THE PREVALENCE OF PARVOVIRUS B19 INFECTION IN A COHORT OF HIV INFECTED PATIENTS WITH SEVERE ANAEMIA

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A Research Report submitted to the Faculty of Health Sciences, University of Witwatersrand, Johannesburg in part fulfilment of the requirements for the degree of Masters of Medicine in the branch of Haematology

Johannesburg, 2017

DECLARATION

I, Nadia Glatt, declare that this Research Report is my own work. It is being submitted for the degree of Master of Medicine (in the branch of Haematology) to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

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Nadia Glatt

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ABSTRACT

Parvovirus B19, a single stranded deoxyribose nucleic acid (DNA) virus, is known to cause anaemia in the setting of immune suppression such as Human Immunodeficiency Virus (HIV) infection. It is typically associated with a severe, isolated, normochromic normocytic anaemia and reticulocytopenia. The bone marrow classically shows a pure red cell aplasia (PRCA) with absence of maturing erythropoiesis, giant pronormoblasts and a variable presence of erythroid viral inclusions. Parvovirus B19 infection is a treatable cause of anaemia using red cell transfusions, intravenous immunoglobulin (Ig) therapy and in the setting of HIV, antiretroviral therapy. In the setting of HIV infection, testing for Parvovirus B19 infection using molecular techniques such as polymerase chain reaction (PCR) are preferred over serological methods, as antibodies are either not made or are dysfunctional. In South Africa, the prevalence of Parvovirus B19 infection in the HIV infected population with severe anaemia is not known. The aim of this study was to assess the prevalence of Parvovirus B19 in a cohort of HIV infected patients with severe anaemia.

The Inclusion criteria for specimens into the study included all specimens submitted for a bone marrow examination submitted for routine diagnostic workup between January 2012 and November 2013 at two academic hospitals in Johannesburg. The study population included HIV infected patients with severe anaemia, defined as haemoglobin levels <8 g/dl for men and non-pregnant women. Real-time PCR using the PrimerDesign™ genesig® Kit for Human Parvovirus B19 (Southampton, United Kingdom) was performed on DNA extracted from bone marrow aspirate slides of these patients. The Parvovirus B19 results (qualitative and semi-quantitative values) were assessed in conjunction with various Parvovirus B19-related clinical and laboratory parameters obtained from the laboratory information system (LIS).

The prevalence of Parvovirus B19 in this cohort of patients was 13.3% (19/143). PCR testing was possible even in samples that were suboptimal for morphological assessment, with 36.8% (7/19) of the Parvovirus B19 infection being observed in these samples. Of note, 31.6% (6/19) of the positive

samples were not requested for Parvovirus B19 testing by the clinician or pathologist, indicating that it is being under diagnosed in this population. PRCA was not observed in all Parvovirus B19 positive samples, with a sensitivity and specificity of 60.0% and 85.1% respectively. Alternate causes of anaemia were present in 42.1% (8/19) of the Parvovirus B19 positive samples, including 21.1% (4/19) of cases which showed *Mycobacterium Tuberculosis* infection, 5.3% (1/19) with iron deficiency and 15.8% (3/19) of cases with marrow infiltration by malignancy. This highlights the importance of excluding Parvovirus B19 infection even in the setting of alternate causes of anaemia.

In patients with severe anaemia and both HIV infection and Parvovirus B19-positivity, there was no statistically significant correlation between Parvovirus B19 viral load and HIV viral load, haemoglobin (Hb) level or CD4 count. Parvovirus B19 positivity was higher than expected in HIV virally suppressed patients, with a prevalence of 18.5% (5/27). However the CD4 counts in these samples were low (<350 cells/µl), suggesting that although viral suppression had been achieved, there was inadequate immune reconstitution to mount an effective humoral response to control the Parvovirus B19 infection.

Serology for IgM as a method for diagnosing Parvovirus B19 infection showed poor sensitivity (60%) but good specificity (100%) suggesting that this is an inadequate screening test in the setting of HIV infection.

The Parvovirus B19 positive samples had statistically significant lower reticulocyte production index (RPI) than the Parvovirus B19 negative samples. The negative predictive value of an RPI was 100%.

Although this is a retrospective pilot study, notable findings were observed. In the setting of HIV infection and severe anaemia, Parvovirus B19 infection may be diagnosed by PCR even in the following scenarios: a negative IgM serology result, no morphological evidence of a PRCA, presence of other causes to explain the anaemia and confirmed HIV viral suppression. Parvovirus B19 is a treatable cause of anaemia and therefore an important entity to exclude. The

cost of molecular diagnosis of parvovirus B19 is relatively higher than using serological methods, therefore should only be performed in the correct clinical setting. In HIV infected patients with grade four anaemia (Hb <6g/dl) and a reduced RPI, these findings support the use of molecular diagnosis for Parvovirus B19 infection regardless of other clinical and laboratory findings.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to the following people who assisted me with this research report:

- *Dr Robyn Marshall, Dr Michelle Bronze* and *Dr Sergio Carmona* for their continued support, advice and encouragement.
- The PCR laboratory staff, in particular *Vibha Kana,* for their assistance.
- *Dr Jenifer Vaughan* for her assistance in data collection.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

3TC	lamivudine
ACTB	beta actin
ARV	anti retroviral therapy
AZT	zidovudine
BM	bone marrow
BMA	bone marrow aspirate
BMT	bone marrow trephine
CHBAH	Chris Hani Baragwanath Academic Hospital
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
Ct	cycle threshold
DLBCL	diffuse large B cell lymphoma
DNA	deoxyribose nucleic acid
EBV	Epstein-Barr virus
ELISA	enzyme linked immunosorbent assay
FBC	full blood count
FNR	false negative rate
FRET	fluorescent resonance energy transfer
Hb	haemoglobin
HIV	human immunodeficiency virus
IC	internal control
Ig	immunoglobulin
IL	interleukin
INH	isoniazide
K_2EDTA	dipotassium ethylenediaminetetraacetic acid
KS	Kaposi sarcoma
LIS	laboratory information system
MCHC	mean cell haemoglobin concentration
MCV	mean cell volume
NHLS	National Health Laboratory Service
NPV	negative predictive value
ORF	open reading frame
PCR	polymerase chain reaction
PLT	platelet
PPV	positive predictive value
PRCA	pure red cell aplasia
RNA	ribonucleic acid
RPI	reticulocyte production index
RQ PCR	real time polymerase chain reaction
SD	standard deviation
TGF-β	tumour growth factor beta
VL	viral load
VP	viral protein
WCC	white cell count

ETHICS

This study was approved by the Medical Ethics Committee of the University of Witwatersrand, Johannesburg, South Africa. Ethics clearance number: M121143.

1 INTRODUCTION

Parvovirus B19, a single stranded deoxyribose nucleic acid (DNA) virus belonging to the genus *Erythrovirus* of the family *Parvoviridae* [1], infects humans and causes various health sequela depending on the person's age and general health condition. In the setting of immunsuppression such as human immunodeficiency virus (HIV) infection, it is known to cause anaemia [2]. The cause of anaemia in HIV infected persons is most often multifactorial. As South Africa has the highest prevalence of HIV infection in the world [3] and since cytopenias, in particular anaemia [4], are known to be an independent prognostic indicator of outcome in HIV infected patients [5], it is especially important to assess in detail all the causes of anaemia in this setting.

1.1HIV infection and Anaemia

HIV is a single-stranded, enveloped ribonucleic acid (RNA) virus and a member of the genus *Lentivirus* of the family *Retroviridae*. It is transmitted via blood and other body fluids and targets host immune cells, specifically $CD4^+$ T cells, macrophages and dendritic cells. The virus evades immune detection by incorporating itself into the target cell genome: viral RNA is reverse transcribed into double-stranded DNA by a virally encoded reverse transcriptase and is then integrated into the host cell DNA by a virally encoded integrase and host co-factors. The virus then becomes latent or is transcribed, producing new RNA genomes and viral proteins that are packaged and released from the cell as new virus particles that are capable of continued replication [6]. Infection with HIV leads to immune dysfunction through various mechanisms of CD4+ T-cell depletion and abnormal immune activation. The result is a paralysed immune system that is susceptible to opportunistic infections and malignancies. As immune dysregulation progresses, all organs are affected directly or indirectly by the virus [6]. This immune dysregulation has a number of

consequences which include the common finding of persistent and often multiple cytopenias as demonstrated by the full blood count (FBC) parameters.

Cytopenias are well described in HIV infection [7-9], with the degree of cytopenia correlating with the degree of infection [8]. Anaemia, the most common cytopenia, is also the most common haematological complication of HIV infection [4, 5] and, affects at least 10-20% of people at presentation and 70-95% during the course of infection [4, 7]. Studies have demonstrated that anaemia is an independent prognostic indicator of outcome [5] and is associated with increased morbidity and mortality independent of CD4 count and HIV viral load (VL) [4, 8, 9]. A large observational cohort study including 19,213 HIV infected people throughout the United States showed that median survival was significantly shortened (21.5 months compared to 30.3 months respectively, $P < 0.05$) in anaemic (Haemoglobin (Hb) <10g/dl) versus non-anaemic controls when matched for other parameters regardless of the underlying cause [10].

Anaemia can be classified according to red cell indices or, preferably, according to (BM) bone marrow activity which is indirectly measured by the reticulocyte production index (RPI) where RPI = %reticulocytes/(reticulocyte maturation time x $\text{Haematocrit}^{\text{patient}}/\text{Haematocrit}^{\text{control}}$)[11] (Table 1.1).

Table 1.1: Anaemia and reticulocyte production index (RPI)

RPI	Bone marrow findings	Aetiology
1-2	Normocellular	-
>2.5	Hypercellular/effective erythropoiesis	Peripheral cause
<1	Hypocellular/ineffective erythropoiesis	Central cause

In HIV infection, the cause of anaemia is often multi-factorial in origin [8, 12] (Table 1.2) with anaemia of chronic inflammation almost always a contributing factor [7]. The pathogenesis of this anaemia is due to increased serum hepcidin in response to pro-inflammatory cytokines such as interleukin (IL)-1, IL-6 and transforming growth factor-β (TGF-β). Increased hepcidin results in

decreased iron absorption from the gastrointestinal tract and decreased iron release from macrophages to erythrocytes (Figure 1.1). This reticulo-endothelial iron blockade usually manifests as a mild, normochromic normocytic anaemia, low serum iron, increased ferritin and low transferrin. The pro-inflammatory milieu seen in anaemia of chronic disease also causes a blunted erythroid response to erythropoietin and decreased red blood cell life span [13]. Nutritional deficiencies and infection related ineffective erythropoiesis are other common causes of anaemia [4].

In immune compromised patients, normal haemopoiesis may be displaced by infective granulomata and macrophage proliferation causing cytopenias. Common organisms include *Mycobacterium Tuberculosis*, Mycobacterium other than Tuberculosis, Histoplasmosis, Cryptococcus Neoforms and Candidasis*[5]*.

All causes of anaemia should be considered and investigated with the aim of providing both supportive and definitive management where appropriate. The active management of anaemia is associated with improved quality of life and decreased mortality [4, 12, 14]. It is therefore imperative, in conjunction with treatment of HIV itself, to diagnose and appropriately manage treatable causes of anaemia, such as Parvovirus B19, in HIV positive patients.

Table 1.2: Causes of anaemia in HIV infection

Decreased production	Increased production
Drugs	**Haemolysis**
Zidovudine	Thrombotic thrombocytopenic purpura
Trimethoprin-sulfamethoxale	G6PD deficiency
Amphotericin B	Autoimmune haemolytic anaemia
Ganciclovir	**Bleeding**
Dapsone	**Drugs**
Deficiencies	**Infection**
Erythropoietin	*Cytomegalo virus*
Iron	*Candida*
Folate	**Hypersplenism**
Vitamin B12	Infection
Infection	Lymphoma
HIV	Haemophagocytic lymphohistiocytosis
Parvovirus B19	Cirrhosis
Mycobacterium avium	
Mycobacterium tuberculosis	
Cytomegalo virus	
Epstein-Barr virus	
Cryptococcus neoformans	
Pneumocystis carinii pneumonia	
Histoplasma capsulatum	
Neoplasms	
Non-Hodgkin lymphoma	
Multiple myeloma	
Castleman's disease	
Hodgkin lymphoma	
Other	
Anaemia of chronic disease	
Haemophagocytic lymphohistiocytosis	
Pre-exisiting condition	

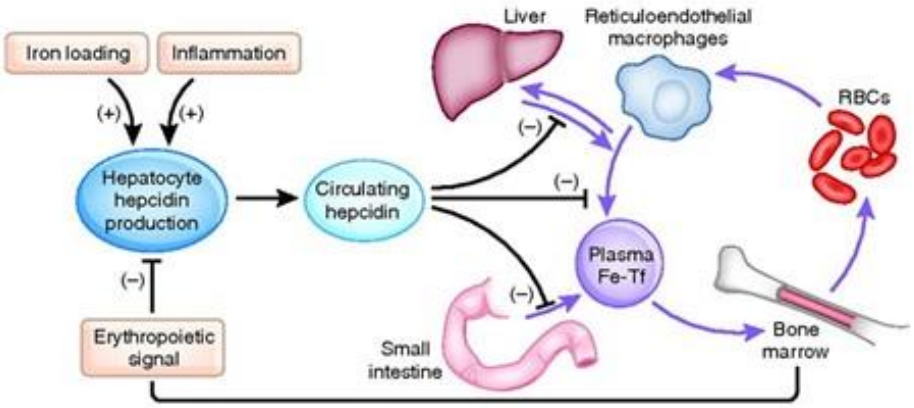

Figure 1.1: Hepcidin and anaemia of chronic inflammation

Hepcidin is the main regulator of body iron. Increased inflammation as well as increased iron and erythropoiesis cause increased hepcidin production leading to decreased iron absorption from gastrointestinal tract and decreased iron release from macrophages. This image was taken with permission from Clinical Journal of American Society of Nephrology [15].

