggacaag gtgataagag gaagaatctg
 27961 cctcacagga cagtgtgaat tcactgtgaa aggaaatccc tgtgatactg ggtagaagct

Exon12

28501 tactagaaag atacacaccg gaagtgtgag gctttaagtg aggatgtgtg aggcaccca
 28561 cctcctacac caca**ccaaca** **gcatgacctc** **tc**ctctctgtt tcag**GACTCA** **TATCCAGGCT**
 28621 CAAATATCCA GTGTCTCAAC AAAACAAGAA TGCACCTTCC ACTGCAGGCC TGGGATACGG
 28681 TAACTCCTTA TTTCTCTGGG GTAGGGTGGA CTGGCCAGTT GCAAAA**ACTA** **CCTTTGCTGg**
 28741 **ccttgcctta** gggagtgtcc ttgaggtaca ctgttctgca gcagctgctc caaggacgtc
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 28861 actgcaaaaa catagattca taaaggcctt tgtcgttgtc ttgggtcttt ttgtctttta

Exon13

29101 aaagacagat tagtacttct aaaaatttcc cttttactag ttttctatt tctaccccag
 29161 tagggatttt tgtctattgt aagaattata cattcat**gac** **cccaaagaat** **cacac**caaga
 29221 ctttattggt aggatcatgg ga**AATTGATC** **CAAAGGACCT** **GACCTTCTTG** **AAGGAGCTGG**
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 29341 TCAAGATGAT CAAAGAAGGC TCCATGTCTG AAGATGAATT CATTGAAGAA GCCAAAGTCA
 29401 TGATgtgagt tat**agcccaa** **actcaactct** **caatctattt** gctggagtct aggaattcac
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Exon14

29761 agcacttttg agatttaggg tgggaaagag gaaaataaac ttgctggtgt gacccttat
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Exon15

30121 CCTTCACCGA GACCTGgtgg gaccttagaa ggattggcct aggacaaagg gctga**tgggg**
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 31621 **aggtgttaca** **g**ttttaa**ctc** tctccttttc tttttttct cattctagcg ttttctgcc

Exon16

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 32281 **TAGCAAGTTC** **AGCAGCAAAT** **CTGACATTTG** **GGCTTTTGT**gt aagtggataa gattacacag
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Exon17

32701 agaaatthgg gcaaaatata aaaagcacag agcggggaac caactgattc taatctttga
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 33001 tgctttatta ggatctctta aattatthcc ttgaatctac ttgccattt agctggccat
 33061 tca**gccacca** **aagactgata** **g**ctgcaacaca ttcttttttt tccttttttt ttttttttaa

Exon18

36121 atgctactgg gcatagagca tatatthaat aaacatthgc tgcttactca ttgcattthc
 36181 cttgaatt**ct** **ggattctagc** **cactct**aaca ctttacttht tctttgtht tagaaag**CAG**
 36241 **ATGAGCGTCC** **CACTTTCAAA** **ATTCTTCTGA** **GCAATATTCT** **AGATGTCATG** **GATGAAGAAT**
 36301 **CCTGAGCTCG** **CCAATAAGCT** **TCTTGGTTCT** **ACTTCTCTTC** **TCCACAAGCC** **CCAATTCAC**
 36361 **TTTCTCAGAG** **GAAATCCCAA** **GCTTAGGAGC** **CCTGGAGCCT** **TTGTGCTCCC** **ACTCAATACA**
 36421 **AAAAGGCCCC** **TCTCTACATC** **TGGGAATGCA** **CCTCTTCTTT** **GATTCCTTGG** **GATAGTGGCT**
 36481 **TCTGAGCAA** **GGCCAAGAAA** **TTATTGTGCC** **TGAAATTTCC** **CGAGAGAATT** **AAGACAGACT**
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 36661 **ATTTGGCAAG** **AATGAAATGG** **TGTCATAAAG** **ATGGGAGGGG** **AGGGTGTFTT** **GATAAAATAA**
 36721 A //

Exon19

Primer Sequences

Primer Pairs from Conley *et al.* (1994)

