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INVESTIGATION OF THE ROLE OF HUMAN PARVOVIRUS B19 IN CHRONIC ANAEMIA OF HIV INFECTED TB PATIENTS

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A dissertation submitted to the Faculty of Medicine,

University of the Witwatersrand, in partial fulfilment

of the requirements for the degree

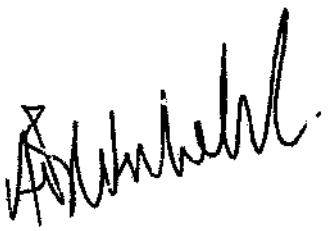
Master of Medicine (Virology)

ABSTRACT

This study was undertaken to determine the role of human parvovirus B19 (B19) in chronic anaemia of HIV infected TB patients. Patients were selected from an existing databank of 307 patients included in a MRC HIV/TB study. Twenty-nine patients, 15 coinfected with HIV/TB and 14 infected with TB only, were identified for further evaluation. These patients' sera were subjected to serological and DNA detection studies using IgG and IgM ELISA methods and a nested polymerase chain reaction (PCR) assay. The selection of the nested PCR was based on comparative evaluation of a new rapid 99 cycle PCR method recommended for hepatitis B DNA detection and the nested PCR method established for B19. The nested assay was shown to be the more sensitive system in the context of B19 DNA detection. Serological evaluation of these 29 patients suggested that a greater proportion of HIV/TB patients with chronic anaemia had evidence of recent or past exposure to B19 than those not experiencing anaemia. The nested PCR demonstrated the presence of circulating B19 DNA in 2 coinfected individuals with haematological pictures compatible with persistent B19 infection. B19 DNA was also demonstrated in a TB only patient without anaemia; further haematological and serological evidence in this patient suggested recent exposure to B19. The serological and DNA amplification assay results of these 29 patients would suggest a possible role - either causal or co-factorial - for persistent B19 infection in the establishment of chronic anaemia in HIV/TB patients.

DECLARATION

I declare that this dissertation is my own, unaided work and has not been submitted for any degree or examination at any other university.



Albertus Bernhardus Willer van Niekerk

September 30, 1994

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TABLE OF CONTESTS

1 INTRODUCTION 1

1.1 History 1

1.2 Classification 1

1.3 Morphology and antigenic structure 4

1.4 Genome organization 5

1.5 Pathogenesis 6

1.5.1 Cytolytic infection 6

1.5.2 Immunological response 6

1.6 Epidemiology 7

1.7 Clinical associations 8

1.7.1 *Erythema infectiosum* (E.I.)/Fifth disease 9

1.7.2 Aplastic crises in predisposed individuals 10

1.7.3 Complications during pregnancy 10

1.7.4 B19-associated arthropathy 11

1.7.5 Anaemia in immunocompromised patients 11

1.7.6 Anaemia in HIV disease 12

1.8 Diagnostic issues 13

1.8.1 Viral culture 13

1.8.2 Viral detection 13

1.8.2 Serology 14

1.8.3 DNA hybridization 14

1.8.4 DNA amplification 15

2 OBJECTIVES 16

2.1 EVALUATION OF DNA AMPLIFICATION TECHNIQUES IN THE DETECTION OF B19 DNA IN SERUM SAMPLES 16

2.2 USE OF SEROLOGY AND DNA AMPLIFICATION TECHNIQUES TO EVALUATE THE ROLE OF B19 IN INDIVIDUALS COINFECTED WITH HIV AND TB WHO HAD CHRONIC ANAEMIA 16

3 PATIENT SELECTION 17

3.1 Data extraction 17

3.2 Subjects 17

4 MATERIALS AND METHODS 19

4.1 Serological Methods 19

4.1.1 Detection of anti-B19 IgG and IgM antibodies 19

4.1.1.1 Detection of anti-B19 IgG antibodies 19

4.1.1.1.1 Principle of the assay 19

4.1.1.1.2 Interpretation of results 19

4.1.1.2 Detection of anti-B19 IgM antibodies 20

4.1.1.2.1 Pretreatment step 20

4.1.1.2.2 Principle of the assay 20

4.1.1.2.3 Interpretation of results 21

4.2 Polymerase chain reaction (PCR) 21

4.2.1 Nucleic acid extraction 21

4.2.1.1 Method 21

4.2.2 Primer selection 22

4.2.3 PCR buffer and reaction mixture 23

4.2.4 PCR protocols 24

4.2.4.1 Nested PCR protocol 24

4.2.4.1.1 First round amplification 24

4.2.4.1.2 Second round amplification 24

4.2.4.2 Rapid 99 cycle PCR assay 25

4.2.5 Sensitivity determination 25

4.2.5.1 Serum dilutions 25

4.2.5.2 Plasmid dilutions 25

4.2.6 PCR assay controls 26

4.2.6.1 Positive controls 26

4.2.6.2 Negative controls 26

4.2.7 Agarose gel electrophoresis 26

4.2.8 General considerations for PCR assays 27

4.2.8.1 Designated laboratories were used for designated reactions 27

4.2.8.2 Autoclaving of tubes and reagents 28

4.2.8.3 Storage of reagents 28

4.2.8.4 Disposable gloves 28

4.2.8.5 PCR reagent mix 28

4.2.8.6 General precautions 29

5 RESULTS 30

5.1 Sensitivity determination of the different PCR assays 30

5.1.1 Serum dilutions 30

5.1.2 Plasmid dilutions 30

5.2 Serological results 33

5.2.1 Detection of anti B19-IgG antibodies 33

5.2.2 Detection of anti B19-IgM antibodies 33

5.3 Polymerase chain reaction results 34

5.3.1 First round amplification 34

5.3.2 Second round amplification of the nested PCR protocol 35

6 SUMMARY OF LABORATORY DATA OF THREE PATIENTS WITH DEMONSTRABLE CIRCULATING B19 DNA 38

6.1 Introduction 38

6.2 Discussion 38

6.2.1 Patient No.1; 1182/91, 54 years old, male 38

6.2.2 Patient No. 2; 453/92 41 years, female 40

6.2.3 Patient No. 3; 103/92, 26 years old, female 40

7 DISCUSSION 43

7.1 Sero-epidemiology 43

7.2 IgM assays 44

7.3 DNA Amplification 46

7.4 Sensitivity evaluation 47

7.5 Role of B19 in HIV/TB patients with anaemia 48

REFERENCES 50

1 INTRODUCTION

1.1 History

Human Parvovirus B19 (B19) was first described in 1975 (Cossart *et al*, 1975) when electronmicroscopic investigation of sera yielding discrepant hepatitis B surface antigen results, demonstrated virus-like particles with a diameter of approximately 23nm. Further morphological evaluation and buoyant density studies strongly suggested that these particles were parvoviruses. For the next couple of years it was generally assumed that B19 infection was common, and mostly asymptomatic. However, since 1981 when B19 was identified as the cause of aplastic crises in children with sickle cell anaemia (Pattison *et al*, 1981), B19 has been associated with a variety of other clinical syndromes. The clinical syndromes shown to be most commonly associated with B19 infection include the following: *erythema infectiosum* (Anderson *et al*, 1983; Anderson *et al*, 1985(a); Plummer *et al*, 1985), polyarthralgia (Reid *et al*, 1985), intrauterine infection (Mortimer *et al*, 1985; Gray *et al*, 1986; Bond *et al* 1986; Lefrere *et al*, 1986) and anaemia in immunocompromised individuals (Kurtzman *et al*, 1987; Kurtzman *et al*,1988).

1.2 Classification

B19 is a member of the family *Parvoviridae*. The name "Parvo" was derived from the Latin word "Parvus" meaning small.

Characteristics of the *Parvoviridae* family are the following:

a) single-stranded DNA, 5,5kb, mostly negative sense;

b) non-enveloped icosahedral particles, 18-22 nm in diameter;

c) a buoyant density of 1.39-1.42 g/cm^3 in CsCl, stable in lipid solvents and pH 3-9;

d) most members of the parvoviridae family replicate independently in the nucleus of infected cells. Helper virus coinfection, with either herpesviruses or adenoviruses, is however required for efficient replication by members of the genus Dependovirus (Francki *et al* (eds.), 1991).

The *Parvoviridae* family is divided into the following 3 genera (see Fig.1):

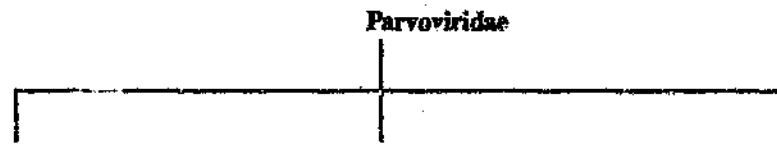
(a) The Dependoviruses, e.g. adeno-associated virus (AAV) type 1. Characteristically the members of this genus are dependent on coinfection with herpesviruses or adenoviruses for effective replication, although limited independent replication has been demonstrated under very specific conditions. Infection by members of this genus has been demonstrated in cattle, chickens, dogs, horses, monkeys, sheep and man, although AAV infection has not been established as a causal link of disease in man (Francki *et al* (eds.), 1991).

(b) The Densoviruses, e.g. galleria densovirus. Members of this genus are not dependent on coinfection by helper viruses for effective replication. Replication is marked by the aggregation of dense, intranuclear inclusions and hypertrophic nuclei in infected cells. Densoviruses infect insects of the orders Diptera, Lepidoptera and Arthoptera and have also been demonstrated in crustaceans such as crabs and shrimps (Francki *et al* (eds.), 1991).

(c) The Parvoviruses, e.g. human parvovirus B19. This group of viruses is not reliant on coinfection with helper viruses but effective replication is usually restricted to actively proliferating cells which provide essential helper functions. Vertical transmission of members of this genus by means of placenta and ova (goose) has been demonstrated. The host range of this genus is restricted to vertebrates and

infection has been demonstrated in cats, cattle, chickens, dogs, geese, man, rats, mice, mink, raccoons, rabbits and pigs. (Francki *et al* (eds.), 1991).