Fe-Tf: iron-transferin complex

1.2Parvovirus B19 infection

1.2.1 Definition of Parvovirus B19

Parvovirus B19 is a small non-enveloped, single stranded DNA virus with a genome size of 5.6kb belonging to the genus *Erythrovirus* of the family *Parvoviridae* [1]. There are three genotypes of Parvovirus B19 that are known to cause anaemia in humans [16, 17]. While genotype 1 is the prototype [18, 19] and the most common genotype tested for in the United States and European countries [20], all three genotypes are present in varying levels in South Africa [21].

1.2.2 Pathogenesis of Parvovirus B19

The Parvovirus B19 genome contains three open reading frames (ORF). The first ORF encodes the non-structural protein NS1 which is involved in viral DNA replication and transcription. The second ORF encodes two structural proteins – viral protein 1 (VP1) and viral protein 2 (VP2) (Figure 1.2). The third ORF encodes another non-structural protein which is involved in the synthesis of structural proteins [22].

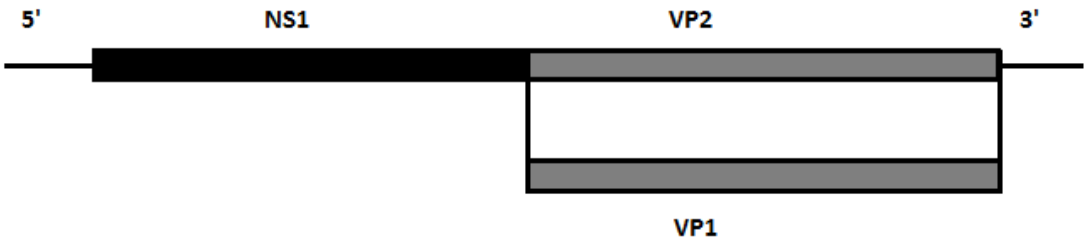

Figure 1.2: Schematic representation of Parvovirus B19 genome

NS: non-structural protein, VP: viral protein.

Parvovirus B19 shows marked tropism for the erythroid lineage and attaches to the host cell via a capsid protein and then integrates into the host cell nucleus where its genome is replicated [2]. Using its structural proteins, it induces arrest at G1 and G2 phases of the cell cycle [23] with subsequent apoptosis [24].

Although any host cell may be targeted by Parvovirus B19, as evidenced by the presence of viral DNA found in various tissues [25], viral replication only occurs in the erythroid lineage in the BM.

In Parvovirus B19 infection in immune competent hosts, erythropoiesis is transiently suppressed and the RPI falls to zero. However, the Hb levels remain stable because erythrocytes have a long lifespan of approximately 120 days and therefore there are adequate circulating erythrocytes to compensate for the transient absence of production [26]. Thus in most cases, individuals are asymptomatic with normal Hb levels. The immune competent host is able to mount an effective humoral response to this primary infection and repeated infection of erythrocytes does not occur on subsequent exposure as lifelong immunity is achieved [2].

In contrast, Parvovirus B19 infection in patients with a reduced erythroid lifespan and those with an impaired immune system is more severe. The pathophysiology and degree of severity are however different in these two categories.

A transient aplastic crisis may occur in patients whose erythrocytes have a reduced lifespan for example in sickle cell anaemia and hereditary spherocytosis. In this situation the shortened erythrocyte lifespan is unable to compensate for the transient absence of erythrocyte production, with a resultant severe anaemia. While this is often severe, it is self-limiting and confers life-long immunity to Parvovirus B19 infection [2].

In the case of the immune deficient host, Parvovirus B19 infection causes a persistent infection as protective antibodies are either not sufficiently produced or are non-functional. In HIV infected individuals and other immunocompromised states, erythroid-lineage cells are continuously destroyed by the active Parvovirus B19 infection and a chronic and persistent pure red cell aplasia (PRCA) develops [27].

Other causes of PRCA include HIV itself, Epstein-Barr virus (EBV), viral hepatitis, drugs including zidovudine (AZT), lamivudine (3TC), isoniazide (INH) and sulphonamides, autoimmune diseases, pregnancy and haematological malignancies.

1.2.3 Prevalence of Parvovirus B19

The prevalence of Parvovirus B19 infection in non-HIV populations has been widely published in local and international studies. Locally the prevalence of Parvovirus B19 infection in pregnant women [28, 29] and young haemophiliacs [30] has been reported as 3.4% and 5.1% respectively. International studies have described prevalence data in blood donors [31-40] ranging from 0.12% to 7.53%. Retrospective record reviews of anaemic patients performed in Taiwan [41] and Kenya [42] reported a prevalence of Parvovirus B19 infection ranging from 0 to 3% using serology for Immunoglobulin (Ig) M or molecular methods. HIV status however was not assessed in these studies. Further, it is difficult to compare the results of these published studies because of the different diagnostic tests used to diagnose Parvovirus B19 infection.

While there are no published studies on the prevalence of Parvovirus B19 infection in the South African HIV infected population with severe anaemia, there are numerous case reports of HIV infected patients with severe anaemia and Parvovirus B19 infection which have been reported internationally [43-56]. In addition, two large multi-centre studies have been performed: The study conducted in Brazil in 2012 looked at 88 HIV infected patients with and without anaemia and found the prevalence of concomitant anaemia and Parvovirus B19 infection to be 3.4% [57]. A study conducted in France in 2010 of 428 HIV positive anaemic patients, reported a similar prevalence of Parvovirus B19 infection of 3.73% [58].

Studies performed in the developing world in HIV infected patients in whom the Hb level was not assessed, reported a wide prevalence range of 0 to 13.1% [57, 59-64]. This variability may be attributed to the different diagnostic tests used (IgM or molecular methods), the patient populations (studies were conducted in Iran, Ghana, China and Brazil) and their HIV prevalence as well as the

study designs (single-centre, multi-centre studies with and with-out case-controls, retrospective and prospective study designs).

The above studies thus have several limitations precluding extrapolation of their results to the SA HIV infected patient cohort

1.2.4 Clinical presentation of Parvovirus B19 in the HIV infected host

Parvovirus B19 infection in HIV infected adults may be asymptomatic such as seen in HIV immune competent persons. It may also present with mild exanthematous disease associated with a viral prodrome and erythematous, reticular rash or with a PRCA which traditionally presents as a severe normocytic anaemia associated with reticulocytopenia [27]. Patients may also present with symptoms and signs of anaemia such as fatigue, dizziness, weakness, shortness of breath, palpations, tachycardia and high output cardiac failure. A bicytopenia or pancytopenia may also be noted. This is seen less commonly and although Parvovirus B19 specifically targets the erythroid lineage in the bone marrow, resulting in an isolated anaemia, immune mediated destruction of neutrophils and platelets (PLT) by Parvovirus B19, resulting in a neutropenia (neutrophil count of less than 1.6 $x10^9$/l [65]) and thrombocytopenia (platelet count of less than $171x10^9$/l [65]) have also been described [48, 66, 67].

1.2.5 Investigations

Preliminary investigation of the cause of anaemia includes an FBC with a differential count and peripheral blood smear assessment, RPI, substrate deficiency testing which includes iron studies, vitamin B12 and red cell folate levels and possibly a haemolytic work up which includes haptoglobin, lactate dehydrogenase and a Coombs test if haemolysis is suspected as an alternative cause for the severe anaemia. More specific investigations into the cause of the anaemia would include bone marrow investigations and testing for Parvovirus B19 itself.

1.2.5.1 Bone marrow investigation

A bone marrow aspirate (BMA) and bone marrow trephine biopsy (BMT) are often performed as part of the diagnostic work-up of anaemia to provide information regarding the haemopoietic activity of the bone marrow in general, degree and nature of erythropoiesis and presence or absence of malignant or infective infiltrates.

The morphological examination of the BMA and BMT in patients with a PRCA secondary to Parvovirus B19 reveals markedly under-represented erythropoiesis relative to the other haemopoietic cell lines present, with a resultant increased myeloid to erythroid ratio [68] and giant pronormoblasts with notable absence of maturing erythroid precursors known as a maturation arrest or PRCA(Figures 1.3 and 1.4). Viral inclusions in the erythrocytes may also be observed on BMA and BMT (Figure 1.4). Atypical cases where erythroid maturation occurs have also been described[69].

Immunohistochemical staining on BMT using a monoclonal antibody to Parvovirus B19 capsid protein VP1 or VP2 antigens may be used to confirm the presence of erythroid Parvovirus B19 inclusions (Figure 1.5) when suspected morphologically. This is not a frequently used technique as it is dependent on many factors including processing methods used for paraffin embedding, available instrumentation and the quality of the anti-Parvovirus B19 antibody used [70]. It is however available in our laboratory in the form of a nuclear stain to capsid protein VP2 using polyclonal IgG rabbit anti-Parvovirus B19 antibody (DakoCytomation, Glostrup, Denmark). There are no published studies comparing the sensitivity and specificity of this method with other diagnostic methods.

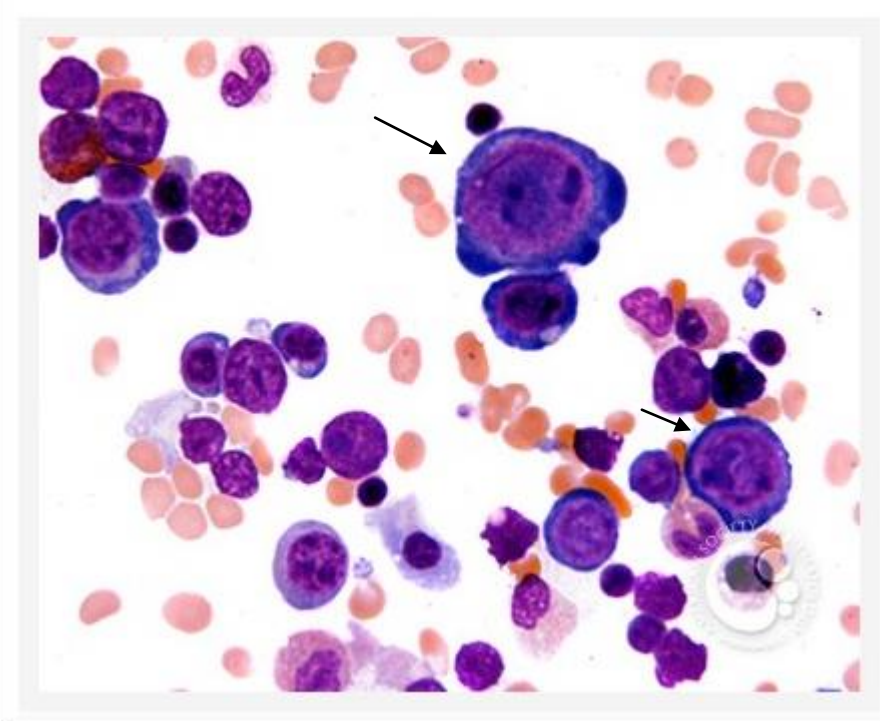

Figure 1.3: Bone marrow aspirate in a patient with Parvovirus B19 infection

50x magnification, giemsa stain shows giant pronormoblasts (black arrows) in a bone marrow aspirate of a patient with acute Parvovirus B19 infection and symptomatic anaemia. This image was taken with permission from ASH image bank[71]

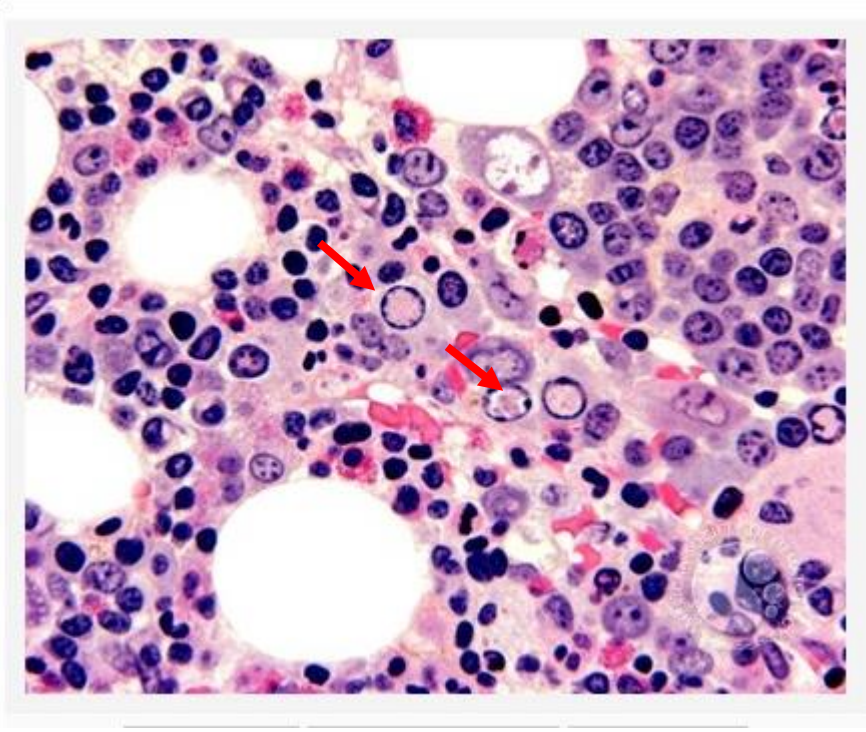

Figure 1.4: Bone marrow trephine biopsy in a patient with Parvovirus B19 infection

40x magnification, haematoxylin and eosin stain showing Parvovirus B19 inclusions (red arrows) in the erythrocytes of a bone marrow trephine biopsy of a patient with Parvovirus B19 infection. This image was taken with permission from ASH image bank [71]

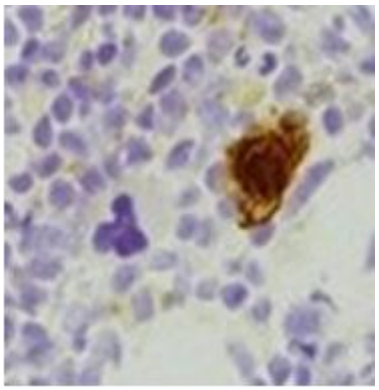

Figure 1.5: Bone marrow trephine biopsy with immunohistochemical staining in a patient with Parvovirus B19 infection

40x magnification, Immunohistochemical staining for Parvovirus B19 using a monoclonal antibody to Parvovirus B19 antigen in a bone marrow trephine biopsy of a patient with Parvovirus B19 infection. This image was taken with permission from ASH image bank[72]

1.2.5.2 Serological and Molecular Testing for Parvovirus B19 testing

Serological and molecular testing are currently available for the diagnosis of Parvovirus B19 infection [73].