Primer Pair	5' Primer	3' Primer	Exon	Domain	Size (bp)	Annealing Temp (°C)
1	TCAGACTGTCCTTCCTCT	AGGTACACATTGCTGCTGCT	1	5' Untranslated	160	55
2	TGGGTCTCAGGAACTTC	CAAGAGAAACAGGCGCTT	2	5' Unique	155	58
3	CTGGAGAGCATCTTTTCTG	GATCTAAGGCCAAGTCCT	2	5' Unique	186	53
4	CCATTTTCTCCTCATTC	GTTCCCATCTCAGACAT	3	5' Unique	160	56
5	GGCTTCTAGTACCTAAGG	CCTTTCGAGATTGGTGA	4	5' Unique	176	56
6	CCTAACTGCTGAAGTCTG	TCCAAATAATTCTCACCG	5	5' Unique	160	53
7	CCTCCATGTCAGATGTGA	TGGAAACAGTCAAAGGAG	6	5' Unique	220	56
8	TCCATATCACTGGCT	GTGTCTTAGGGCTTGAC	7	5' Unique	164	56
9	CCCTCCTACCTTTTCTCC	TTCCGCAGCTGTAGATCA	8	5' Unique/ SH3	148	50
10	AGGTTGTGGCCCTTTATG	AAGGGCTGGTGTGGACTC	8	SH3	142	50
11	CTGGATGAACTGCCACAT	AGAGAGAGTTCCTCCTGG	9	SH3	140	55
12	TCTGCTTATGACCAGGAG	CCTCACTTATGCAAGGAG	10	SH2	150	56
13	ACTTCCTCCTACAGACAG	CAAGGAGCTATTAGGAGG	11	SH2	180	56
14	TCCTGGGTTCTGTTGACC	GTAATACTGGCTCTGAGG	12	SH2	118	53
15	GACCCTCAAGGGGTGATA	AACTCCCTTCTCAGTTGC	12	SH2	155	58
16	CCAACAGCATGACCTCTC	GGCAAGGCCAGCAAAGGT	13	SH2/ SH1	170	60
17	GACCCCAAAGAATCACAC	GAGAGTTGAGTTGGGCT	14	SH1	230	60
18	GGACAATTTGGGGTAGTG	GAGAGTTGAGTTGGGCT	14	SH1	155	58
19	CTAGTTCCTTGCCTTTCC	TAGCAGCTGAGTCTG	15	SH1	170	51
20	AACTACCTGAGGGAGATG	ACTGCCTACTCCACCCA	15	SH1	180	58
21	TCACTGGTCTCTGTTTGC	CTGTAACACCTACCCATG	16	SH1	160	58
22	GCAACAAGTCTGAATCC	TGGAGTTGCAAAGTGTG	17	SH1	175	56
23	TCTAGGAAGACTAGGACC	TTGGGCAATGTGTTGAGC	18	SH1	155	53
24	AGGTGAGACTGCTGAACAC	CTATCAGTCTTTGGTGGC	18	SH1	186	53
25	CTGGATTCTAGCCACTCT	AGAAGCTTATTGGCGAGC	19	SH1/ 3' Untranslated	130	56

Identification of the Novel Mutation and the effects thereof.