Fig. 1 **Classification of Parvoviridae.**



Genus:	Dependoviruses	Densoviruses	Parvoviruses
		Galleria densovirus	Human parvovirus B19
Members:	Adeno-associated virus (AAV) type 1		
	(AAV) type 2	Junonia densovirus	Aleutian mink disease parvovirus
	(AAV) type 3	Agraulis densovirus	Bovine parvovirus
	(AAV) type 4	Bombyx densovirus	Feline parvovirus
	(AAV) type 5		Goose parvovirus
	Avia AAV		H1
	Bovine AAV		Lapine parvovirus
	Canine AAV		Lu III
			Porcine parvovirus
			Rat virus
			RT
			TVX

1.3 Morphology and antigenic structure

B19 virions are non-enveloped and display the following characteristics:

a) icosahedral symmetry (Fig. 2);

b) range in diameter from 20-25 nm with a mean of 23 nm (Cossart *et al,* 1975);

c) the buoyant density ranges from 1.41-1.45, mean of 1.43, in CsCl (Clewley *et al,*1984);

d) the capsid consists of two definite capsid proteins:

   i) VP1 with an approximate molecular weight of 83 kilodaltons (kd);

   ii) VP2 with an approximate molecular weight of 60 kd;

VP2 is the predominant protein and accounts for approximately 80% of the total protein mass (Cotmore *et al,* 1986). Isolated reports have described the existence of a third capsid protein of 48 kd (Clewley *et al,* 1984).

e) stable and resistant to ether and chloroform. Infectivity is compromised by acid (0,5 N HCl), alkali (0,05 N Na OH) and heating at 56°C for 5 minutes (Anderson, 1991).

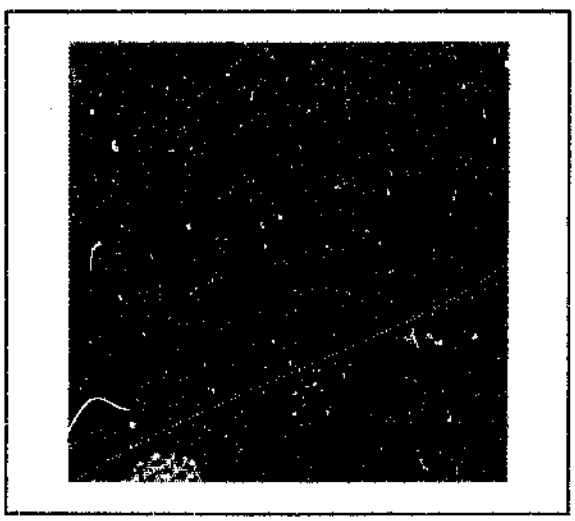


Fig. 2 Electron micrograph of B19 virus particles.

1.4 Genome organization

The B19 genome is single-stranded DNA and 5,5 kb in length with hairpin structures at the 5′ and 3′ ends (Francki *et al* (eds.), 1991). The mature virions of most members of the genus contain minus-strand DNA (Francki *et al* (eds), 1991), but B19 encapsidates both plus and minus strands into separate virions with almost similar efficiency (Clewley *et al*, 1984). The B19 genome has 2 open reading frames (ORFs), situated at the 3′ end and 5′ end, coding for the nonstructural and structural proteins respectively (Cotmore *et al*, 1986) (Fig. 3). Five B19 genotypes have been found when using restriction endonuclease analysis but no antigenic differences have been described and a single stable antigenic type is thought to exist (Sargeant *et al*, 1981; Paver *et al*, 1976).

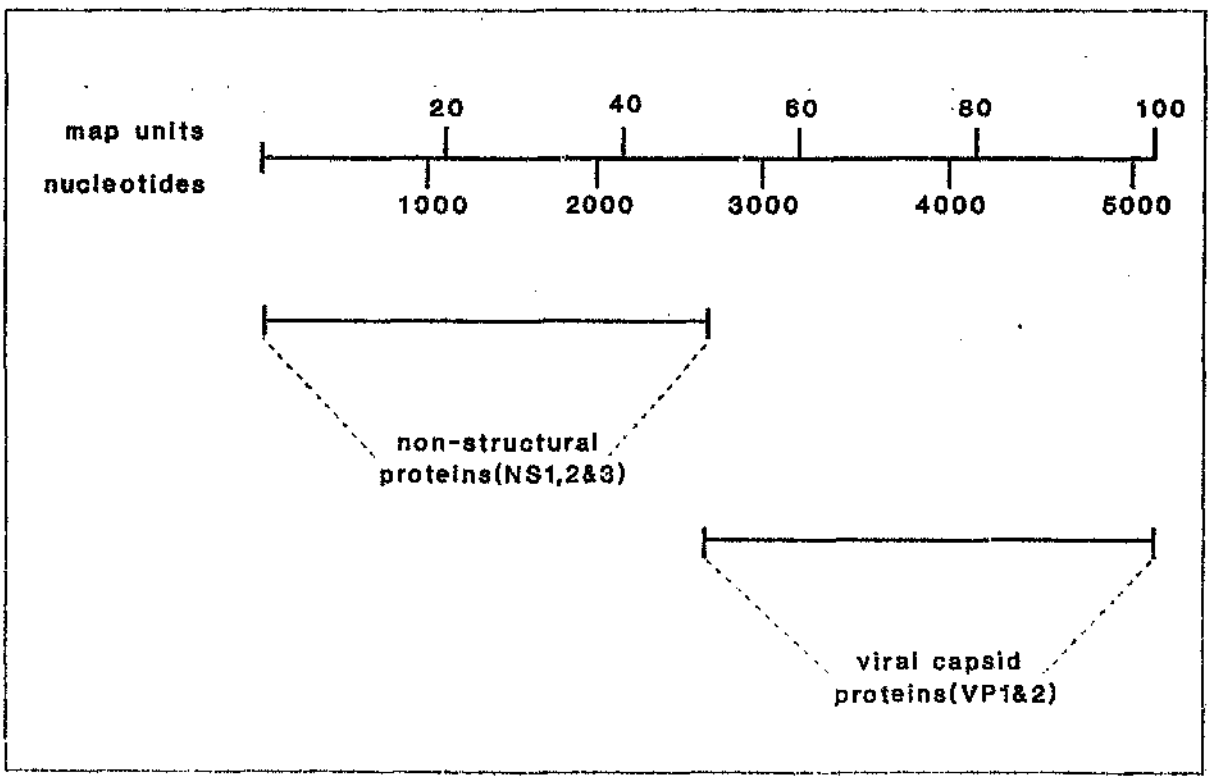


Fig. 3 Genome organization of B19.

1.5 Pathogenesis

The pathogenesis of B19 associated disease can be divided into two components:

a) a cytolytic infection in rapidly dividing susceptible cells and

b) an immunological response to viral products.

1.5.1 Cytolytic infection

A productive B19 infection is dependent on helper functions being supplied by infected cells and needs to infect rapidly dividing cells. B19 infects erythroid precursor cells in bone marrow, interrupts erythrocyte production (Mortimer *et al*, 1983; Potter *et al*, 1987) and is responsible for cytolysis. This transient erythrocyte precursor infection is usually of little or no clinical significance in haematologically competent individuals (Potter *et al*, 1987) but may, in patients with a shortened red blood cell survival time, lead to aplastic crises (Pattison *et al*, 1981). B19 infection can persist in the foetus due to the immaturity of the foetal immune response, resulting in cessation of erythrocyte production and eventually leading to the development of *hydrops foetalis* (Bond *et al*, 1986; Anderson *et al*, 1988). Persistence of B19 infection with a resultant chronic anaemia is also demonstrated in immunocompromised individuals, including AIDS patients (Kurtzman *et al*, 1987; Kurtzman *et al*, 1988; Frickhofen *et al*, 1990).

1.5.2 Immunological response

An immunological response to viral products in the form of anti-IgM and IgG antibodies is usually seen 12 to 18 days after exposure and coincides with the appearance of a maculo-papular rash in infected individuals (Anderson *et al*, 1985(a); Plummer *et al*, 1985) (Fig.4). In immunocompromised individuals with chronic B19 infection, the administration

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of immunoglobulin containing anti-B19 antibodies can also lead to the appearance of signs and symptoms of *erythema infectiosum* or fifth disease (Kurtzman *et al,* 1988), further suggesting that the humoral immune response is partially responsible for the clinical signs following B19 infection.

1.6 Epidemiology

Widespread B19 seroprevalence studies had been limited until recently due to difficulties in obtaining B19 antigen from viraemic individuals for use in serological assays. A limited number of serological studies performed worldwide, reported adult seroprevalence rates of 30-60% in the developed world (MMWR, 1989). Reports from the developing world found adult seroprevalence rates of 25% (Schoub *et al,* 1993) and 46% (De Freitas *et al,* 1990) respectively. Recent reports also suggested an increased B19 seroprevalence rate in HIV infected individuals. (Frickhofen *et al,* 1990; Nigro *et al,* 1992; Arakelov *et al,* 1993).

B19 infection usually occurs during late winter, spring and early summer in temperate climates, leading to outbreaks in primary schools; infection can also subsequently spread to susceptible adult contacts of infected children. This pattern of infection was demonstrated in observations regarding the epidemiology of *erythema infectiosum* and in limited serological studies where the acquisition of anti-B19 antibody occurred mostly between the ages of 4 and 10 years and although antibodies against B19 are still acquired after this age, it takes place at a much slower rate (Anderson, 1991).

Transmission of B19 occurs mainly via the respiratory route (Anderson *et al,* 1985(a)) as a result of viral presence in the throat for approximately 5 days. The appearance of virus in the throat, usually a week after exposure, coincides with the intense viraemia of 10^{11} particles per millilitre blood seen during the incubation period of B19 infection (Fig. 4).

Transplacental and parenteral transmission of B19 infection are also associated with this viraemic stage (Mortimer *et al*, 1983(a); Bond *et al*, 1986).