Serological results report both IgM and IgG. IgM positivity suggests the presence of neutralizing antibodies to a current infection whereas IgG positivity suggests antibodies present due to previous exposure to a now cleared infection.

Molecular methods detect the presence or absence of the viral genome.

In general, serology is more widely available, less technically challenging, less labour intensive and lower cost than molecular methods and is therefore used more often. However, antibodies to Parvovirus B19 are usually non-functional or absent in the setting of HIV infection and therefore serology is an unreliable diagnostic tool in this setting [43]. Prevalence data based on serological diagnostic tests therefore does not accurately reflect the prevalence reported. Rather, viral DNA detection using molecular testing, namely PCR, is indicated. It is imperative to establish accurate cut-off values for positive and negative samples to distinguish between a low viral load which indicates previous exposure and absence of acute current infection, known as 'viral dust', and a high viral load indicating current disease [58]. Previous studies using molecular methods reported a positive PCR at a lower than recommended cut-off level. Therefore some samples reported as positive may in fact represent viraemia but not infectivity or active disease [58].

Molecular testing may be performed on peripheral blood, BMA and BMT samples at the request of the clinician or as a recommendation by the pathologist reviewing the BM. BM is the preferred specimen because it is more likely to yield a positive result in the setting of Parvovirus B19 and HIV infection as compared to peripheral blood testing.

BM erythroid progenitors are the target cells of the Parvovirus B19 infection which are present on BMA and BMT sections. Further, the BMA and BMT sections assist with the diagnostic interpretation and exclusion of an associated infiltrate. Classic BM findings in Parvovirus B19 infection of a PRCA with absent/markedly reduced erythropoiesis and giant pronormoblasts with occasional erythroid viral inclusions have been well described. A BM, however, is an invasive procedure which requires a

specialist diagnostic service for interpretation. This is an important consideration in the developing world with limited access to resources.

1.2.6 Treatment

Persistent infection with Parvovirus B19 is clinically relevant because it is a potentially treatable cause of anaemia [27, 52].

Treatment of Parvovirus B19 induced PRCA in HIV infected patients includes specific and supportive measures. Improvement of the immunosuppression caused by HIV with anti-retroviral (ARV) therapy is a cornerstone of treatment, as reported in various studies [44-48, 51, 74]. Intravenous immunoglobulin administration is used as a form of passive immunity against Parvovirus B19 [43, 75, 76].

Supportive measures include steroid therapy and transfusion of packed red cells with iron chelation therapy to prevent or treat iatrogenic iron overload secondary to the chronic transfusions that are often required.

1.2.7 Monitoring response to therapy

Monitoring of response to therapy includes clinical and laboratory features: improvement of the symptoms of anaemia, Hb level and RPI. Currently, monitoring of Parvovirus B19 VL by quantitative PCR is not routinely performed. PCR testing is expensive and does not offer more information than the routine surrogate markers used to monitor response to therapy (Hb, RPI). In addition, when multiple pathologies contribute to the anaemia, Hb level and RPI are a better reflection of clinical status than Parvovirus B19 PCR. Perhaps for specific cases who fail to respond to therapy, monitoring Parvovirus B19 VL by PCR testing would be of value [77] to determine if the persistent anaemia is due to the Parvovirus B19 itself or another pathology.

1.2.8 Prognosis

Prognosis varies in different clinical settings. Excellent results have been described, with 90% of cases of PRCA in HIV positive patients fully recovering in less than three months [44-47, 51]. Interestingly, reports have shown that in some cases, being on ARV therapy alone may result in adequate immunological recovery and therefore in clearance of the Parvovirus B19 and improvement of anaemia [46, 47, 51, 78]. This has considerable clinical and cost implications as blood transfusions are costly and associated with significant risks (immune suppression, iron overload, alloimmunity and infections) [79]. In addition, intravenous immunoglobin therapy is not always readily available in our setting and is expensive [80].

However, in the South African setting results have not been as promising, with many patients requiring repeated administration of intravenous immunoglobin often accompanied by multiple blood transfusions [81]. The reason for this poor response is uncertain, but may be due to patients late presentation, that is severe HIV infection and very low haemoglobin levels due to delayed access to medical care as well as co-existing diseases contributing to the anaemia [81].

The CD4 count in an HIV infected person is used as a marker of immune status. A lower CD4 count is associated with more severe immune suppression. PRCA secondary to Parvovirus B19 in HIV positive patients has been shown to be associated with very low CD4 levels, ranging from a median of 24 – 42 cells/µL [43, 59, 76]. It has been suggested that the CD4 count may be used as a prognostic marker to predict the development of a chronic PRCA in an HIV infected patient exposed to Parvovirus B19, with very low counts associated with PRCA and anaemia and high counts associated with adequate humoral response and absence of severe anaemia.

1.3Aim of the study

The aim of this study was to assess the prevalence of Parvovirus B19 infection in the HIV infected population group in South Africa with severe anaemia in order to assess the role of routine

Parvovirus B19 PCR screening. This has implications for the testing and management of this subset of patients and treatment guidelines.

2 RESEARCH QUESTIONS

- To determine the prevalence of Parvovirus B19 infection in HIV infected patients with severe anaemia at two academic hospitals in Johannesburg who had a bone marrow examination submitted for routine diagnostic workup between January 2012 and November 2013.
- To determine the association between Parvovirus B19 VL and 1) the degree of anaemia and 2) HIV immune suppression as indicated by high HIV VL and low CD4 count
- To evaluate the clinical utility of laboratory parameters in anaemic patients to determine the most suitable markers for distinguishing Parvovirus B19 positive and Parvovirus B19 negative patients
- To assess the role of bone marrow morphology as a diagnostic tool for Parvovirus B19 infection

3 MATERIALS AND METHODS

3.1Study design and population

A retrospective study was performed at two academic hospitals in Johannesburg which are serviced by the National Health Laboratory Service (NHLS): Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) and Chris Hani Baragwanath Academic Hospital (CHBAH).

The Inclusion criteria for specimens into the study included all specimens submitted for a bone marrow examination for routine diagnostic workup between January 2012 and November 2013. The study population included HIV infected patients with severe anaemia, defined as haemoglobin levels <8 g/dl for men and non-pregnant women [82]. Patients aged 18 years and older were included. Exclusion criteria included insufficient BM material for PCR analysis and absence of BMA or BMT slides for analysis. Real-time PCR for Parvovirus B19 was performed on all samples (Figure 3.1).

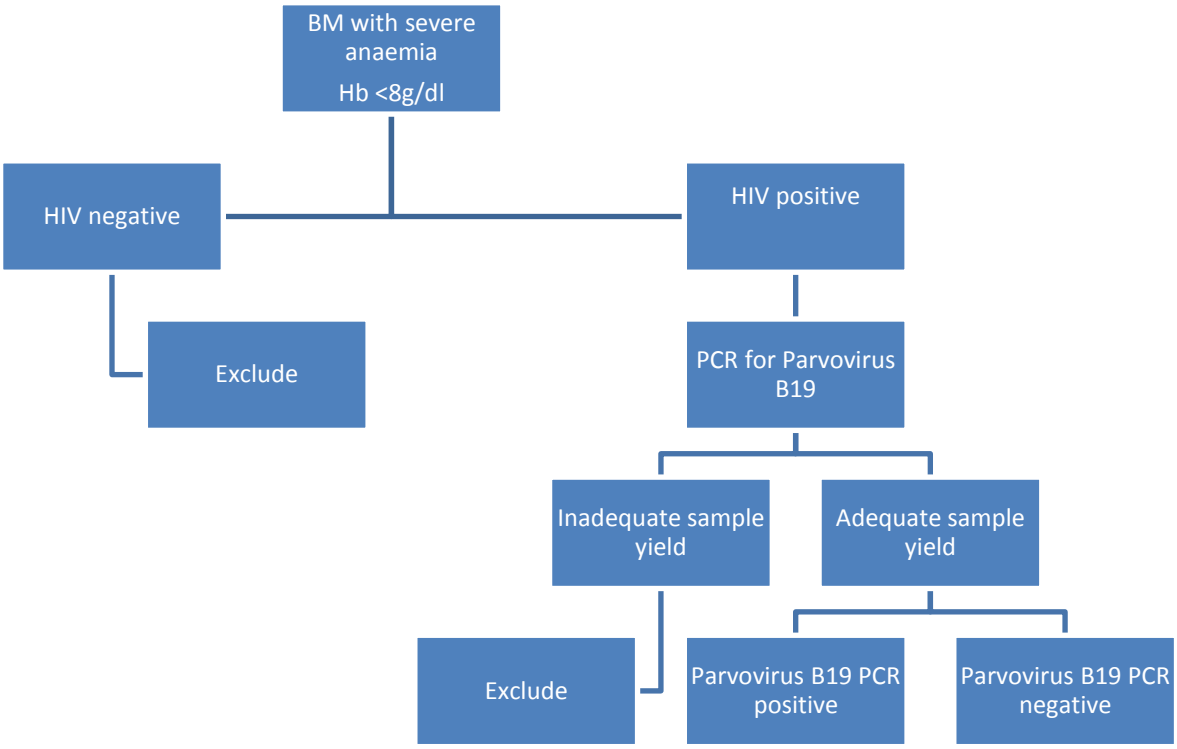

Figure 3.1: Study population

Bone marrow samples from HIV infected patients with an Hb <8g/dl were included in this study. Parvovirus B19 PCR was performed on all samples with sufficient material for testing.

BM: bone marrow, Hb: haemoglobin, HIV: Human Immunodeficiency Virus, PCR: polymerase chain reaction

3.2Data collection

Demographic information (gender and age), clinical information (ARV regimen, known medical conditions, infections and malignancies) and laboratory information (HIV VL, CD4 count, haemoglobin, mean cell haemoglobin concentration (MCHC), mean cell volume (MCV), white cell count (WCC), neutrophil count, PLT, RPI, haematinics (iron, vitamin B12, red cell folate) and Parvovirus B19 serology), where available, were obtained from the laboratory information system (LIS) and recorded on data collection sheets (Appendix A, Table A1)

Parameters were assessed according to reference ranges and definitions presented in Appendix A (Tables A2 and A3).

3.3Diagnostic testing

3.3.1 FBC, Differential count and RPI

Blood samples were collected indipotassium ethylenediaminetetraacetic acid (K_2EDTA) tubes which contain between 1.5–2.2 mg of K_2EDTA dihydrate per millilitre of blood.

The ADVIA 2120 Hematology System (Siemens Diagnostics, Deerfield, IL, USA) and SLS XT-series (Sysmex, Kobe, Japan) were used to measure the FBC, differential count and RPI parameters. The samples analysed on the ADVIA 2120 had cells counted and sized by light scatter (flow cytometry) technology using white light for white cells and laser light for red cells and platelets. Hb was measured by the conventional cyanmethaemoglobin method. The six-part differential analysis was performed in two channels. Cells in the peroxidase channel were measured by peroxidase staining intensity and light scatter. Cells in the basophil/lobularity channel were measured by dual laser light scatter, nuclear density and lobulation index. Reticulocytes were stained with oxazine 750. The SLS XT-series (Sysmex, Kobe, Japan) uses impedance, light scatter (flow cytometry) and fluorescent dye technology. The WCC, erythrocytes and platelets were counted by 'impedance technology' in which

cells pass between two sensing electrodes and cause impedance to the current which is proportional to the volume of the cell passing through. Flow cytometry with appropriate fluorescent dyes was used for the white cell -5 part differential count, nucleated erythrocytes and reticulocyte count. Hb was measured with cyanide-free sodium lauryl sulphate reagent.