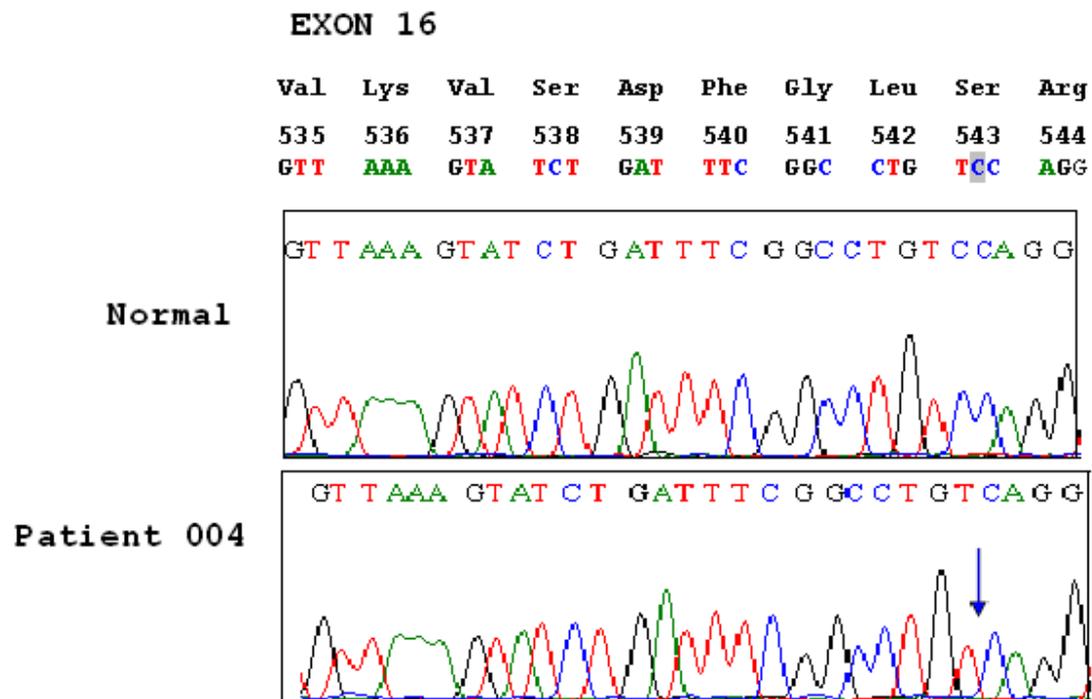


Figure 1. Nucleotide Sequence of the genomic DNA of the Normal exon (PubMed MIM300300) Mutated nucleotide is indicated in bold print and the the variant sequence found in Patients 003 and 004

540				543	545				550				555			
F	G	L	S	R	Y	V	L	D	D	E	Y	T	S	S	V	G
TTC	GGC	CTG	TCC	AGG	TAT	GTC	CTG	GAT	GAT	GAA	TAC	ACA	AGC	TCA	GTA	GGC

Figure 2. An amino acid and nucleotide representation of a section of normal exon 16 and 17 of the Btk gene.

Area of interest in **Red**

NORMAL SEQ	... TTC GGC CTG TCC AGG TAT GTC CTG GAT GAT GAA TAC ACA AGC TCA GTA GGC ...
MUTATED SEQ	... TTC GGC CTG TCA GGT ATG TCC TGG ATG ATG AAT ACA CAA GCT CAG TAG

Figure 3. Nucleotide sequence of a section of normal Btk gene and the truncated Btk gene produced due to the mutation found in Patient 004.

Affected amino acid in **red**

Stop Codon in **brown**

(...) indicate that only part of the sequence is represented.

Abbreviations

Primary Immunodeficiency disorder	PID
X-linked Agammaglobulinaemia	XLA
Bruton's tyrosine kinase	Btk
Intravenous gammaglobulin replacement therapy	IVIg
National Research Foundation	NRF
National Health Laboratory Services	NHLS
B cell receptors	BCR
T cell receptors	TCR
T Helper cells	T _H
Cytotoxic T cells	T _c
	AIDS
	BLNK
B cell linker protein	
Immunoglobulins	Ig
Alpha	α
Beta	β
Mu	μ
Lambda	λ
Subcutaneous treatment	SCIg
Cytoplasmic protein-tyrosine kinases	PTKs
Bone Marrow kinase gene on the X chromosome	Bmx
IL2-inducible T cell kinases	Itk
Tec protein tyrosine kinase	Tec
Progenitor B cells	Pro-B cells
Precursor B cells	Pre-B cells
Phospholipase C γ 2	PLC- γ 2
Nuclear factor kappa-light-chain-enhancer of activated B cells	NF- κ B
Pleckstrin homology domain	PH
Tec homology domain	TH

Src homology 3 domain	SH3
Src homology 2 domain	SH2
Catalytic kinase domain	SH1/ Kinase
Common Variable Immunodeficiency disorder	CVID
Primary Immunodeficiency South Africa Registry	PISA
Polymerase Chain Reaction	PCR
Immunodeficiency mutation database	IDbase
Not determine	N.D
Ethylenediaminetetraacetic acid	EDTA
Normal	N
Deletion	Del
Arginine	R
Histidine	H
Tryptophan	W