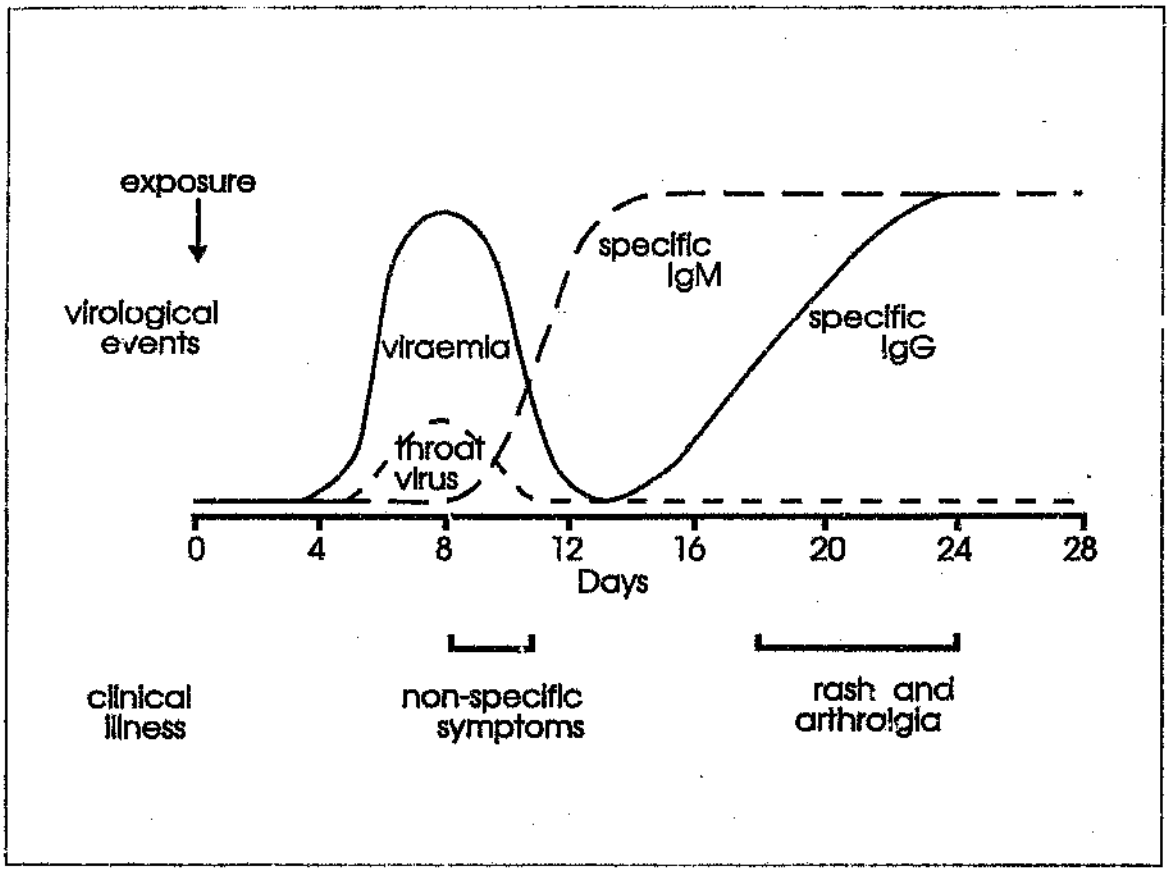


Fig. 4 Schematic presentation illustrating the clinical, virological and immunological characteristics following on B19 infection.

.7 Clinical associations

B19 infection, as is the case in other common viral childhood infections, ranges from being clinically asymptomatic (Chorba *et al*, 1986) to causing a life threatening anaemia in a small number of predisposed individuals (Pattison *et al*, 1981).

Since its description in 1974, a number of clinical syndromes have been associated with B19 infection and include the following:

a) *erythema infectiosum*/fifth disease;

b) aplastic crises in predisposed individuals;

c) complications during pregnancy;

d) B19-associated arthropathy;

e) anaemia in immunocompromised individuals.

1.7.1 *Erythema infectiosum* (E.I.)/Fifth disease

B19 infection was associated with an outbreak of *erythema infectiosum* or fifth disease in London in 1983 (Anderson *et al,* 1984) and a causal role for B19 in EI has since been described (Anderson *et al,* 1985(a)).

Classical EI is seen in children aged 4-10 years and presents as a marked erythema of the cheeks - the so-called "slapped-cheek" appearance. This usually follows 5-7 days after a prodromal illness of malaise, pyrexia and upper respiratory symptoms which is associated with an intense viraemia (Fig. 4). The erythematous maculopapular rash subsequently spreads to cover large areas on the trunk and limbs, usually lasting 2-3 days but may recrudesce up to three weeks after the initial episode upon exposure to sunlight, exercising or bathing (Plummer *et al,* 1985; Anderson, 1991). In older individuals B19 infection is less frequently associated with *erythema infectiosum* but manifests more commonly with an arthropathy (Woolf *et al,* 1989).

1.7.2 Aplastic crises in predisposed individuals

B19 infection of erythrocyte precursors is of little or no clinical significance in haematologically competent individuals (Potter *et al,* 1987), but poses a significant threat to patients with a shortened red blood cell survival time and may precipitate aplastic crisis in these patients (Pattison *et al,* 1981; Sargeant *et al,* 1981).

In 1981 infection with B19 was demonstrated to cause aplastic crisis in children with sickle cell anaemia (Pattison *et al,* 1981) and these findings were also confirmed in Jamaica on sickle cell anaemia patients (Sargeant *et al,* 1981). Subsequent reports demonstrated that B19 infection may be responsible for aplastic crisis in patients suffering from a variety of chronic haemolytic anaemias such as pyruvate kinase deficiency (Duncan *et al,* 1983), hereditary spherocytosis (Green *et al,* 1984), dyserythropoietic anaemia (West *et al,* 1986) and autoimmune haemolytic anaemia (Bertrand *et al,* 1985).

1.7.3 Complications during pregnancy

B19 infected individuals demonstrate a high-titre viraemia 7 days after exposure (Anderson *et al,* 1985(a)) and this may, in pregnant females, result in virus crossing the placenta and infecting the foetus. Infection by B19 during the first trimester can result in spontaneous abortion and foetal loss (Mortimer *et al,* 1985; Gray *et al,* 1986). *Hydrops foetalis* is associated with maternal B19 infection during the second trimester of pregnancy. The development of *hydrops foetalis* may follow up to 12 weeks after maternal infection and is the result of B19 persistence in the erythrocyte precursors due to the failure of the immature foetal immune response to clear the infection (Bond *et al, 1986*; Anderson *et al,* 1988). Third trimester infections by B19, although not extensively documented, are also thought to

be associated with *hydrops foetalis* (Porter *et al,* 1988). Teratogenicity has not been associated with B19 infection (Cramp *et al,* 1977; PHLS, 1993).

1.7.4 B19-associated arthropathy

B19-associated arthropathy is the most common complication in immunocompetent and haematological normal individuals, with approximately 8% of children and up to 80% of adults reporting arthralgia (Ager *et al,* 1966; Anderson *et al,* 1984).

These observations were confirmed by serological evidence of B19 infection in patients suffering from arthritis after an outbreak of *erythema infectiosum* (Reid *et al,* 1985), this investigation also confirmed the preponderance of females to suffer from B19 associated arthropathy. Arthritis following B19 infection is usually symmetrical, affecting the wrist, small joints of the hands, knees and ankles, and mostly resolves within 2-4 weeks (Reid *et al,* 1985; White *et al,* 1985). In extreme cases, B19 associated arthropathy may continue for months and even years (White *et al,* 1985).

1.7.5 Anaemia in immunocompromised patients

In immunocompetent individuals the development of neutralizing antibodies, approximately 12-18 days after infection, is associated with the clearance of B19 from the circulation (Anderson *et al,* 1985(a)).

Immunocompromised individuals infected with B19 may not mount an adequate immune response, subsequent viral persistence follows which could lead to chronic anaemia. B19 persistence is well described in patients with Nezelof's syndrome (Kurtzman *et al,* 1987), combined immunodeficiency (Kurtzman *et al,* 1989) and leukaemia (Kurtzman *et al,* 1988; Coulombel *et al,* 1989; Davidson *et al,* 1989); the clinical picture has been that of a chronic

or relapsing anaemia coinciding with B19 viraemia, usually in the presence of a weak humoral response (Kurtzman *et al*, 1988). Immunoglobulin has been used in the treatment of these chronic B19 infections but with limited success (Kurtzman *et al*, 1988; Kurtzman *et al*, 1989).

1.7.6 Anaemia in HIV disease

HIV infection, as well as late-stage HIV disease, have been associated with granulocytopenia, thrombocytopenia and anaemia. Of these three, anaemia is most commonly associated with HIV infection and HIV disease and was shown to be present in 10-23% of recently infected HIV infected patients and in approximately 70-80% of patients during late stage disease. Reduced haematopoiesis as a result of anti-HIV chemotherapy, neoplastic disease and various infections were shown to be largely responsible for anaemia during HIV infection (Doweiko, 1993).

HIV infection inhibits haematopoiesis by means of infection of various haematopoietic progenitor cells and by infection of bone-marrow stromal cells. Opportunistic infections due to HIV mediated immunosuppression also plays a major role in causing anaemia in these patients. Opportunistic B19 infection in other immunocompromised patients has been shown to be associated with chronic anaemia. A recent report has demonstrated the presence of B19 in the bone marrow of HIV infected patients with anaemia (Frickhofen *et al*, 1990). Subsequent reports have also demonstrated an increase in B19 seroprevalence in HIV infected individuals (Nigro *et al*, 1990; Arakelov *et al*, 1993) providing evidence that opportunistic B19 infection may be an important cause of chronic anaemia in HIV infected individuals.

1.8 Diagnostic issues

B19 infection can be diagnosed by means of the following laboratory techniques:

a) Viral culture

b) Viral detection

c) Serology

c) DNA hybridization

e) DNA amplification

1.8.1 Viral culture

Routine *in vitro* viral culture is not undertaken as conventional cell lines do not support the growth of B19. The absence of suitable cell lines for the propagation of B19 infection has been a limiting factor in the diagnosis of B19 infection and has also, until recently, hampered the development of serological assays for the detection of B19 antibodies.

B19 has been cultured in the research setting using specialized cell lines. These cell lines include human umbilical cord blood erythroid progenitor cells (Sosa *et al*, 1992) and a human erythroid leukaemia cell line (Takahashi *et al*, 1993). The availability of such cell cultures for B19 propagation could in future be used for the further investigation of viral replication and pathogenesis (Sosa *et al*, 1992; Takahashi *et al*, 1993).