The films were spread manually or on the Advia autoslide slide maker and stained on the Hema-Tek slide stainer.

3.3.2 Haematinic studies

Iron studies, Vitamin B12 and red cell folate assays were analysed using the Advia 1800 chemistry analyser (Siemens Diagnostics, Deerfield, IL, USA) at CMJAH and the cobas-8000 (Roche, Basel, Switzerland) at CHBAH.

3.3.3 Serology Parvovirus B19

Serological testing for Parvovirus B19 IgM and IgG was performed on samples at the request of the treating clinician. Serology was performed manually using an enzyme linked immunosorbent assay (ELISA) method (IBL international, GMBH, Hamburg, Germany). Diluted serum samples were added to Microtiter strip wells precoated with Parvovirus B19 antigens. After washing the wells to remove all unbound sample material horseradish peroxidase labelled anti-human IgG conjugate was added. This conjugate binds to the captured Parvovirus B19 specific antibodies in the sample serum. The immune complex formed by the bound conjugate was visualized by adding tetramethylbenzidine substrate which gives a blue reaction product. The intensity of this product is proportional to the amount of Parvovirus B19 specific IgG antibodies in the specimen. Sulphuric acid was then added to stop the reaction. This produces a yellow endpoint colour. Absorbance at 450 nm was read using an ELISA microwell plate reader. A positive, negative and cut-off control, supplied by the manufacturer, were used in each run. A run was considered valid if the cut-off control absorbance value was between 0.150 and 1.30, the negative control absorbance value was less than the cut-off control value and the positive control absorbance value was greater than the cut off control value. A result

was considered positive if its absorbance value was more than 10% higher than the cut-off absorbance value. All results were reported as either positive or negative. No indeterminate results were reported. Testing was performed in one central location.

3.3.4 CD4 counts

Single platform PanLeucogate[83] CD4 counts were prepared using the ImmunoprepTM lyse-no-wash method (Beckman Coulter, USA). Post preparation, Flow Count TM beads were added and samples acquired and analysed on a FC 500 MPL flow cytometer (Beckman Coulter, USA). Briefly, 10µl of Beckman Coulter FlowCareTM CD45/CD4 monoclonal antibody was added to 100 µl of whole blood. The samples were incubated for 10 minutes during which the sample reacted with fluorochrome labelled CD4 and CD45 monoclonal antibodies (contained in the Beckman Coulter Flow Care PLG CD4 kit) that are specific for the cell surface antigens CD4 and CD45. Post incubation, ImmunoprepTM reagents (lysing agent, stabiliser and fixative) were added to haemolyse red blood cells while maintaining the integrity of the white blood cells and the white blood cell surface antigens. Prior to acquisition and analysis on the flow cytometer, 100µl of beads was added to each sample. Cells that had bound the fluorochrome labelled antibody were identified on the basis of their specific fluorescence emission related to the specific fluorochrome attached to either the CD45 or the CD4 antibody. A sequential automated gating strategy was used to include all CD45+ cells and isolate the bright CD4+ lymphocytes from dimly expressing CD4+monocytes. The CD4+ lymphocytes were further separated from the rest of the white cells on the basis of their complexity (side scatter) and their specific CD45 and CD4 expression. The CD4 absolute count was subsequently calculated by comparing the CD4+ lymphocyte events in the CD4 region to the bead events in the bead region and referencing the bead calibration factor supplied with each bottle of Flow Count TM beads using the formula: CD4 events/ bead events x calibration factor. CD4 count was reported as the number of CD4 cells per µl.

3.3.5 Morphologic analysis

3.3.5.1 Bone marrow slides preparation

Preparation of BM slides for morphologic assessment was performed in the same way at both CMJAH and CHBAH. Bone marrow aspirate slides were fixed for ten minutes in methanol, air dried and stained twice using the Hematek stainer and Hematek Stain Pak (Siemens Diagnostics, Deerfield, IL, USA) at a pH of 6.8. The Hematek Stain Pak comprises three solutions: stain solution (methanol >99%, polychrome methylene blue-eosin stain), buffer solution (phosphate buffers, surfactant, preservative) and rinse solution (methanol 10%, phosphate buffers, surfactant, preservative). Haemoglobin molecules and eosinophilic granules are basic and take up the eosin dye (acidic dye). Nucleic acid, proteins of cell nuclei and cytoplasm of primitive cells are acidic and take up methylene blue (basic dye).

Bone marrow trephine biopsies were prepared for morphologic assessment at the Department of Anatomical Pathology for samples received from both CMJAH and CHBAH. These samples were received in 10% buffered formal saline and fixed in this solution for at least 24 hours, after which they were placed on a labelled cassette which offers a support structure and is optimised to obtain maximum fluid exchange during processing. Cores were measured and those of 1cm or longer were cut in half or quarters depending on the length. Samples in their cassettes were decalcified in EDTA for a minimum of 48 hours and then processed in an automated tissue processor. Samples were inserted into the processing chamber and processed overnight with formalin at different concentrations, dehydrated in alcohol, xylene cleared and wax embedded. They were then embedded in small moulds and ribbon cut at 1μ by the microtome. Three levels were cut for each block with 6-8 sections per slide. Slides were manually stained with haematoxylin for nuclear staining and eosin phyloxine for cytoplasmic staining and were then dehydrated in absolute alcohol, mounted on coverslip machine and labelled.

All BMA and BMT samples were reviewed as part of the routine assessment. They were considered

inadequate for morphological assessment if they were not representative of BM *i.e.* if the BMA sample comprised no marrow elements such as particles and background stromal cells and if the BMT samples did not have bony spicules and intact marrow expanses present or were too traumatised for accurate assessment. All BMA and BMT of the Parvovirus B19 positive samples were reviewed again during this study.

3.3.5.2 Ziehl-Neelsen stain

Ziehl-Neelsen staining for acid fast bacilli on trephine biopsy slides was performed as part of routine workflow, briefly, as follows: slides were stained with filtered Carbol Fuchsin for ten minutes, washed with tap water, flooded with 95% alcohol to remove excess carbol fuchsin without altering the bacilli staining, washed in tap water, differentiated in 1% acid alcohol, washed in tap water and counterstained with 1% methylene blue for less than 10 seconds, following which they were washed again in tap water, air dried and cover slipped. The mycolic acid present in the cell wall of *Mycobacterium tuberculosis* is resistant to penetration by the acid alcohol so that the background appears pale blue and the acid fast bacilli appear red (due to the carbol fuchsin) when viewed under the microscope. A positive control, obtained from a tissue with known *Mycobacterium tuberculosis* infection, was performed with each sample.

3.3.5.3 Immunohistochemical stain for Parvovirus B19

Immunohistochemical staining using polyclonal rabbit anti-Parvovirus B19 IgG (Code: B-0091) (DakoCytomation, Glostrup, Denmark) was performed as part of routine workflow, briefly, as follows: slides were washed for five minutes in hydrogen peroxide and then rinsed with buffer. The primary antibody to the Parvovirus B19 capsid protein VP2 was added and left to incubate at room temperature for 20 minutes and then rinsed with buffer. The envision kit was applied and incubated for 20 minutes at room temperature and then rinsed with buffer. For visualization, the substrate and chromogen (3,3'-Diaminobenzidine) were added and incubated at room temperature for ten minutes. Slides were then rinsed thoroughly and counterstained in haematoxin. They were then

dehydrated in alcohol and zylene and cover slipped. A positive control from a known Parvovirus B19 positive sample was performed with each sample.

3.4PCR for Parvovirus B19

All samples in this study were tested for Parvovirus B19 infection with the PrimerDesign™ genesig® Kit for Human Parvovirus B19 (Southampton, United Kingdom) [84] in the PCR laboratory at the NHLS, CMJAH.

The primers and probe in this kit have 100% homology with all reference sequences currently in the NCBI database including the three genotypes known to be present in South Africa ((AY083234, DQ333428, DQ225151). The assay results were measured using both qualitative (positive or negative) and semi-quantitative (Parvovirus B19 viral load) outputs. The amount of house-keeping gene, beta actin, in each sample was used as a reference value to give a semi-quantitative Parvovirus B19 viral load. Under optimal PCR conditions genesig® HPVB19 detection kit has a sensitivity of >95% and can detect less than 100 copies of target template in 5µl of sample.

3.4.1 The TaqMan principle

The PCR reaction used in this study exploits the TaqMan principle which uses primers and fluorescently labelled probes. During PCR amplification, forward and reverse primers hybridise to the Parvovirus B19 DNA. A fluorogenic DNA probe labelled with a 5'-fluorescent dye and 3'-non-fluorescent quencher is included in the same reaction mixture. In the intact probe, the non-fluorescent quencher and the fluorescent dye are in close proximity and as such the quencher quenches the fluorescence energy emitted by the dye through the fluorescent resonance energy transfer (FRET) effect.

During PCR amplification, the 5' exonuclease activity of the Taq polymerase cleaves the probe in a 5' to 3' direction, separating the reporter dye and quencher. The resulting increase in fluorescence is measured in real-time since it is read after each PCR cycle, with the amount of fluorescence directly proportional to the amount of DNA at the start of that cycle.

3.4.2 DNA extraction

A total of 250µl of phosphate buffered saline was used to wash the bone marrow aspirate from their slides. DNA Extraction was performed using the NucliSENS® EasyMag® (bioMérieux) system, (Boxtel, Netherlands) with an input volume of 196µl and an elution volume of 25µl. Four micro-litres (4µl) of internal control (IC) was spiked into the sample prior to extraction. Briefly, buffer containing guanidine thiocyanate and Triton X-100 was added to each sample in an extraction cartridge vessel. This disrupts all cellular matter or viral particles and inactivates RNAses and DNAses in the sample. Fifty microlitres of magnetic silica was then manually added to the sample. Nucleic acids present in the sample bound to the magnetic silica dioxide particles under high salt conditions. The magnetic silica was washed several times and subsequently the nucleic acids were eluted in a volume of 25µl and were available for the downstream PCR reaction.

3.4.3 PCR set-up

Real time PCR (RQ-PCR) using the PrimerDesign™ genesig® Kit for Human Parvovirus B19 (Southampton, United Kingdom)[84] was used to detect Parvovirus B19 DNA.

The commercial assay includes:

- Parvovirus B19 specific primer/probe (proprietary information)
- Parvovirus B19 positive control with a viral copy number of 2000000 copies/5µl
- Internal extraction control DNA
- Internal extraction control primer/probe mix
- Endogenous beta actin (ACTB) primer/probe mix
- RNAse/DNAse free water

Two separate PCR reactions were performed – the first to detect the presence of Parvovirus B19 DNA and the second to detect the presence of a house-keeping gene ACTB.

The pathogen detection reagent mix was prepared as per standard procedure (Table 3.1). Each well was filled with 15µl of this mix before 10µl of extracted DNA template was added with a final volume of 25µl.

Table 3.1: Reagent preparation for Parvovirus B19

Component	Volume (µl)
2 x precision master mix	10
Pathogen primer/probe mix	1
Internal control primer/probe mix	1
RNAse/DNAse free water	3
Final volume	15

3.4.4 Standard curve

A standard curve (Figure 3.2) was generated by serial dilutions of the positive control (from $2x10^5$ copies/µl to $2x10^0$ copies/µl) followed by a PCR reaction. These VL copies were plotted on the y-axis, against the cycle threshold (Ct) value on the x-axis using Microsoft Excel 2013 from which a logarithmic equation was then generated $y=1E10^{(-0.66x)}$. Based on the results of the standard curve a Ct of 30 ($2x10^0$ copies/µl) was derived as the lower limit of detection. This equation was then used for test samples to calculate Parvovirus B19 VL from Ct values.

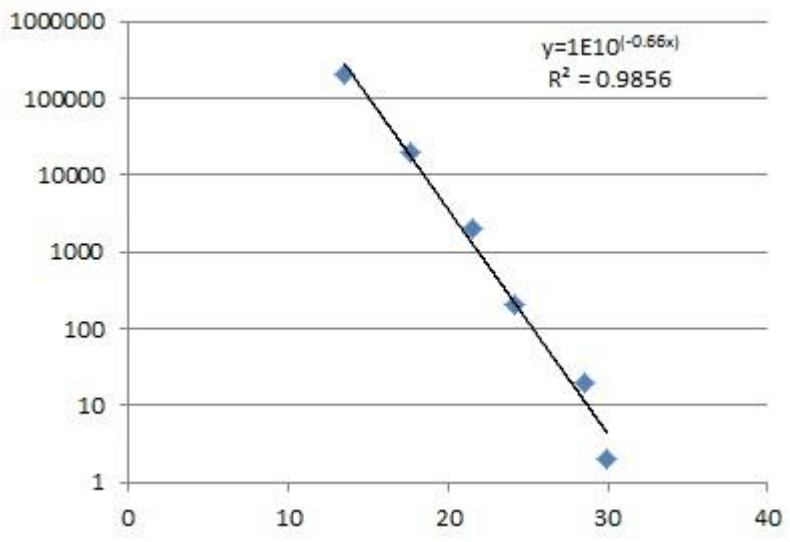

Figure 3.2: Standard curve generated by serial dilutions of the positive control

3.4.5 Positive and Negative controls

A negative control, in which the template DNA was replaced with RNAse/DNAse free water, was added to each PCR run to exclude nucleic acid contamination. A positive control, provided within the kit, was added to each run to ensure validity of negative results and was also used to set up the standard curve described above.

A run was considered valid if the positive control amplified between cycle 16 and 23 and the negative control showed no amplification or amplified after cycle 35. If the positive control was negative or if the negative control amplified before cycle 35, the run was considered invalid and the test was repeated.

3.4.6 Internal control

The PCR assay contains two sets of specific forward and reverse primers which were amplified together in a PCR reaction: one for Parvovirus B19 and one for an IC which is an exogenous source of DNA template that is spiked into the lysis buffer. The IC is used to prevent false negative results owing to inadequate extraction or amplification steps. In a Parvovirus B19 negative sample, the IC must be positive. In a Parvovirus B19 positive sample with a high VL, the IC may be negative due to competitive inhibition.

The Parvovirus B19 fluorescence is detected in the FAM channel (excitation wavelength 452-488nm, emission wavelength 505-545nm) and the IC fluorescence is detected in the VIC channel (excitation wavelength 542-582nm, emission wavelength 665-705nm).

3.4.7 PCR amplification and detection

Amplification protocols were followed as per standard procedure (Table 3.2):

Table 3.2: Amplification protocols

Step	Time (S)	Temperature (°C)
Denaturation	10	95
Data collection	60	60

PCR amplification and detection was performed on the 7900HT fast real-time PCR system (Applied Biosystems, inc. Foster city, CA). The resultant output from this instrument is Ct values, which can be used as input into the logarithmic equation $y=1E10^{(-0.66x)}$ to calculate Parvovirus B19 VL or give an indication of IC amplification.

3.4.8 PCR for the house keeping gene Beta Actin (ACTB)

A second PCR reaction (detected in the FAM channel) was performed, as a separate reaction, on all samples with specific primers and probes provided in the kit to detect an endogenous housekeeping gene ACTB (Table 5). This was used to confirm the extraction of valid biological templates and to exclude false negative results on the basis of insufficient sample input. Samples were run in the same way as described in 3.4.3 and 3.4.4 above. An ACTB standard curve was calculated and samples in which the Ct was greater than 30 were excluded, as Ct values higher than this were beyond the lower limit of detection of this assay.

Table 3.3: Reagent preparation for Beta Actin

Component	Volume (µl)
2 x precision master mix	10
Endogenous ACTB primer/probe mix	1
RNAse/DNAse free water	4
Final volume	15

3.5Statistical analysis

Inorder to accurately estimate the prevalence of Parvovirus B19 positivity in this cohort, sample size was calculated using a confidence level of 95% and a confidence interval of 5-15%. This yielded a suggested sample size of 96. A larger sample size of 170 was used to account for any technical errors or inadequate sample yield.

Data were captured in Microsoft Excel 2013 and analysed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diago, CA). Statistical comparisons were performed using the Chi-squared and Fisher's exact tests as appropriate for categorical variables and the parametric paired t-test or non-parametric Wilcoxon Matched Pairs test for continuous variables depending upon the normal distribution. The degree of linearity between the Parvovirus B19 VL levels and the laboratory tests (CD4 count, HIV VL and haemoglobin) were illustrated in a scatter plot with an appropriately fitted regression line. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and false negative rate (FNR) were calculated as necessary for serological results, presence of PRCA, presence of viral inclusions and RPI. Statistical significance was set at a $P < 0.05$.

4 RESULTS

4.1Study population

During the study period, 170 BM samples were eligible for inclusion in the study. Twenty seven samples were later excluded based on inadequate sample yield and the final cohort therefore had 143 samples from HIV infected patients with severe anaemia (Figure 4.1).

The prevalence of Parvovirus B19 in this cohort was 13.3% (19/143).

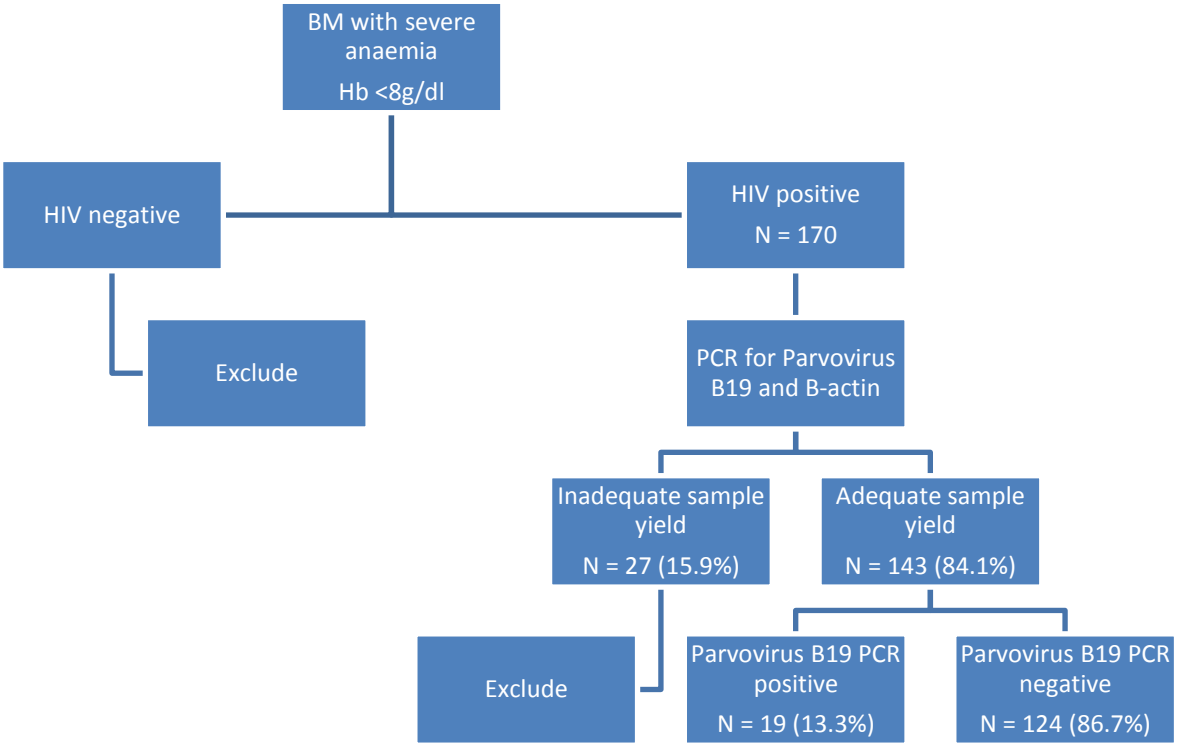

Figure 4.1: Flow diagram of sample selection

Of the 170 samples included in this study, 143 were adequate for assessment. Of these 143, 19 tested positive for Parvovirus B19 and 124 tested negative.

4.2Patient demographics and haematology findings

Demographic details, laboratory results and clinical information available from the LIS for Parvovirus B19 positive and negative groups are presented in Table 4.1 and described below. Extra information regarding the Parvovirus B19 positive group is presented in Appendix A, Table A4.

Table 4.1: Patient demographics and haematology findings- comparison of pertinent demographic, clinical and laboratory findings of Parvovirus B19 positive and negative groups

	Parvovirus B19 + (n=19)	Parvovirus B19 - (n=124)	Total (n=143)	P-value
Gender –n (%)				0.12
Male	9 (47.4)	36 (29.0)	45 (31.5)	-
Female	10 (52.6)	88 (70.9)	98 (68.5)	-
Age –mean (±SD)	29.3 (±9.9)	38.2 (±9.9)	33.76 (±9.9)	0.21
ARV experienced –n (%)	16 (84.2)	67 (54.0)	83 (58.0)	0.01*
***Mycobacterium tuberculosis* infection –n (%)**	4 (21.1)	38 (30.6)	42 (29.4)	0.59
Malignancy –n (%)	3 (15.8)	7 (5.6)	10 (7.0)	0.16
Hb -g/dl	3.7 (±1.6)	4.5 (±1.5)	4.4 (±1.6)	0.03*
MCV –fl	86.6 (±9.9)	87.7 (±12.6)	87.6 (±12.2)	0.69
MCHC -g/dl	32.0 (±3.4)	31.1 (±2.6)	31.2 (±2.7)	0.17
WCC -x10^9/l	5.5 (±3.2)	5.1 (±4.0)	5.1 (±3.9)	0.64
Neutrophil -x10^9/l	3.8 (±2.0)	3.7 (±4.1)	3.7 (±4.3)	0.56
PLT -x10^9/l	305.3 (±210.3)	175.1 (±151.7)	192.4 (±165.8)	0.00[#]
CD4 -cells/µL	88(8-806)	69(1-1424)	73 (1-1424)	0.42
HIV VL[1] -copies/ml	2564 (390-222715)	24387 (71-1000000)	12231 (71-1000000)	0.21
Undetectable HIV VL –n (%)	5 (26.3)	22 (17.7)	27 (18.9)	0.36
RPI	0.0 (±0.1)	0.3 (±0.4)	0.3 (±0.1)	0.00[#]
Ferritin -µg/l	731 (6-1898)	1343 (15-24116)	1037 (6-24116)	0.13

Data are expressed as mean (±SD) for parametric tests, median (range) for non-parametric tests and n(%) of total for categorical values.

[1]: This excludes undetectable viral loads as they were not represented by numerical values

*: Statistically significant (P <0.05)

[#]: P = 0.00 represent P-values <0.001

ARV: antiretroviral therapy, Hb: haemoglobin, HIV VL: Human Immunodeficiency virus viral load, MCHC: mean cell haemoglobin concentration, MCV: mean cell volume, PLT: platelet, RPI: reticulocyte production index, SD: standard deviation, WCC: white cell

4.2.1 Study Population Demographic

In the study population there was a predominance of females, with 68.5% (98/143) of the samples collected from female patients. Of note however, in the Parvovirus B19 positive group, the male to female ratio was ~1:1.

The mean (±standard deviation (SD)) age in the Parvovirus B19 positive and negative groups was 29.3 (±9.9) and 38.2 (±9.9) respectively (p=0.21).

4.2.2 Clinical information

A total of 58.0% (83/143) of the study population were on ARV therapy with 84.2% (16/19) of the subjects in the Parvovirus B19 positive group on ARV therapy, of which 31.3% (5/16) were virally suppressed (HIV VL <40copies/mL).

In the Parvovirus B19 positive group who were on ARV therapy, the ARV regimen was documented in 62.5% (10/16) of the patients, of which 50.0% (5/10) were on AZT and 100% (10/10) were on 3TC as part of a three-drug regimen.

4.2.3 Laboratory investigations

4.2.3.1 Full blood count

All patients in the Parvovirus B19 positive group had a grade four anaemia (Hb <6g/dl) [82].

The mean Hb was significantly lower in the Parvovirus B19 positive group compared to the Parvovirus B19 negative group (p<0.05).

There was no difference for MCV, MCHC, WCC and neutrophil count in the two groups.

The mean platelet count was significantly lower in the Parvovirus B19 negative group (p<0.05).

In the Parvovirus B19 positive group, 26.3% (5/19) had associated thrombocytopenia and 31.6% (6/19) had associated neutropenia of which 40.0% (2/5) and 66.7% (4/6) had another documented cause for the cytopenia respectively.

4.2.3.2 CD4 count and HIV viral load

The median (range) for CD4 count was 88 (8-806)cells/µl and 69 (1-1424)cells/µl in the Parvovirus B19 positive and negative groups respectively. There was no significant difference in CD4 or HIV VL in the Parvovirus B19 positive and negative groups (p= 0.42 and 0.21 respectively).

Of the Parvovirus B19 positive group, 26.3% (5/19) had HIV viral suppression and had a mean (±SD) Hb of 3.7 (±1.6) g/dl. There was no statistically significant difference between the Hb, CD4 count and Parvovirus B19 viral load in this group compared to those not virally suppressed. In addition, all of the CD4 counts in this group were less than 350 cells/µl and all had classical PRCA findings on BM analysis.

4.2.3.3 Reticulocyte production index

This test was requested on 95 of the 143 samples in this study, 15 of which were in the Parvovirus B19 positive group (defined according to the Parvovirus B19 PCR diagnostic test). The mean RPI was significantly lower in the Parvovirus B19 positive group ($p<0.05$) as compared to the Parvovirus B19 negative group. The diagnostic performance of the RPI was compared with the Parvovirus B19 PCR test by ROC analysis. The area under the curve was 0.81 with a 95% confidence interval of 0.72 to 0.89 for the RPI. The best RPI cut-off was ≤0.1, which corresponds to a sensitivity of 93.3% and a specificity of 57.5%. A single patient in the Parvovirus B19 positive group and 45 patients in the Parvovirus B19 negative group had an RPI of >0.1.

4.2.3.4 Haematinics

The median(range) ferritin level was 731 (6-1898)µg/l and 1343 (15-24116)µg/l in the Parvovirus B19 positive and negative groups respectively. This was not found to be statistically different ($p=0.13$). Anaemia of chronic inflammation (as defined in Appendix A, Table A3) was a contributing factor in 85.3% (122/143) of the specimens, with no statistically significant difference in the Parvovirus B19 positive and negative groups ($p=0.64$). Iron deficiency anaemia was present in 5.3% (1/19) and 5.7% (7/124) of the Parvovirus B19 positive and negative samples respectively. Megaloblastic anaemia secondary to deficiencies of Vitamin B12 and folate were documented in 3.7% (4/107) and 3.7% (4/109) respectively of the parvovirus B19 negative group. There was no documented megaloblastic anaemia in the Parvovirus B19 positive group.