1.8.2 Viral detection

A high titre viraemia with levels of 10^{11} particles per millilitre has been demonstrated at the onset of the non-specific prodromal symptoms (Anderson, 1991); this makes serum samples the specimens of choice for viral detection. A wide variety of assays has been used to demonstrate the presence of B19 in sera and these included electron microscopy, immune-

electron microscopy, radioimmunoassays and enzyme immunoassays (Anderson, 1991). Counter-current immunoelectrophoresis (CIE) was also frequently used to demonstrate the presence of B19 and also had the advantage of offering a very rapid result (Sargeant *et al*, 1981; Anderson, 1991).

1.8.2 Serology

Difficulties in the propagation of B19 in suitable cell lines for the production of B19 antigen had been the major obstacle in the development of B19 serological assays and serum or plasma of infected individuals had until recently been the source of antigen for antibody assays (Cohen *et al*, 1990; Anderson *et al*, 1991). Recombinant B19 protein and synthetic peptides are now the major source of antigen for use in serological assays. These antigens were used in an immunofluorescence assay for the detection of anti-B19 IgG and IgM antibodies (Brown *et al*, 1990) and enzyme linked immunosorbent assay (Patou *et al*, 1991; Söderlund *et al*, 1992); a baculovirus expression system has also been used for the development of antibody capture haemadherence tests for parvovirus B19-specific IgM and IgG (Hilfenhaus *et al*, 1993).

1.8.3 DNA hybridization

B19 DNA has been demonstrated in serum samples and tissue specimens by means of DNA-DNA hybridization techniques (Anderson, 1991). Dot-blot hybridization, using radio-labelled probes (Anderson *et al*, 1985(c); Clewley, 1985) and colorimetric and chemiluminescent reactions (Azzi *et al*, 1990; Zerbini *et al*, 1993), were shown to be more sensitive than direct viral detection in serum (Anderson *et al*, 1985(c)).

In situ hybridization for the detection of B19 DNA in formalin-fixed, paraffin-embedded tissues of non-immune *hydrops foetalis* was well described. This technique did not only provide a diagnosis but also demonstrated the cellular distribution of B19 in positive cases (Morey *et al*, 1992).

1.8.4 DNA amplification

DNA amplification in the form of the polymerase chain reaction, (PCR) has largely replaced DNA hybridization in demonstrating B19 in clinical specimens due to its greater sensitivity (Koch *et al*, 1990; Erdman *et al*, 1991; Schwarz *et al*, 1992). The nested PCR, which incorporated a second round of DNA amplification, has further increased the sensitivity of this assay (Carrière *et al*, 1993). This technique could be especially of value in immunocompromised patients where a low level of circulating B19 DNA is the only evidence of B19 infection (Musiani *et al*, 1993).

The major obstacle to successful DNA amplification is DNA contamination of equipment and/or reagents used during these assays and special precautions have been advocated to limit contamination to a minimum (Kwok *et al*, 1989). Recently, a new DNA amplification technique was described for hepatitis B DNA amplification. This assay relied on a single round of amplification consisting of 99 cycles and has demonstrated the same sensitivity as a nested PCR assay for hepatitis B amplification (Vandenvelde *et al*, 1993).

2 OBJECTIVES

THE OBJECTIVES OF THE STUDY WERE AS FOLLOWS:

2.1 EVALUATION OF DNA AMPLIFICATION TECHNIQUES IN THE DETECTION OF B19 DNA IN SERUM SAMPLES.

2.2 USE OF SEROLOGY AND DNA AMPLIFICATION TECHNIQUES TO EVALUATE THE ROLE OF B19 IN INDIVIDUALS COINFECTED WITH HIV AND TB WHO HAD CHRONIC ANAEMIA.

3 PATIENT SELECTION

3.1 Data extraction

Data extraction was performed on the existing databank of 97 patients included in a MRC study using the following parameters.

a) Anaemia on admission and lasting for 3 months (Hct <45 in males; Hct <40 in females)

b) HIV status

c) Age

d) Patients were identified by their hospital number

Data extraction results suggested that coinfected HIV/TB patients were more likely to develop chronic anaemia than patients infected with TB only.

3.2 Subjects

A total of 29 patients was identified for further evaluation of B19's role in persistent anaemia in coinfected HIV/TB patients.

The 29 patients were divided into 2 matching groups (Tables 1a and 1b):

a) group 1 consisted of 15 coinfected HIV/TB patients;

b) group 2 consisted of 14 TB only infected patients.

Each of these groups were divided into two subgroups:

a) a group with chronic anaemia and

b) a group in which anaemia was not demonstrated over a three month period.

Table 1a: Group 1: Characteristics of HIV/TB patients

	Patients with chronic anaemia		Patients without anaemia	
	Males	Females	Males	Females
Ages	23 - 54 (34,5)	20 - 53 (32,6)	24 - 48 (36)	20 - 36 (28)
Hct on admission: range (mean)	20,1 - 42,6 (33,6)	25,0 - 39,1 (31,5)	46.8 - 52,9 (49,9)	45,1 - 47,6 (46,4)
HCT after 3 months: range (mean)	37,1 - 43,9 (41,3)	31,9 - 39,8 (37,0)	55,8 - 56,2 (56)	48,3 - 51,6 (50,0)
Number	6	5	2	2
Total number of patients	15			

Table 1b: Group 2: Characteristics of TB only patients

	Patients with chronic anaemia		Patients without anaemia	
	Males	Females	Males	Females
Ages	25 - 51 (33,7)	23 - 50 (31,8)	25 - 47 (36,8)	26 - 40 (33)
Hct on admission: range (mean)	30,9 - 44,5 (37,2)	29,1 - 36,2 (33,4)	45,1 - 55,2 (49,1)	47,2 - 51,0 (49,1)
HCT after 3 months: range (mean)	39,1 - 44,5 (42,3)	32,8 - 39,8 (37,6)	51,6 - 61,8 (51,7)	47,2 - 59,4 (53,3)
Number	5	4	3	2
Total number of patients	14			

4 MATERIALS AND METHODS

4.1 Serological Methods

4.1.1 Detection of anti-B19 IgG and IgM antibodies

Sera from all 29 patients were evaluated for the presence of antibodies to B19.

4.1.1.1 Detection of anti-B19 IgG antibodies

A commercially available enzyme-linked immunosorbent assay (ELISA) for the detection of IgG antibodies (Genoclin, GmBH Laboratories, Hamburg) against B19 was used.

4.1.1.1.1 Principle of the assay

Recombinant B19 capsid antigen is coated onto a solid phase (microtiter wells). The patient's serum sample is added to the microtiter wells and if IgG antibodies against B19 are present, they will bind to the B19 antigen, forming antigen-antibody complexes.

These complexes are demonstrated by incubation with an anti-human IgG horseradish peroxidase (HRP) conjugate followed by exposure to an O-phenylendiamine-2HCl (OPD) substrate. The intensity of the colour reaction is measured by a spectrophotometer with a filter set at 492 nm.

4.1.1.1.2 Interpretation of results

Cut-off value

$$\text{Cut-off} = \frac{\text{Absorbance of negative control} + \text{absorbance of positive control}}{2}$$

Specimens with optical density (O.D.) values > cut-off value +10% were considered as positive for anti-B19 IgG antibodies.

Specimens with O.D. values < cut-off value were considered negative for anti-B19 IgG antibodies.

Specimens with an O.D. value within the range of cut-off +10% were considered equivocal and repeated.

4.1.1.2 Detection of anti-B19 IgM antibodies

A commercially available ELISA assay for the detection of IgM antibodies (Genoclin, GmBH Laboratories, Hamburg) against B19 was used.

4.1.1.2.1 Pretreatment step

All specimens for anti-B19 IgM antibody detection were initially subjected to a pretreatment step. Patient's serum was diluted with a specimen diluent (Biowhitaker, Maryland, U.S.A.) that contained and absorbent to remove IgG. The absorbent prevented IgG/rheumatoid factor complexes from forming and prevented interfering competition from IgG during the test procedure.

4.1 1.2.2 Principle of the assay

Recombinant B19 capsid antigen is coated onto a solid phase (microtiter wells). The patient's sample is added to the microtiter wells and if IgM antibodies are present, they will bind to the B19 antigen, forming antigen-anti' ody complexes.

These complexes are demonstrated by incubation with an anti-human IgM HRP conjugate followed by exposure to an OPD substrate. The intensity of the colour reaction is measured by a spectrophotometer with a filter set at 492 nm.

4.1.1.2.3 Interpretation of results

Cut-off value

$$\text{Cut-off} = \frac{\text{Absorbance of negative control} + \text{absorbance of positive control}}{2}$$

Specimens with O.D. values > cut-off + 10% were considered positive for anti-B19 IgM antibodies.

Specimens with O.D. values < cut-off + 10% were considered negative for anti-B19 IgM antibodies.

Specimens with O.D. values in the range cut-off + 10% were considered equivocal and repeated.

4.2 Polymerase chain reaction (PCR)

4.2.1 Nucleic acid extraction

DNA extraction was done by using a rapid and simple method described by Patou *et al*, 1991.

4.2.1.1 Method

50μl of each specimen was added to a 100μl solution containing 10 mmol Tris HCl (pH 8.3), 0,45% Nonidet-P40 (BDH chemicals, England), 0,1 mg/ml gelatin, 50 mmol potassium

chloride and 0,006 mg proteinase K (Boehringer Mannheim GmbH, W Germany) Proteinase-K was separately aliquotted and added to the serum just before extraction was started.

This mixture was incubated at 65°C for 60 min, followed by incubation at 95°C for 10 min to inactivate the proteinase-K. The formed precipitate was further pelleted by centrifuging at 14000 rpm for 15 minutes. The supernatant was aspirated into a separate tube and 5µl of this was used in the PCR assay.

4.2.2 Primer selection

Primer selection was based on results obtained by Koch *et al* (1990) and was restricted to identifying complementary sequences in the VP1 region of the parvovirus genome. Numbering of nucleotides is according to Shade *et al* (1986) (Fig. 5).

Three oligonucleotide primers, (B1 B2 and B3) were identified for use in the nested and rapid 99 cycle PCR assays (Fig. 5). The primers were used in the following combinations:

a) Primers B1 and B2 were used for first round amplification of the nested PCR assay and were also used for the rapid 99 cycle assay. The target sequence of this primer combination was 275 base pair (b.p.) in length.

b) Primers B3 and B2 were subsequently used for second round amplification during the nested PCR assay. The target region amplified by these primers was 202 base pairs.