4.2.3.5 Parvovirus B19 serology

Parvovirus B19 serology (IgM and IgG) was available in 25.9% (37/143) subjects (5 in the Parvovirus B19 positive group and 32 in the Parvovirus B19 negative group).

IgM was positive in 60% (3/5) and negative in 40% (2/5) of the Parvovirus B19 positive group with sensitivity and specificity (when using Parvovirus B19 PCR as the gold standard) of 60.0% and 100% respectively and positive and negative predictive value of 100% and 94.1% respectively. In the Parvovirus B19 negative group all IgM results were negative (32/32).

In the Parvovirus B19 positive group all IgG results were negative (5/5), while in the Parvovirus B19 negative group 31.3 % (10/32) were IgG negative and 68.8% (22/32) were IgG positive.

4.2.3.6 Correlation between viral load of Parvovirus B19 eluted from the bone marrow and haemoglobin level

There was no statistically significant correlation between the viral load of Parvovirus B19 (as a ratio to beta actin) and the degree of anaemia (r^2=0.03, p=0.46) (Figure 4.2).

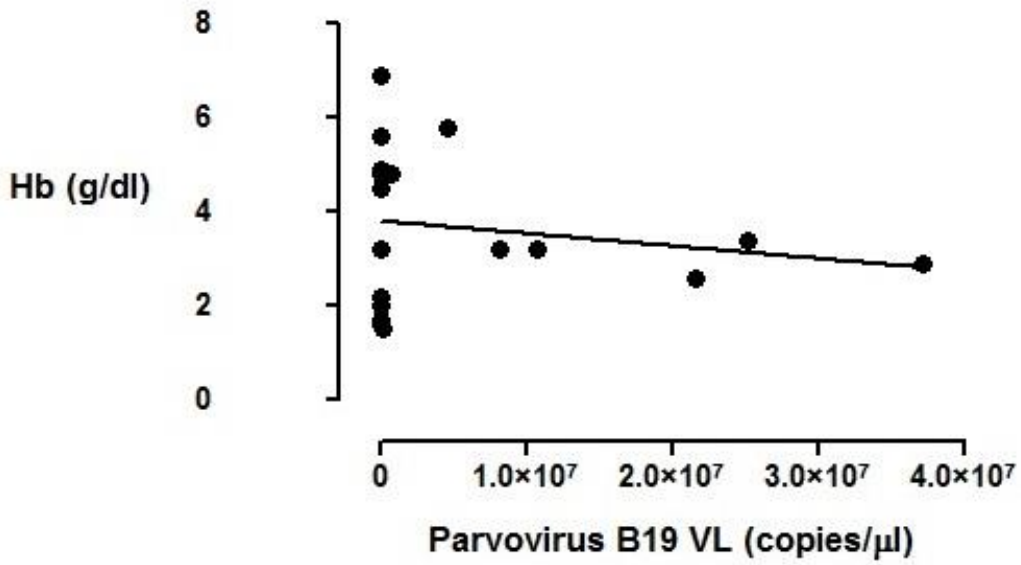

Figure 4.2: Parvovirus B19 viral load (VL) versus haemoglobin (Hb) level

4.2.3.7 Correlation between viral load of Parvovirus B19 eluted from the bone marrow and CD4 count

The Parvovirus B19 viral load (as a ratio to beta actin) was assessed in conjunction with the CD4 count with no statistically significant correlation between the two parameters (r^2=0.02, p=0.58) (Figure 4.3).

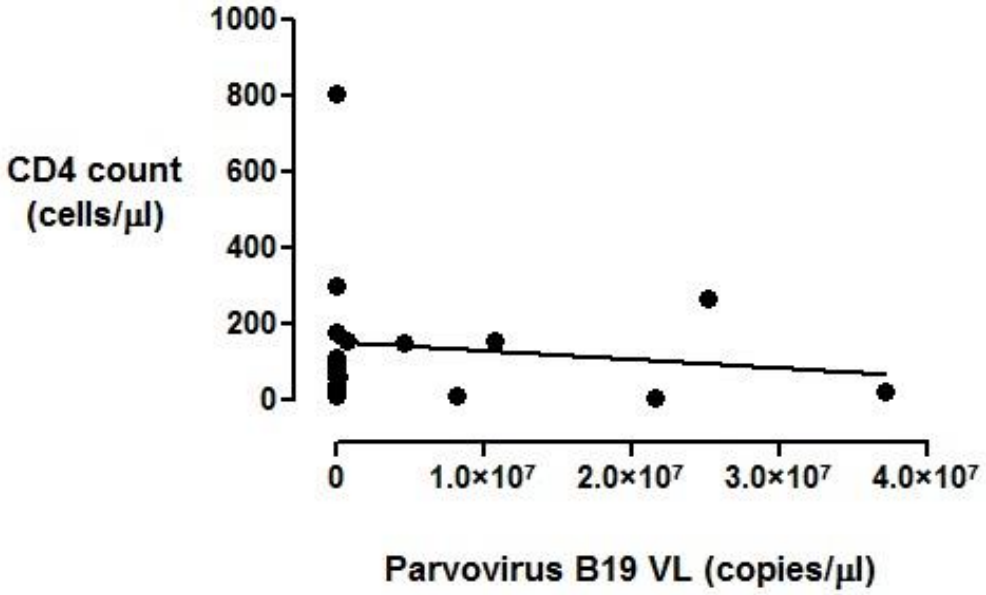

Figure 4.3: Parvovirus B19 viral load (VL) and CD4 count

4.2.3.8 CD4 count in Parvovirus B19 positive and negative samples

There was no statistically significant difference in the CD4 counts of Parvovirus B19 positive and negative samples with median (range) of 88 (8-806)cells/µl and 69 (1-1424)cells/µl respectively (p=0.84) (Figure 4.4).

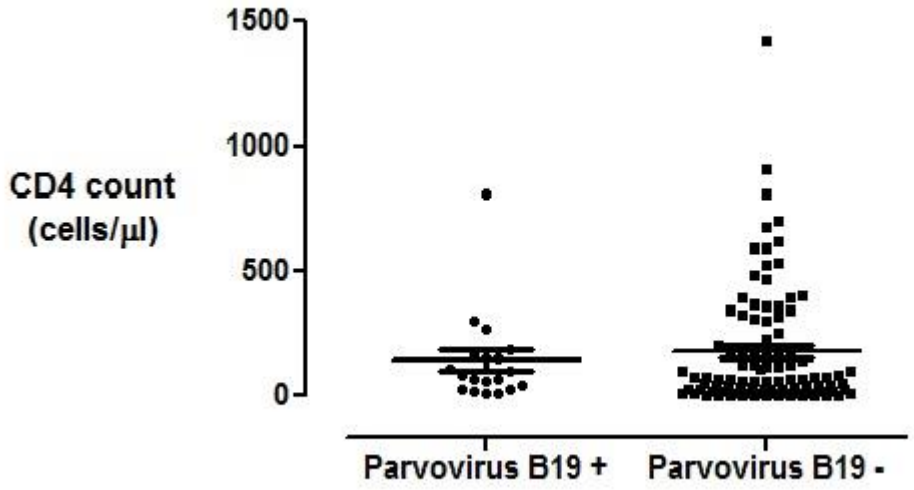

Figure 4.4: CD4 count in Parvovirus positive and negative samples

4.2.3.9 Parvovirus B19 viral load and HIV viral load

There was no statistically significant correlation between the HIV VL and the Parvovirus B19 VL (r^2=0.02, p=0.63) (Figure4.5). In addition, when the patients with suppressed HIV VLs were compared to those without HIV VL suppression, there was no statistically significant difference in the Parvovirus B19 viral load (r^2=0.03, p=0.77).

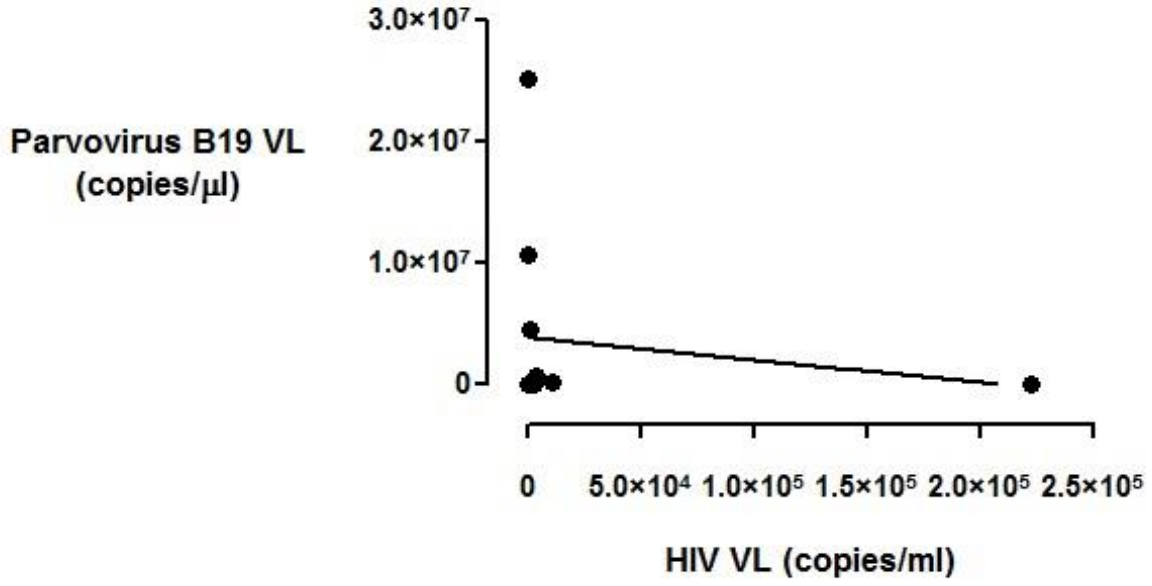

Figure 4.5: Human Immunodeficiency Virus viral load (HIV VL) and Parvovirus B19 viral load (VL)

4.2.4 Bone marrow aspirate and trephine findings

4.2.4.1 Bone marrow aspirate and trephine samples that were inadequate for morphological assessment

Of the Parvovirus B19 positive samples, 21.1% (4/19) had poor quality aspirates that were inadequate for morphological assessment and 36.8% (7/19) had poor quality trephines that were inadequate for assessment. In the Parvovirus B19 negative cohort, 18.6% (23/124) of the aspirates and 16.1% (20/124) of the trephines were of poor quality and were inadequate for morphological assessment. The presence or absence of PRCA or Parvoviral inclusions could therefore not be assessed in these samples however a molecular diagnosis was still possible.

4.2.4.2 PRCA in Parvovirus B19 positive and negative samples

In the Parvovirus B19 positive samples, PRCA was diagnosed in 47.3% (9/19) of the BMA and BMT samples, while 31.6% (6/19) of the BMA and 15.8% (3/19) of the BMT showed adequate erythropoiesis through all stages of maturation (the remainder of the samples were inadequate for

assessment). There was no statistically significant difference in the Hb (p=0.26), HIV VL (p=0.87) and Parvovirus B19 VL (p=0.77) in cases with and without PRCA.

In the Parvovirus B19 negative samples, PRCA was diagnosed in 12.1% (15/124) of the BMA and 16.1% (20/124) of the BMT samples.

In summary, a total of 20.3% (29/143) of the BM samples examined had evidence of a PRCA on either the aspirate or trephine or both, 31.0% (9/19) of which were Parvovirus B19 positive.

BMA as a diagnostic test for PRCA yielded a sensitivity and specificity of 60.0% and 85.1% respectively and a PPV and NPV of 37.5% and 93.5% respectively when using Parvovirus B19 PCR as the gold standard. Similarly, BMT as a diagnostic test for PRCA yielded a sensitivity and specificity of 75.0% and 80.8% respectively and a PPV and NPV of 31.0% and 96.6% respectively when using Parvovirus B19 PCR as the gold standard.

4.2.4.3 Parvovirus B19 inclusions

Parvovirus B19 inclusions were observed in 10.5% (2/19) of BMT of Parvovirus B19 positive samples and in none of the Parvovirus B19 negative samples, with a sensitivity and specificity (when using Parvovirus B19 PCR as the gold standard) of 15.38% and 100% respectively.

4.2.4.4 Immunohistochemical stains for Parvovirus B19

Two Parvovirus B19 positive samples also had a Parvovirus immunohistochemical stain performed on the BMT (as a routine test), both of which were positive. Both of these samples had evidence of PRCA on BMT.

4.2.4.5 Morphologic evidence of other pathology

Additional causes of anaemia were found in a subset of Parvovirus B19 positive patients (Table 4.2). A total of 29.4% (42/143) had documented evidence of *Mycobacterium Tuberculosis* infection (granulomata and acid fast bacilli on Ziehl-Neelsen stain). Of the Parvovirus B19 positive samples, 21.1% (4/19) had documented evidence of co-existing *Mycobacterium Tuberculosis* infection. There

was no statistical difference in the prevalence of *Mycobacterium Tuberculosis* infection between the Parvovirus B19 positive and negative samples (p=0.58).

In the Parvovirus B19 positive group, 15.8% (3/19) had bone marrow involvement by a malignancy including Kaposi Sarcoma (KS) (2/19) and Diffuse large B cell lymphoma(DLBCL) (1/19). In the Parvovirus B19 negative group, 5.6% (7/124) had evidence of a malignancy including KS (3/124), Hodgkin Lymphoma (2/124) and carcinoma of the breast (2/124). There was no statistical difference in the prevalence of malignancies in the Parvovirus B19 positive and negative groups (p=0.16). Chemotherapy treatment status of both groups of patients was not known.

Table 4.2: Alternate causes of cytopenias in Parvovirus B19 positive patients

Alternate cause of anaemia	Number (%) of samples
Mycobacterium Tuberculosis	4 (21.05)
Kaposi sarcoma	2 (10.53)
Diffuse large B-cell lymphoma	1 (5.26)
Iron deficiency	1 (5.26)

4.2.5 Parvovirus B19 PCR requested as routine investigation by clinician/pathologist

Of the 143 samples assessed a total of 69.2% (99/143) did not have routine Parvovirus B19 PCR testing, these samples had Parvovirus B19 PCR as part of this study. Of note, 31.6% (6/19) of the samples that were found to be Parvovirus B19 positive in this study, were not routine requests. These six samples have a mean (±SD) Hb of 4.2 ±(1.80)g/dl and median CD4 count of 74 (14-806) cells/µl which were not statistically different from the Parvovirus B19 positive samples that were routinely requested (p=0.21 and p=0.66 respectively). The 13 Parvovirus B19 positive samples that were requested as a routine investigation by either the clinician (30.8%) or the pathologist (69.2%) were prompted by clinical or morphological/histological findings respectively.

5 DISCUSSION

5.1Prevalence of Parvovirus B19 in this cohort

This is the first documented study, to our knowledge, showing the prevalence of Parvovirus B19 in HIV infected patients with severe anaemia in South Africa.

The prevalence of Parvovirus B19 in this study, at 13.3% is much higher than what was expected compared to a study with similar inclusion criteria reporting a prevalence of 3.4% [57, 78]. This may be because in the South African setting patients often present later to health care institutions and have more immune suppression predisposing them to opportunistic infections such as Parvovirus B19.

In the Parvovirus B19 positive group, the male to female ratio was equal, however in the total study population there was a predominance of females. This is in keeping with findings seen in health care services in South Africa [85] and perhaps reflects different health seeking behaviour of the different genders.

31.6% (6/19) of the samples positive for Parvovirus B19 were not performed as a routine request by the attending clinician. These six patients who were not diagnosed with Parvovirus B19 at the time of their hospital admission were tested as part of the study only. On review, the aspirate and trephine findings of these cases revealed atypical findings, namely adequate erythroid maturation in contrast to an erythroid maturation arrest. Giant pronormoblasts and viral inclusions were not reported. These six cases highlight the fact that Parvovirus B19 is not exclusively associated with a classical PRCA. Parvovirus B19 specifically targets the erythroid lineage in the bone marrow, resulting in an isolated anaemia. Parvovirus however also causes immune mediated destruction of neutrophils and platelets and can present with neutropenia or thrombocytopenia. Although this is less commonly seen, parvovirus testing should also be considered in this clinical setting. Parvovirus B19

infection in this cohort is therefore being under-diagnosed. This diagnosis is extremely important as it may cause severe, life threatening cytopenias which are treatable and curable.

5.2 The association between Parvovirus B19 VL and the degree of anaemia and HIV immune suppression

5.2.1 Association between Parvovirus B19 VL and anaemia

Although the Parvovirus B19 positive group had a statistically significant lower Hb than the Parvovirus B19 negative group, there was no correlation between Parvovirus B19 viral load and degree of anaemia suggesting that Hb level cannot be used to assess the severity of the Parvovirus B19 infection. This may be due to the effect of other contributors to the Hb level, such as baseline Hb level, nutritional status, co-existent causes of anaemia and when in the course of the Parvovirus B19 infection the test was performed.

5.2.2 Association between Parvovirus B19 VL and CD4 count

After exposure to Parvovirus B19, an adequate humoral immune response is required to prevent chronic infection [43]. Persistent infection occurs when protective antibodies are either not produced or are non-functional such as in HIV infection and other immunocompromised states [27] . In this setting the CD4 count, as a measure of immune response, is likely to be suppressed.

PRCA secondary to parvovirus B19 has been described to be associated with very low CD4 levels, ranging from 24 – 42 cells/µL [19, 20], however this study noted a higher median CD4 count of 88(8-806) cells/µL in the Parvovirus B19 positive patients. In agreement with the findings in previous studies [20, 21], this study did not show a statistically significant difference in the CD4 counts in the Parvovirus B19 positive and negative groups.

Possible reasons for the difference in median CD4 counts in the different study groups as well as lack of statistical significance between the Parvovirus B19 positive and negative groups include: CD4 count not being an accurate reflection of immune status, in that a high CD4 count may still be associated with immune dysregulation and a low CD4 count may be found in the setting of adequate HIV viral suppression.

This study shows that there is no association between CD4 count and Parvovirus B19 status.

5.2.3 Association between Parvovirus B19 VL and HIV VL

The HIV VL in an HIV infected person is a quantitative measure of the viraemia and is used clinically as a surrogate marker of immune status [14]. A high HIV VL is associated with severe immune suppression and an increased risk of development of, or persistence of, diseases that use immune dysregulation as part of their pathogenesis for example opportunistic infections including *Mycobacterium Tuberculosis* and pneumocystis jerovici as well as malignancies such as DLBCL and KS. An undetectable HIV VL is generally associated with immune reconstitution [14].

This is the basis of using ARV therapy in the treatment of many illnesses associated with HIV including Parvovirus B19. Elsewhere it has been shown a 70% response rate from ARV therapy alone and a 90% response rate with the addition of blood transfusion [46, 47, 51, 78]. In South Africa, results are not as promising with patients requiring not only ARV therapy but also other supportive and specific therapy [81]. Speculative reasons for this include the fact that this patient population often present later in the course of their disease with more severe immune suppression and more co-morbidities [77].

The proportion of total patients on ARV therapy in this study (58.0%) is comparable to national findings, as is the proportion on treatment who are virally suppressed (31.3%) [85].

In this study, 26.3% (5/19) of the patients with positive Parvovirus B19 PCR and a documented HIV VL were on ARVs and had undetectable HIV VLs, however they still had severe anaemia with a Hb mean (±SD) of 3.72(±1.55) and persistent Parvovirus B19 infection. This suggests that the HIV VL is not a reliable marker for prediction of development of PRCA after exposure to Parvovirus B19 infection in HIV positive patients and therefore there is likely no association between Parvovirus B19 VL and HIV VL. It is important to note, however, that the length of time of HIV viral suppression (and therefore the level of immune reconstitution) was not known in these patients. These findings may differ in patients with long-term HIV viral suppression who, in theory, are able to mount a sufficient immune response to destroy the Parvovirus B19 infection.

There was no statistically significant difference between the Hb level and Parvovirus B19 viral load in the group with HIV viral suppression compared to those not virally suppressed and all showed classic features of PRCA on BM. Of note however, all of the CD4 counts were less than 350 cells/µl, possibly indicating that although viral suppression had been achieved, this has not yet translated into immune reconstitution. This highlights the importance of achieving adequate immune reconstitution to prevent and manage Parvovirus B19 infection in this setting.

5.3 Clinical utility of laboratory parameters to distinguish Parvovirus B19 positivity

5.3.1 Parvovirus quantification

This study reported both a qualitative and quantitative Parvovirus B19 result. The qualitative result reflected the prevalence of Parvovirus B19 infection in this cohort. The quantitative result was analysed in conjunction with the CD4 count, HIV VL and Hb level and there was no correlation found between VL of Parvovirus B19 infection and degree of anaemia, CD4 count or HIV VL suggesting quantification of Parvovirus B19 VL is of no clinical value.

Routine testing in our laboratory, and in other laboratories internationally using the same assay, only report a qualitative result. For accurate semi-quantitative results, a housekeeping gene must be added to each sample. This increases cost, labour and turn-around time. In addition, primers and probes for this gene are not routinely supplied in the kit.

Perhaps a semi-quantitative reporting may have a role in monitoring of response therapy in a patient with persistent anaemia, with a decrease in Parvovirus B19 VL suggesting disease eradication and an alternate cause of the persistent anaemia. A negative result on a qualitative assay however, would likely be just as valuable. In summary, this study suggests that for routine testing and management, a qualitative Parvovirus B19 result will suffice.

5.3.2 Contributing factors to anaemia in this cohort

Anaemia is most often multifactorial in the setting of HIV infection as was found in this study where anaemia of chronic inflammation, iron deficiency and megaloblastic anaemia contributed in 85.3%, 5.26% and 3.7% respectively.

5.3.2.1 Parvovirus B19 positivity and co-existing causes of anaemia

There was a statistically significant difference in the Hb levels of the Parvovirus B19 positive and negative groups, with all Parvovirus B19 positive samples having grade 4 anaemia (Hb <6g/dl) [82]. In addition, an important finding of this study was the presence of more than one pathology in a significant proportion of Parvovirus B19 positive samples 42.1% (8/19).

In the Parvovirus B19 positive group, 21.1% (4/19) had *Mycobacterium Tuberculosis* infection, 5.3% (1/19) had iron deficiency and 21.1% (3/19) had a malignancy which included DLBCL and KS, all of which could have contributed to the anaemia. Although this study lacks comparison with a control group without severe anaemia, the above findings suggest that in the correct clinical setting, testing for Parvovirus B19 infection should be considered even when the severe anaemia may be explained by another pathology.

5.3.2.2 Anaemia and associated cytopenias

Classically, Parvovirus B19 infection is associated with an isolated severe anaemia. In this study the mean PLT and neutrophil counts (3.85±2.03) fell within the laboratory's normal reference ranges in the Parvovirus B19 positive group, indicating that the majority of these patients had an isolated severe anaemia. In contrast, the Parvovirus B19 negative group had a significantly lower PLT count albeit that the mean cell count was still within the laboratory's normal reference range. This is perhaps a reflection on the different underlying causes of anaemia in the two groups. While Parvovirus B19 most often results in an isolated anaemia [27], some of the other causes of anaemia in HIV infection are more likely to affect other haemopoietic cell lines too, with more patients in Parvovirus B19 negative group presenting with a thrombocytopenia.

Concurrent autoimmune mediated destruction of neutrophils and platelets by Parvovirus B19, resulting in a neutropenia and thrombocytopenia have also been described [48, 66, 67]. Thrombocytopenia and neutropenia were seen in a proportion of the Parvovirus B19 positive samples (26.3% and 31.6% respectively) however 40.0% and 66.7% respectively were in the setting of additional causes of cytopenias. In this setting the anaemia may be multifactorial and be caused by both pathologies present while the other cytopenias may be unrelated to the Parvovirus B19 infection *i.e.* secondary to marrow infiltration by *Mycobacterium Tuberculosis* or chemotherapy for malignancy. It is difficult however to confirm with any certainty which is the cause of these cytopenias.

5.3.3 Reticulocyte production index

Perhaps the most consistent finding in all Parvovirus B19 positive samples was the significantly low RPI level, with a mean (±SD): 0.04 (±0.08) ($p<0.05$), in keeping with the pathogenesis of Parvovirus B19 infection. Importantly, the RPI level was independent of the morphology findings of the absence or presence of PRCA.

The RPI had NPV of 97.8% in this study. This suggests that an RPI should be performed as a first line investigation for anaemia secondary to Parvovirus B19. Parvovirus B19 PCR should be performed as a second line investigation if the RPI result is low in view of the PPV of 22.01%. Currently in this clinical setting the RPI is not routinely being performed as a screening test. In this study a RPI was requested in 66.4% (95/143).An RPI should be requested as part of the initial work up of all patients with severe anaemia. It is important to educate clinicians about the predictive value of this inexpensive test. In contrast to a Parvovirus B19 PCR which is performed on BM, an RPI is performed on peripheral blood. Further it gives important information about the aetiology of anaemia.

5.3.4 Parvovirus B19 PCR and serology (IgM) testing

Parvovirus B19 serology (IgM) tests the presence of a humoral response to Parvovirus B19 infection, while Parvovirus B19 PCR tests for the presence of viral DNA.

In immunodeficient states such as HIV infection, antibodies to Parvovirus B19 may be non-functional or absent [43] which may explain the poor sensitivity of Parvovirus B19 serology (IgM) in this cohort (60.0%). Serology (IgM) should therefore not be used as a screening tool due to its high false negative rate.

It is important for clinicians to understand the pathogenesis of Parvovirus B19 infection in HIV infected persons so that the correct testing will be requested. Unlike in the immunologically competent state in which IgM negativity correlates with absence of current infection, serological testing for acute infection of Parvovirus B19 has limited usefulness in the presence of HIV infection when the humoral response may be suppressed.

5.4Bone marrow morphology as a diagnostic tool for Parvovirus B19 infection

5.4.1 Bone marrow aspirate and trephine findings

Classically the bone marrow aspirate and trephine findings in Parvovirus B19 infection are described as a PRCA with absent/markedly reduced erythropoiesis and giant pronormoblasts [43]. Occasionally erythroid viral inclusions can be appreciated on the trephine biopsy [26].