The three primers used in the PCR assays were as follows:

1. Sense primer PVB1 (29mer):

   5 CGTACAACTACCCGGTACTAACTATGTTG 3' (position 2809→2837)

2. Antisense primer PVB2 (30mer):

5 GCAAACTTCCTTGAAAATGGGCCACAGGGG3' (in position 3054←3083)

3. Sense primer PVB3 (29mer):

5 GACAGTGCTGCAAGGATTCATGACTTTAGG3' (position 2882→2910)

These primers were designed in the NIV laboratory (S. Barr - unpublished research).

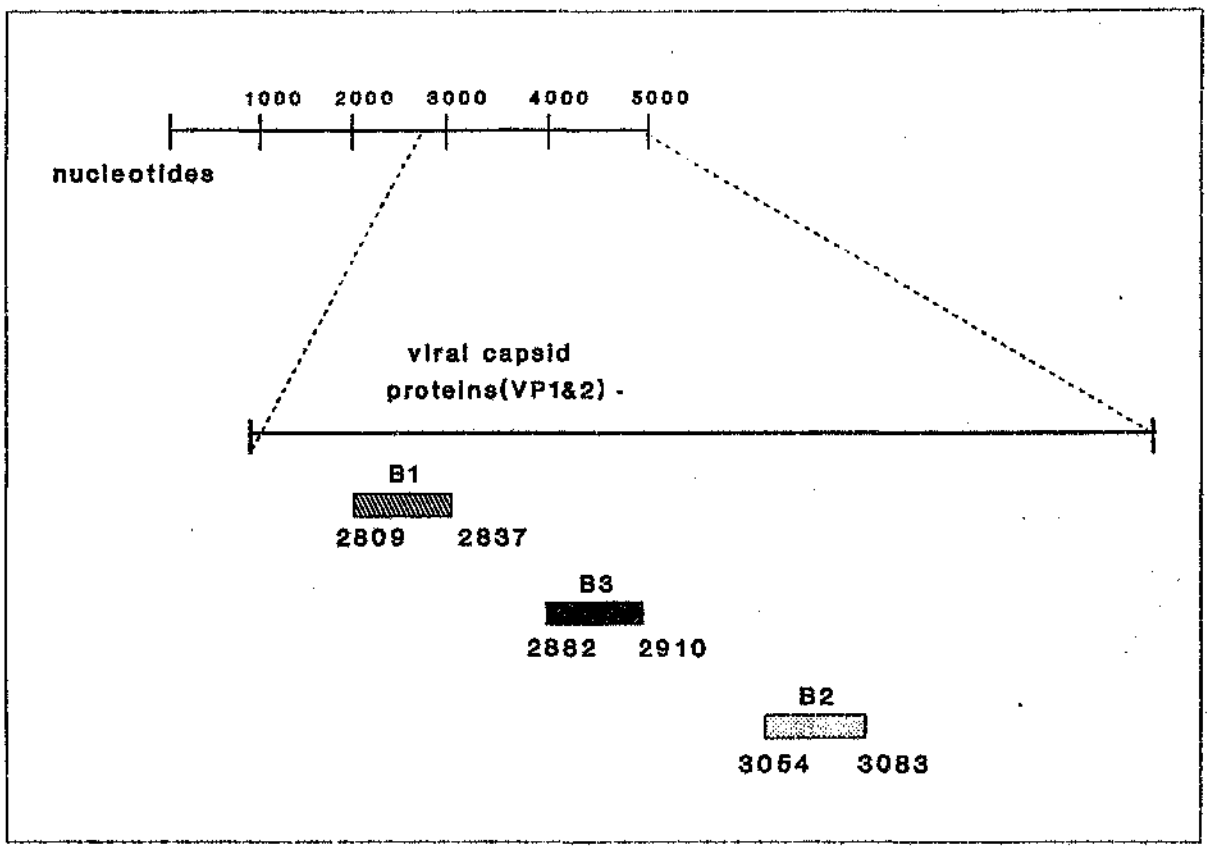


Fig. 5 Location of primers used in nested PCR and rapid 99 cycle PCR assays.

4.2.3 PCR buffer and reaction mixture

Extracted serum (5μl) or product from first round amplification (5μl) was added to a 45μl reaction volume containing 50 mmol potassium chloride, 10 mmol Tris HCl (pH 8,3), 1,5

mmol magnesium chloride, 0,01% gelatin, 0,1% Triton X-100, 1,5 units Taq DNA polymerase (Promega Corporation, Maddison, USA), 0,3 mmol of each dNTP and 0,25 mmol of each primer. This mixture was covered with two drops of liquid paraffin, thoroughly mixed on a vortex mixer and then, briefly centrifuged.

4.2.4 PCR protocols

4.2.4.1 Nested PCR protocol

4.2.4.1.1 First round amplification

This reaction was performed with the primer pair B1 and B2, the PCR buffer and reaction mix as described. Using a programmable thermal cycler (TRIO-Thermoblock, Biometra, Göttingen, FRG), samples were initially pre-heated at 95°C for 3 min to facilitate complete denaturation of any double stranded DNA which may have been present. After this initial denaturation step, samples were incubated at 72°C for 3 minutes (primer annealing and extension) and then reheated to 94°C for 1 min (denaturation of DNA). During first round amplification, 35 such cycles were used, following which specimens were kept at 4°C until used in the second round amplification step of the nested PCR protocol.

4.2.4.1.2 Second round amplification

Conditions during this round of amplification were exactly as described for first round amplification, except primer pair B2 and B3 was used and the number of cycles was limited to 25. After the 25 cycles, specimens were kept at 4°C until run on an agarose gel.

4.2.4.2 Rapid 99 cycle PCR assay

Specimens were prepared for amplification using the same primer pair (B1 and B2), PCR buffer and reaction mix as previously described for first round amplification. The 99 cycle amplification reaction was performed using a programmable thermal cycler equipped with a facility which offers tube temperature monitoring (Hybaid Ltd, U K). B19 DNA was amplified using 99 cycles of the following sequence: denaturation of DNA at 95°C for 1 second, then cooling to 50°C for 1 second to allow primer annealing and then heated to 72°C for 1 second for primer extension. Once the 99 cycles were completed, samples were further incubated at 72°C for 10 minutes to allow further primer extension, this was then followed by storing the samples at 4°C until they were run on an agarose gel.

4.2.5 Sensitivity determination

4.2.5.1 Serum dilutions

Serum derived from a patient with hereditary spherocytosis suffering from an aplastic crisis previously shown to be positive by first round amplification was aliquotted and used in the evaluation of the sensitivity of the two amplification assays. B19 DNA amplification in serial 10 fold dilutions of the abovementioned serum using the nested and rapid 99 cycle assays were evaluated.

4.2.5.2 Plasmid dilutions

Serial 10 fold dilutions of the plasmid PV-MT1, consisting of the entire B19 genome and at a concentration of 13 pg DNA/ml, were also used to determine the sensitivity of the nested and rapid 99 cycle PCR assays.

4.2.6 PCR assay controls

4.2.6.1 Positive controls

Serum derived from a patient with hereditary spherocytosis suffering from an aplastic crisis previously shown to be positive by first round amplification was aliquotted and used as an extraction and as a positive control (1.100 000 dilution) during the nested PCR assays done on the specimens of the 29 patients.

4.2.6.2 Negative controls

Sera previously shown to be negative for B19 DNA was included in each DNA extraction round and included in each PCR assay.

The ratio of specimens to negative controls in each run was 2:1.

4.2.7 Agarose gel electrophoresis

Agarose gels were prepared by adding 2,5 gram agarose (High strength analytical 1 grade agarose; BioRad Laboratories, Hercules, California) to 100 ml 0,5 Tris-borate (TBE). The solution was heated using a microwave oven until the agarose was completely dissolved.

The agarose solution was cooled to ±60°C and 5µl of stock ethidium bromide solution (10 mg/ml ded. The solution was poured into a mould with the appropriate comb properly in place.

When the gel was completely set (±40 min at room temperature), the comb was removed and the gel placed into an electrophoresis tank and covered by electrophoresis buffer (0,5 TBE).

Samples of B19 DNA were mixed with the gel loading buffer and loaded into the individual wells in the gel. The electrophoresis tank was closed, the electrical leads attached and a voltage of 3V/cm was applied for 90 minutes.

After 90 minutes, the current was turned off, the gel removed from the tank and examined by UV light at 302nm, for the presence of B19 DNA. Photographs of the gels, using Polaroid film were then taken.

4.2.8 General considerations for PCR assays

In order to reduce the chances of generating false positive reactions during the nested and rapid 99 cycle assays due to contamination, general guidelines were followed (Kwok *et al*, 1989). These guidelines were as follows:

4.2.8.1 Designated laboratories were used for designated reactions

Three laboratories were used during the preparation of the PCR assays:

a) **Laboratory No 1** - DNA extraction of samples and controls took place in this laboratory.

b) **Laboratory No 2 or the pre-PCR laboratory** - in this laboratory preparation for the first round and 99 cycle assays were carried out in a biosafety hood.

c) **Laboratory No 3 or post PCR laboratory** - in this laboratory the agarose gels were run. A biosafety hood was also used for preparation of the second round PCR assay.

All three laboratories were equipped with their own designated reagents and equipment such as pipettes and tubes. Separate laboratory coats were also assigned to each laboratory and special care was taken in changing coats between laboratories.

The biosafety hoods used were equipped with ultraviolet (UV) germicidal lamps, which were used to decontaminate surface areas before and after preparation of the PCR assays took place.

4.2.8.2 Autoclaving of tubes and reagents

All microcentrifuge tubes were autoclaved before use.

Reagents such as de-ionized water and other solutions not affected by autoclaving were routinely autoclaved before use.

4.2.8.3 Storage of reagents

All reagents, where possible, were aliquotted after preparation, properly marked, numbered and then stored in a separate area away from any amplified PCR products.

4.2.8.4 Disposable gloves

Each designated laboratory was equipped with its own supply of disposable gloves. Changing of gloves when moving from one laboratory also coincided with the changing of laboratory coats.