In this study the presence of a PRCA on BMA and BMT showed a sensitivity of 60.0% and 75.0% respectively and specificity of 85.1% and 80.8% respectively for the molecular confirmation of Parvovirus B19 infection. These findings are comparable to a previous study which found 45.5% of the PRCA BM samples to be associated with Parvovirus B19 infection [75]. The low PPV (37.5% and 31.0% respectively for BMA and BMT) shows that there are other causes of a PRCA which need to be considered such as HIV itself, EBV, viral hepatitis, drugs (including AZT, 3TC, INH and sulphonamides), autoimmune diseases, pregnancy and haematological malignancies.

In addition, while not all cases with PRCA were Parvovirus B19 positive, not all Parvovirus B19 positive samples showed evidence of PRCA on BM. As previously mentioned, 31.6% (6/19) were

confirmed positive despite showing adequate erythropoiesis through all stages of maturation. These are important findings because morphologic analysis is currently used as a screening tool for Parvovirus B19, however if Parvovirus B19 PCR testing is only requested by the pathologist when classical features of PRCA are seen, up to one third of cases will be missed.

In the Parvovirus B19 positive group, the morphological findings of the presence or absence of PRCA were independent of the severity of the Parvovirus B19 infection (as assessed by Parvovirus B19 VL), severity of the HIV infection (as assessed by HIV VL) and degree of the anaemia.

This study shows that in HIV infected patients with severe anaemia, PRCA on BM is not sensitive enough as a screening tool for Parvovirus B19 infection, nor does the absence of PRCA in the setting of Parvovirus B19 infection confer a lower viral burden.

5.4.2 Viral inclusions on trephine biopsy and Parvovirus B19 positivity

The presence of Parvovirus B19 inclusions in the erythrocytes on trephine biopsy may be appreciated in Parvovirus B19 infection. This was observed in 10.5% (2/19) of the Parvovirus B19 positive samples, with a high FNR of 89.5%. However, the presence of viral inclusions was highly specific (100%) for the diagnosis of Parvovirus B19 infection on PCR testing. It is important then for the pathologist to know that the presence of these may help to confirm a diagnosis but that their absence should not prevent further Parvovirus B19 testing in the correct clinical and laboratory context.

5.4.3 Diagnosis of Parvovirus B19 infection in suboptimal bone marrow specimens

The PrimerDesign™ genesig® assay for Human Parvovirus B19 (Southampton, United Kingdom) used in this study may be used on both peripheral blood and bone marrow aspirate samples. This is beneficial even in aparticulate and haemodilute bone marrow samples in which morphological examination is not able to contribute to assessing the cause of the anaemia.

Of the samples in which the diagnosis of Parvovirus B19 infection was made, 21.0% (4/19) of the bone marrow aspirates and 36.8% (7/19) of the bone marrow trephines were inadequate for assessment. This highlights the importance of Parvovirus B19 molecular diagnosis in this cohort of patients even when the bone marrow aspirate and trephine morphology/histology is not contributory.

5.5Limitations

This is a retrospective study and some data were therefore missing from the LIS, as not every parameter assessed had been requested by the clinician for each sample. There was no ethical clearance for access to hospital and clinic notes and there was no contact with treating clinicians. These factors limited the interpretation of the data produced.

As this was a retrospective study, there was no opportunity to repeat a suboptimal BM to assess for evidence of PRCA or Parvovirus B19 inclusions nor was it possible to test follow up samples to assess the trend in Parvovirus B19 VL. Although immunohistochemical staining for Parvovirus B19 had excellent PPV, it was only performed on two samples.

A proportion of samples that should have been included had to be excluded due to lack of adequate BM tissue for PCR testing.

There is possible sample selection bias, as only samples from Johannesburg were used, and only from academic hospitals. In addition, if patients fulfilled criteria for study inclusion but their clinician did not perform a BM, they were not included.

This study only looked at the prevalence of Parvovirus B19 infection in HIV infected patients with severe anaemia, with no testing of HIV infected patients without severe anaemia. The overall prevalence of Parvovirus B19 infection in the setting of HIV infection was therefore not established.

HIV infection was an inclusion criteria for this study. As such, the prevalence of Parvovirus B19 in HIV uninfected patients with severe anaemia was not established.

This study looks specifically at molecular testing for Parvovirus B19 and therefore comparison to other available tests, such as serology, is very valuable. Due to the retrospective nature of this study, not all samples had serology testing performed, hindering diagnostic interpretation.

5.6Recommendations

Further improvements on this study may include:

- Assessing a cohort of patients on a prospective basis which will allow for obtaining all laboratory tests which will be of value upfront, as well as ensuring quality BM samples are obtained. Full clinical details will be available and patients can be followed longitudinally to assess response to treatment and patient outcome. In particular serology (IgM and IgG) testing could be included on all patients to guide diagnostic algorithms.
- Although the sample size was sufficient for a pilot study and many interesting observations were made, a larger study would be of value in increasing the sample size of specific subgroups, such as those patients with undetectable HIV VL, those without PRCA on BM morphology and those patients with malignancy or *Mycobacterium Tuberculosis* infection.
- A study comparing the Parvovirus B19 VL of BM and peripheral blood samples taken at the same time will be useful to understand if these yield similar enough results to be used interchangeably.
- Parvovirus B19 PCR testing of HIV infected patients regardless of their Hb level would be of value to establish the overall prevalence in this group, as well as comparing the Parvovirus B19 VL in the anaemic versus non-anaemic groups.
- The prevalence of Parvovirus B19 infection in the setting of severe anaemia regardless of HIV status will be of value. Comparison of the prevalence of Parvovirus B19 infection in HIV infected compared to HIV uninfected patients with severe anaemia would be useful in confirming the proposed pathogenesis of anaemia due to Parvovirus B19 in the setting of immune suppression.

6 CONCLUSION

This is the first documented study showing the prevalence of Parvovirus B19 in HIV infected patients with severe anaemia in South Africa. The prevalence of Parvovirus B19 infection in this cohort of adult patients with HIV infection and severe anaemia was 13.3%. This is higher than reported in other cohorts in the developing world. This highlights the value of diagnostic testing for Parvovirus B19 infection.

An important finding of this study is the diagnostic sensitivity and specificity of the assays for the diagnosis of Parvovirus B19. In the setting of HIV infection, it is imperative that correct testing is performed in order to avoid false negative results. This studied confirmed the poor sensitivity of serology (IgM) owing to the absence or ineffective production of antibodies in the setting of immune suppression associated with HIV infection. This study also showed that an undetectable HIV viral load, at which time one would assume sufficient immune reconstitution, may still be associated with acute Parvovirus B19 infection and testing should still be considered in this subgroup of patients. For accurate diagnosis of Parvovirus B19 infection, PCR testing is required which is expensive and not widely available. Clinical acumen is therefore required to offer the best service to the patient in conjunction with the best use of available resources.

In developing countries with limited resources, the RPI is a useful screening test for HIV infected patients with anaemia. In this study, the RPI yielded an excellent NPV. In contrast to PCR, this test is cost-effective and widely available. Based on the findings of this study a diagnostic algorithm has been proposed. In patients with grade four anaemia and a reduced RPI, PCR testing for Parvovirus B19 infection is recommended. This is regardless of the clinical setting and morphology findings of BMA and BMT.

This study illustrated that Parvovirus B19 infection may occur in the absence of the classical features of PRCA on aspirate or trephine and in the presence of additional cytopenias namely a thrombocytopenia and neutropenia. Further, it can be diagnosed in the setting of, or in association with, other known causes of anaemia. The most common in this study included iron deficiency, *Mycobacterium Tuberculosis* infection and malignancies such as KS and DLBCL.

In South Africa Parvovirus B19 testing and treatment are widely available. Treatment is often extremely effective, and thus it is imperative that this diagnosis is not missed. In conclusion, the findings of this study are important clinically, as Parvovirus B19 is a reversible and curable cause of severe anaemia in the setting of HIV infection. Insights into its prevalence and diagnosis can ultimately improve outcomes of patients in the South African setting. The results of this study contribute significantly to the current body of knowledge in this field and have the ability to change clinical practice.

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7 APPENDIX A

Table A1: Data information sheet containing data collected for Parvovirus B19 positive and negative samples

Parameter	Unit
Gender	Male/Female
Hb	g/dL
MCV	fL
MCHC	g/dL
WCC	$X10^9$/L
Neutrophil count	$X10^9$/L
CD4 count	Cells/µl
HIV VL	Copies/mL
RPI	N/A
Ferritin	µg/L
Vitamin B12	nL
Folate	µg/L
Parvovirus B19 IgM serology	+/-
Parvovirus B19 IgG serology	+/-
PRCA on BMA	+/-
PRCA on BMT	+/-
Viral inclusions on BMT	+/-
ARV therapy	+/-
Which ARVs	List
Evidence of *Mycobacterium Tuberculosis*	+/-
Evidence of malignancy	+/-
Was PCR performed as routine request	+/-

ARV: antiretroviral therapy, BMA: bone marrow aspirate, BMT: bone marrow trephine, Hb: Haemoglobin, HIV VL: human immunodeficiency viral load, MCV: Mean cell volume, MCHC: Mean cell haemoglobin concentration, PCR: polymerase chain reaction, PRCA: pure red cell aplasia, RPI: reticulocyte production index, WCC: white cell count

Table A2: Reference ranges[65] used for parameters assessed in this study

	Reference range male	Reference range female	Units
WCC	3.92-10.40	3.90-12.60	$x10^9$/l
Hb	13.4-17.5	11.6-16.4	g/dl
MCV	83.1-101.6	78.9-98.5	Fl
MCHC	33.00-35.00	32.7-34.9	g/dl
PLT	171-388	186-454	$x10^9$/l
Neutrophil	1.6-6.98	1.6-8.3	$x10^9$/l
Ferritin	15.00-150.00	15.00-150.00	µg/l
Vitamin B12	145.00-637.00	145.00-637.00	pmol/l
RBC folate	924.00-3337.00	924.00-3337.00	nmol/l

Hb: haemoglobin, MCV: mean cell volume, MCHC: mean cell haemoglobin concentration, PLT: platelet, RBC: red blood cell, WCC: white cell count

Table A3: Definitions of terms used in the text

Term	Definition
HIV infected	Positive ELISA or PCR[86]
Severe anaemia	Hb< or = 8 g/dl[82]
Thrombocytopenia	Platelet count <171 $x10^9$/l[65]
Neutropenia	Neutrophil count <1.6 $x10^9$/l[65]
PRCA as seen on morphologic assessment	Absent or markedly reduced erythropoiesis and a lack of serial maturation of erythrocytes on BMA/BMT[68]
Megaloblastic anaemia	Vitamin B12 or red blood cell folate level below the given reference range in conjunction with an anaemia[82]
Iron deficiency	Ferritin level below the given reference range or absent iron stores on a Perl's stain (in an adequate quality bone marrow aspirate)[68]
Anaemia of chronic disease	Increased ferritin, usually in association with decreased transferrin and mild normocytic or microcytic anaemia[7]
HIV viral suppression	HIV VL <40 copies/ml
Sensitivity	True positives/(true positives + false negatives)
Specificity	True negatives/(true negatives + false positives)
Negative predictive value	True negatives/(true negatives + false negatives)
Positive predictive value	True positives/(true positives + false positives)
False negative rate	False negative/(false negative + true positive)

BMA: bone marrow aspirate, BMT: bone marrow trephine, ELISA: enzyme linked immunosorbent assay, Hb: haemoglobin, HIV VL: Human Immunodeficiency Virus VL, PCR: polymerase chain reaction, PRCA: pure red cell aplasia

Table A4: Parvovirus B19 positive data information

Sample	PV VL (copies/µl)	Hb (g/dl)	CD4 (cells/µl)	RPI	BMA PRCA	BMT PRCA	Viral inclusions
1	37 122 276	2.9	23	0.0	YES	YES	YES
2	25 200 000	3.4	268	0.0	YES	YES	NO
3	21 500 000	2.6	8	0.3	NO	NR	NO
4	10 700 000	3.2	154	0.0	NR	YES	NO
5	8 088 212	3.2	11	0.0	YES	NR	NR
6	4 581 903	5.8	152	0.0	NR	YES	NO
7	657 723	4.8	157	0.0	YES	NR	NR
8	199 742	1.5	61	0.0	YES	NR	NR
9	50 415	1.7	103	0.0	NR	YES	NO
10	32 333	2.1	41	0.1	YES	NR	NR
11	7 602	5.6	64	0.0	YES	YES	YES
12	585	2.2	299	0.1	NR	YES	NO
13	500	4.5	111	0.0	YES	NR	NR
14	173	4.8	72	0.	YES	YES	NO
15	124	6.9	806	NR	NO	NO	NO
16	84	4.9	27	NR	NO	NO	NO
17	40	1.6	181	NR	NO	NR	NR
18	26	4.5	14	0.1	NO	NO	NO
19	15	3.2	88	NR	NO	YES	NO

ACTB: beta actin, BMA: bone marrow aspirate, BMT: bone marrow trephine, Hb: haemoglobin, NR: no result (inadequate for assessment), PRCA: pure red cell aplasia, PV VL: parvovirus B19 viral load

Table A5: Turn it in report

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