4.2.8.5 PCR reagent mix

All the PCR reagents used, were premixed in a cocktail and then aliquotted into the separate microcentrifuge tubes. Once this was completed, mineral oil was added, followed by the extracted sample DNA. After DNA was added the tube would be capped and only then would DNA be added to the next tube.

4.2.8.6 General precautions

a) Positive displacement pipettes with their disposable tips and plungers were used in all the PCR assays.

b) Tubes containing sample DNA amplified modules were spun down before opening them. This was done to prevent splashing of liquid containing DNA when the tubes were opened.

c) When amplified bands were demonstrated in any of the negative controls, the results of that run were not recorded. The run was subsequently repeated starting from the extraction step.

5 RESULTS

5.1 Sensitivity determination of the different PCR assays

5.1.1 Serum dilutions

The rapid 99 cycle amplification assay demonstrated the presence of B19 DNA in a 1:100 000 serum dilution, while B19 DNA was demonstrated in a 1:1 000 000 serum dilution using the nested PCR protocol (Fig. 6).

5.1.2 Plasmid dilutions

B19 DNA could be demonstrated in 1:10 000 dilution of plasmid using the 99 cycle amplification assay. The nested amplification assay demonstrated the presence of B19 DNA in plasmid dilutions of 1:100 000 (Fig. 7).

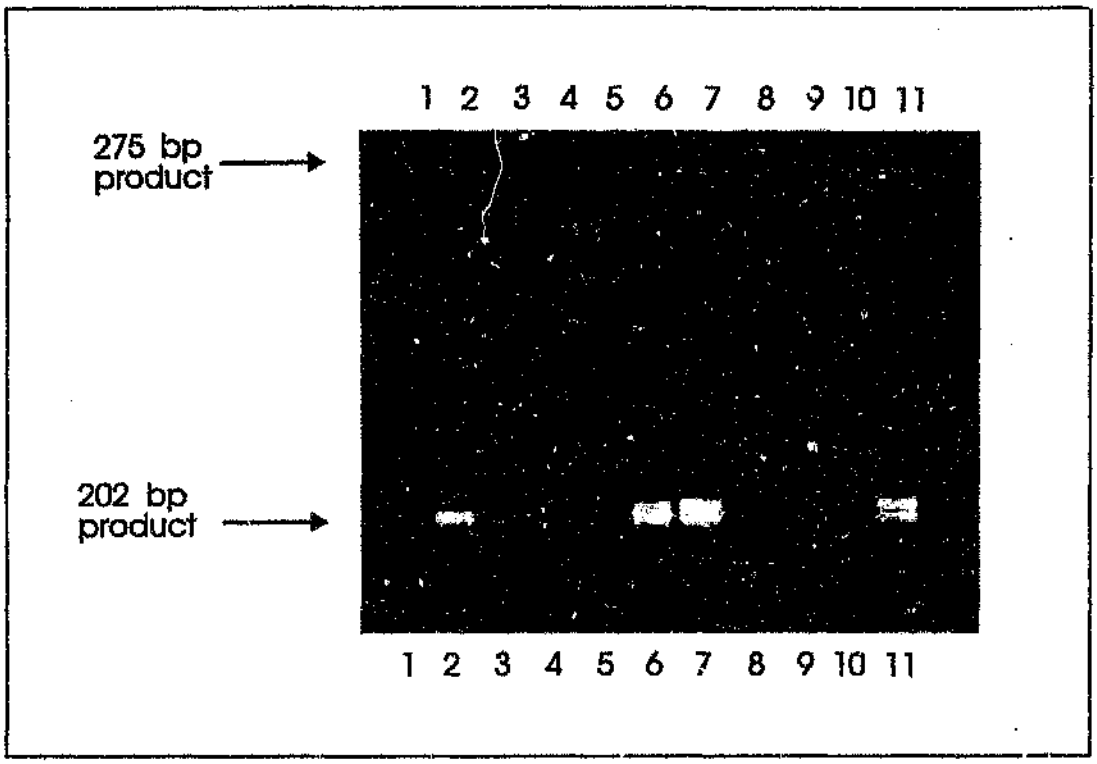


Fig. 6 Gel electrophoresis of amplified products of serum dilutions.
Lane 1 - 1:10 000 000 dilution
Lane 2 - 1:1 000 000 dilution
Lanes 3, 5, 8 and 10 - left open
Lanes 4 and 9 - negative controls
Lane 6 - 1:100 000 dilution
Lane 7 - 1:10 000 dilution
Lane 11 - 1:1 000 dilution

Please note: The molecular weight marker and dilutions 1:10 and 1:100 are not shown, but were included in the gel.

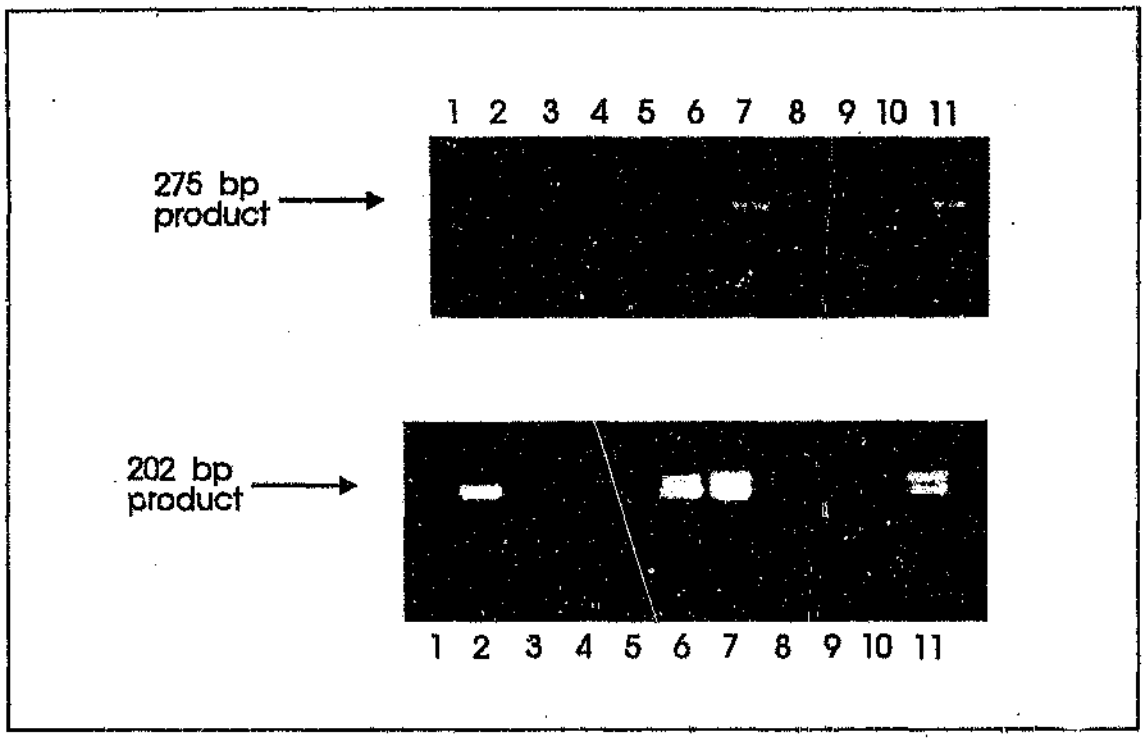


Fig. 7 Gel electrophoresis of amplified products of plasmid dilutions.
Top gel - 99 cycle amplification
Bottom gel - nested amplification
Lane 1 - 1:1 000 000 dilution
Lane 2 - 1:100 000 dilution
Lanes 3, 5, 8 and 10 - left open
Lanes 4 and 9 - negative controls
Lane 6 - 1:10 000 dilution
Lane 7 - 1:1 000 dilution
Lane 11 - 1:100 dilution

Please note: The molecular weight marker and dilutions 1:10 and 1:10 000 000 are not shown, but were included in the gel.

5.2 Serological results

5.2.1 Detection of anti-B19 IgG antibodies

Anti-B19 IgG antibodies were demonstrated in 18 (62,1%) of the 29 patients (Table 2).

Eleven of the 18 patients demonstrating anti-B19 IgG antibodies were dually infected with HIV and TB; 9 of these patients also had a chronic anaemia.

Seven patients infected only with TB were positive for anti-B19 IgG antibodies. Four of these patients suffered from chronic anaemia, while 3 patients did not demonstrate anaemia over the observed period.

Equivocal IgG antibody results were not demonstrated in any of the specimens.

Table 2: Anti-B19 IgG antibody results

		IgG	
		Positive	**Negative**
HIV/TB coinfected (n = 15)	**Anaemic**	9	2
	Not Anaemic	2	2
Pulmonary TB only (n = 14)	**Anaemic**	4	5
	Not Anaemic	3	2
Total		18	11

5.2.2 Detection of anti-B19 IgM antibodies

Positive anti-B19 IgM antibody results were repeatedly demonstrated in specimens from 2 (6,6%) of the 29 patients; both were dually infected with HIV and TB and had chronic anaemia (Table 3).

Equivocal IgM antibody results were demonstrated in 12 (41,4%) of the 29 patients. Seven of the 12 patients were coinfected with HIV and TB and had chronic anaemia. The remaining 5 patients were infected only with TB; two of these patients had chronic anaemia and in 3 patients, anaemia could not be demonstrated over the 3 month period. All patients with equivocal or positive IgM antibody results were positive for anti-B19 IgG antibodies.

Table 3: Anti-B19 antibody results

		IgM		
		Positive	Equivocal	Negative
HIV/TB coinfected (n = 15)	Anaemic	2	7	2
	Not Anaemic	0	0	4
Pulmonary TB only (n = 14)	Anaemic	0	2	7
	Not Anaemic	0	3	2
Total		2	12	15

5.3 Polymerase chain reaction results

5.3.1 First round amplification

All specimens were evaluated twice using the nested PCR assay and were thus also twice subjected to first round amplification. The validity of each run of 1st round amplification was confirmed by:

a) the demonstration of a 275 base pair (b.p.) amplified band in the positive control.

b) The absence of any demonstrable bands in the negative controls.

First round amplification was not demonstrated in any of the specimens evaluated (Fig. 8).

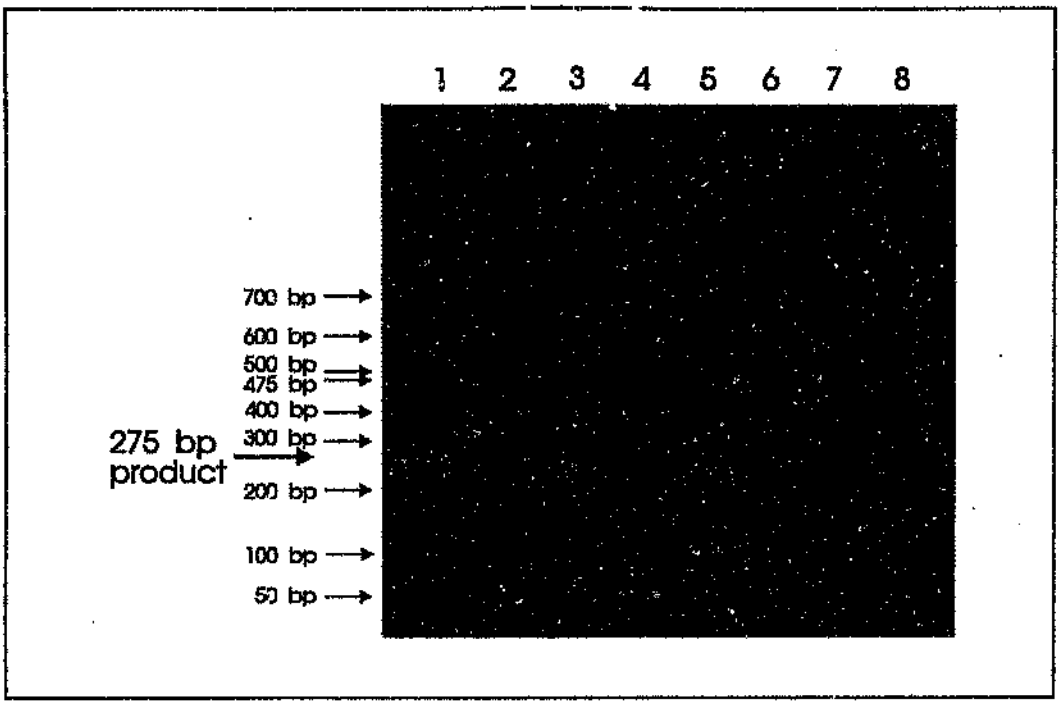


Fig. 8 Gel electrophoresis of 1st round amplification products:
Lane 1 - molecular weight marker
Lane 2 - positive control
Lanes 3 and 6 - negative controls
Lane 4 - patient No. 1182/91
Lane 5 - patient No. 453/92
Lane 7 - patient No. 103/92
Lane 8 - patient No. 1238/91

5.3.2 Second round amplification of the nested PCR protocol

Validity of each 2nd round amplification run was confirmed as follows:

a) the demonstration of a 202 b.p. band in the positive control.

b) no bands were demonstrable in any of the negative controls.

Specimens from three patients were found to be positive for B19 DNA by the nested PCR assay. Two patients (1182/91 and 453/92) were coinfected with HIV and TB and had chronic anaemia (Fig. 9).

The third patient, 103/92, was infected only with TB and did not suffer from anaemia. (Table 4).

All results were reproducible with repeated evaluation of specimens and no discrepant results were demonstrated.

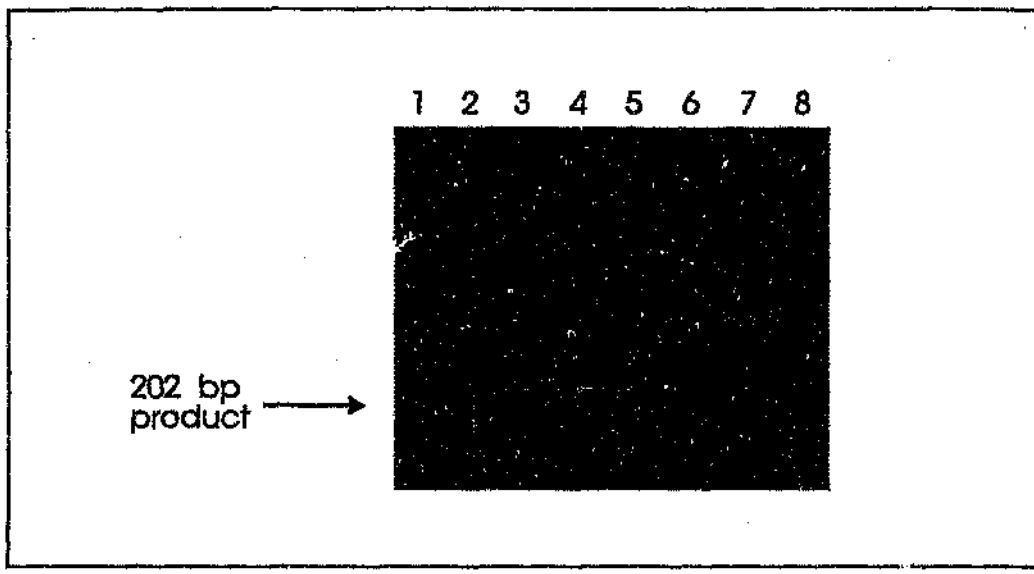


Fig. 9 Gel electrophoresis of 2nd round amplification products
Lane 1 - molecular weight marker
Lane 7 - positive control
Lanes 3 and 6 - negative controls
Lane 2 - patient No. 1182/91
Lane 4 - patient No. 453/92
Lane 5 - patient No. 103/92
Lane 8 - patient No. 1238/91

Table 4 **Summary of DNA amplification results**

		1st Round Amplification		2nd Round Amplification	
		Positive	Negative	Positive	Negative
HIV/TB (μ = 15) coinfected	Anaemic	0	11	2	9
	Not Anaemic	0	4	0	4
Pulmonary TB (μ = 14) only	Anaemic	0	9	0	9
	Not Anaemic	0	5	1	4
Total		0	29	3	26

6 SUMMARY OF LABORATORY DATA OF THREE PATIENTS WITH DEMONSTRABLE CIRCULATING B19 DNA

6.1 Introduction

Circulating B19 DNA was demonstrated in serum specimens of three patients using the nested PCR assay. A brief summary of laboratory data available on these patients will be presented. This will be followed by a short discussion on the possible significance of circulating B19 DNA in each of the patients.

6.2 Discussion

6.2.1 Patient No.1; 1182/91, 54 years old, male

On admission, this patient coinfected with HIV and TB demonstrated anaemia (HCT = 41,4) has evidence of recent exposure or persistent infection with B19 as demonstrated by a positive anti-B19 IgM ELISA result. The presence of low level circulating B19 DNA was indicated by a positive nested PCR assay.

After 3 months of routine TB treatment, the anaemia had slightly improved but was still present (HCT = 43,9); the anti-B19 IgM ELISA was still positive, but no circulating B19 DNA could be demonstrated.

The CD4 count, as an indication of the level of immunosuppression, in this patient was 510 cells/ml on admission and dropped to 330 cells/ml after three months.

The following possible explanations could be offered for these findings:

a) The patient was infected with B19 shortly before admission or had been suffering from persistent B19 infection with the subsequent anaemia on admission partially due to B19. Following admission, the patient cleared the B19 infection which lead to the disappearance of B19 DNA; the detection of anti-B19 IgM 3 months after infection was compatible with recovery from acute infection (Erdman *et al*, 1991). The chronic anaemia was therefore probably due to other factors including HIV infection (Doweiko, 1993). In this instance, B19 infection would have been a coincidental finding and not a co-factor in persistent anaemia in this HIV/TB coinfected patient.

b) Chronic anaemia in this patient was partially due to persistent B19 infection as demonstrated by a low level of B19 DNA detected on admission. This low level of viraemia was also responsible for persistent antibody stimulation leading to a detectable IgM level on admission and 3 months later. B19 DNA was not demonstrated in the second specimen due to the following reasons:

   i) it was present at very low levels in the serum and therefore not always demonstrable when using ethidium bromide stained agarose UV fluorescence but could possibly have been demonstrated using internal probing (Carrière *et al*, 1993);

   ii) it was shed intermittently into the circulation depending on the humoral immune status of the patient (Kurtzman *et al*, 1988). In this event chronic B19 infection would be one of the co-factors in causing persistent anaemia in this HIV/TB coinfected patient.

   Of these, it is probably more likely that B19 infection was a co-factor in causing persistent anaemia in this patient as the level of immunosuppression in this patient, as reflected by the CD4 count, did not improve over the three month period.

As a result, it is unlikely that B19 would have been eliminated over this period and was most probably not detected in the second specimen due to the abovementioned explanations.

6.2.2 Patient No. 2; 453/92 41 years, female

This HIV/TB coinfected patient demonstrated anaemia on admission (HCT = 39,1) and had evidence of previous exposure to B19 (anti-B19 IgG ELISA positive). There was no evidence of circulating B19 DNA (nested PCR assay negative) or recent exposure to B19 (anti-B19 IgM ELISA negative).

Three months after admission, the patient was still anaemic as demonstrated by HCT = 39,5 and circulating B19 DNA was demonstrated by the nested PCR assay. The B19 IgM ELISA was equivocal, suggesting possible a humoral immune response to circulating B19.

These findings could be explained in the following manner:

A persistent B19 infection existed on admission but was not detected initially due to very low levels of circulating B19 or the intermittent shedding of B19 DNA (Kurtzman *et al*, 1988). Three months later, B19 DNA was still present and had also stimulated an IgM response which manifested as an equivocal IgM ELISA test result. In this situation B19 infection could be a co-factor responsible for chronic anaemia in this HIV/TB coinfected patient.

6.2.3 Patient No. 3; 103/92, 26 years old, female

This TB only infected patient shows a dramatic improvement in haematological parameters 3 months after admission, a picture commonly seen in hospitalised TB patients receiving routine TB treatment (Dr B Miller - personal communication). On admission, this patient also has evidence suggestive of recent exposure to B19 infection as demonstrated by an

equivocal anti-B19 IgM ELISA result, detection of circulating B19 DNA and a detectable IgG response. The evidence here therefore, suggests that this patient was infected with B19 shortly before admission, and part of the haematological picture seen on admission was due to the effect of an acute B19 infection (Potter *et al*, 1987). The patient's immune response was subsequently responsible for clearing the infection, and 3 months later no haematological or virological evidence suggestive of persistent B19 infection was present.

Table 5: Summary of laboratory data on each patient

	Patient No 1; 54 yrs, male		Patient No 2; 41 yrs, female		Patient No 3; 26 yrs, female	
	Admission: 18/3	Follow up: 8/6	Admission: 22/1	Follow up: 22/4	Admission: 22/1	Follow up: 22/4
HCT	41,4	43,9	39,1	39,5	47,2	59,4
RBCC	4,07	4,18	3,42	3,64	5,20	5,02
WCC	13,0	6,3	10,2	7,1	8,8	8,9
Total Lymphocytes	5070	2210	2040	1920	1230	1510
Monocytes	520	440	510	500	620	450
Polymorphs	7410	3650	7650	4620	6950	6940
CD4 cells	510	330	820	860	470	510
T8 cells	4770	1770	940	920	540	710
B19 IgM ELISA	+	+	-	Equivocal	Equivocal	-
B19 IgG ELISA	+	+	+	+	+	+
B19 Nested PCR	+	-	-	+	+	-
HIV Status	+	+	+	+	-	-
TB treatment (a)Isoniazid	+	+	+	+	+	+
(b)Rifampicin	+	+	+	+	+	+
(c)Pirazinamide	+	+	+	+	+	+

7 DISCUSSION

Recent reports have demonstrated that HIV infected individuals and AIDS patients were prone to develop chronic anaemia following B19 infection (Frickhofen *et al*, 1990. Nigro *et al*, 1992). The ever increasing numbers of HIV infected individuals and AIDS patients in Africa may thus be at a potential risk of developing chronic anaemia due to persistent B19 infection. In this preliminary study the primary objective was to evaluate the role of B19 in a subgroup of the HIV infected individuals, namely coinfected HIV/TB patients with chronic anaemia using serological methods and DNA amplification assays.

7.1 Sero-epidemiology

Most B19 seroprevalence studies have until recently not addressed B19 seroprevalence in HIV infected individuals. Only lately has interest been generated in the sero-epidemiology of B19 in HIV infected individuals with most of these reports originating in the developed world (Frickhofen *et al*, 1990; Nigro *et al*, 1992; Arakelov *et al*, 1993). These reports suggested that B19 seroprevalence was increased in HIV infected individuals. No reports have been published on the seroepidemiology of B19 in immunocompromised patients in South Africa. In this preliminary study, the following observations were noted when the presence of anti-B19 antibodies were used as evidence of past exposure to B19.

Firstly, a greater proportion of patients in this study group (62%) demonstrated serological evidence of past exposure to B19 when compared to the adult seroprevalence rates recently reported in South Africa (25%) (Schoub *et al*, 1993).

Secondly, in the HIV-TB coinfected group more patients with persistent anaemia demonstrated evidence of past exposure to B19 than those without anaemia, 82% vs. 18% respectively, although the numbers are too small to make any statistical correlation. This tendency was not demonstrated in the group of pulmonary TB patients not infected with HIV. These two observations would suggest the following:

a) Factors or conditions facilitating the transmission of TB may also facilitate the transmission of B19. Poor socio-economic conditions and over-crowding are commonly associated with the transmission of TB and may thus also play a role in B19 transmission as respiratory spread is thought to be the major route of transmission for this virus. Malnutrition with an accompanying immunosuppression is also associated with the acquisition of TB and may theoretically in a B19 infected individual result in viral persistence, a prolonged carriage and excretion of B19 with the resultant increase of chance of transmission to non-immune individuals.

b) Previous evidence of B19 infection may be associated with chronic anaemia in HIV-TB coinfected individuals; this could be the result of B19 persistence (Kurtzman *et al*, 1987, Frickhofen *et al*, 1990) in the immunocompromised patients.

These seroepidemiological results are however from a small, highly selected group of individuals and can therefore not be interpreted as being conclusive of any definite serological trend.

7.2 IgM assays

The performance of IgM assays could be compromised by a variety of factors. These include the following:

a) Repeated freezing and thawing of specimens compromise the integrity of the IgM macromolecule often leading to false negative results.

b) The interference of rheumatoid factor (RF) with the IgM assay. This problem is addressed by the evaluation of all IgM positive specimens for the presence of RF.

c) IgG antibodies could occupy antibody sites in the IgM assay interferring with the result. Currently this can be circumvented by the use of a specific IgG antibody absorbent as pretreatment before performing IgM assays. The development of specific IgM capture antibody assays such as the ELISA (Yaegashi *et al*, 1989; Patou *et al*, 1991; Erdman *et al*, 1991) has now eliminated this problem.

d) Non-specific polyclonal B-cell activation by agents such as EBV, HHV6 and HIV resulting in false positive IgM assay results.

Conditions for the performance of anti-B19 IgM tests were not ideal due to the retrospective nature of the study. These specimens were initially used for other serological assays and freezed and thawed at least twice before being submitted for anti-B19 IgM antibody testing, and results obtained with this assay should be interpreted with this in mind. Positive anti-B19 IgM results over a 3 month period were demonstrated in only 2 patients, both were infected with HIV and TB and had a chronic anaemia. This would suggest that these patients experienced either a primary infection (Erdman *et al*, 1991) or re-infection (Schoub *et al*, 1993) before admission with the subsequent IgM response lasting three months. Alternatively, they were exposed before admission and re-exposed between admission and follow-up to either a reinfection or reactivation with a low-level circulating B19 (Kurtzman *et al*, 1988) which elicited a detectable IgM response. Intermittent low-level circulating B19 could not only stimulate a demonstrable anti-B19 IgM response but would also be compatible

with B19 persistence (Frickhofen *et al*, 1990) and chronic anaemia observed in these patients (Kurtzman *et al*, 1988; Coulombel *et al*, 1989; Nigro *et al*, 1992). Insufficient serum was available for the performance of the urea denaturation test which could have distinguished between primary or secondary infection (Schoub *et al*, 1993).

Interpretation of the equivocal IgM results is impossible in view of the suboptimal conditions which existed for IgM testing. It should also be remembered that the majority of equivocal IgM results occurred in patients infected with HIV which could in itself be responsible for polyclonal B-cell activation resulting in such results. Although small in numbers, the results of the anti-B19 IgM assays nevertheless tend to suggest that a greater proportion of patients coinfected with HIV-TB patients with anaemia were recently exposed to B19 compared to those without anaemia. This would also be compatible with a hypothesis that coinfected anaemia patients were more likely to harbour persistent B19 infection.

7.3 Amplification

DNA amplification techniques, especially the nested PCR assay, have been shown to be highly sensitive in demonstrating low levels of circulating B19 DNA (Koch *et al*, 1990; Carrière *et al*, 1993; Musiani *et al*, 1993). The generation of false positive reactions as a result of contamination during the assays has always been a major obstacle in the reproducibility of results. During this study strict measures (Kwok *et al*, 1989) were taken to prevent contamination and this resulted in the reproducibility of all results.

The presence of circulating B19 DNA could not be demonstrated after first round amplification and was only shown af 2nd round amplification of the nested PCR assay.

The nested PCR assay demonstrated the presence of low level circulating B19 DNA in 3 patients. Two of these patients, both HIV-TB coinfected with chronic anaemia, had haematological pictures compatible with persistent B19 infection, the presence of B19 DNA could not be demonstrated repeatedly over a 3 month period in these patients; this would be consistent with a persistent B19 infection.

The third patient had no clinical, haematological or virological features suggestive of a persistent B19 infection after 3 months of routine TB treatment; suggesting that the results obtained on admission were most probably due to a primary infection of B19 occurring shortly before admission which resolved without the development of sequelae associated with persistent B19 infection.

7.4 Sensitivity evaluation

The other objective of the study was to compare the nested and rapid 99 cycle PCR assays in the detection of B19 DNA in serum samples, as the sensitivity of the 99 cycle assay was shown to be comparable with that of the nested PCR when HBV DNA was detected (Vandenvelde *et al*, 1993). Should the 99 cycle assay be shown to have the same sensitivity as the nested PCR assay in detecting the presence of B19 DNA it would have the advantage of reducing the risk of contamination as only 1 round of amplification is undertaken with this assay.

The sensitivity of the nested PCR assay and the rapid 99 cycle assay was compared using serial 10 fold dilutions of a known positive control serum and a positive plasmid control. Serial 10 fold dilutions of the positive control specimen and the plasmid positive control both demonstrated that the nested assay could detect a 10 fold greater dilution of B19 DNA than

the rapid assay. Both these evaluations thus demonstrated that the nested assay was more sensitive than the rapid 99 cycle assay in demonstrating circulating B19 DNA. It should be pointed out that the conditions for the 99 cycle protocol were not optimized before use in this study and the results obtained here should not be regarded as conclusive until further evaluations have been done to establish optimal performance conditions for this assay. Future consideration should be given to using either radiolabelled, digoxigenin-labelled (DIG) or biotin labelled probes in demonstrating amplified products after PCR assay as this may enhance the sensitivity of these assays, especially in patients with a chronic B19 infection as these patients may have very low levels of circulating B19 DNA (Carrière *et al*, 1993).

7.5 Role of B19 in HIV/TB patients with anaemia

In conclusion, the virological evaluation of these 29 highly selected patients demonstrated several noteworthy trends. Firstly, proportionally more patients infected with TB had evidence of previous exposure to B19 than patients attending an ante-natal clinic (Schoub *et al*, 1993); a greater proportion of patients coinfected with HIV/TB with anaemia demonstrated evidence of post exposure to B19 than those without anaemia. Secondly, a greater percentage of coinfected HIV and TB patients with persistent anaemia demonstrated evidence of recent exposure to B19 when compared to the coinfected group without anaemia. Thirdly, circulating B19 DNA was demonstrated in the serum of 2 coinfected individuals with haematological pictures compatible with persistent B19 infection; the presence of B19 DNA could not be demonstrated in coinfected individuals without anaemia. Together these serological and DNA amplification assays results would suggest a possible role - either causal or cofactorial - for persistent B19 infection in the establishment of chronic anaemia in HIV

of chronic anaemia in HIV and TB coinfected patients. A larger prospective study is needed to provide more conclusive answers.

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