DEVELOPMENT AND APPLICATION OF NESTED PCR FOR THE DETECTION OF PARVOVIRUS B19 DNA

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DEDICATION

This thesis is dedicated to my parents
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continuous support and encouragement.

ABSTRACT

The development and characterisation of nested polymerase chain reaction (PCR) for the detection of human parvovirus B19 DNA is described. Two sets of oligonucleotide primers were used, one set have been described previously and a novel set were developed. The PCR reaction conditions were optimised for magnesium concentration, oligonucleotide primer concentration, extension time, reaction buffer and the serum treatment used for extraction of B19 DNA. The assay was characterised with a series of diagnostic sera validated by dot blot hybridisation for B19 DNA and by class specific capture radio-immunoassays for the detection of B19 IgM and IgG. The PCR assay was also applied to serial sera, throat swabs and peripheral mononuclear cells (PBMNC) collected during an outbreak of parvovirus B19. B19 DNA was detectable in the throat swabs and in the PBMNC fraction of whole blood. *In situ* hybridisation and immunolabelling were used in an attempt to determine the site of B19 DNA within the PBMNC fraction.

B19 PCR was then applied to a number of clinical situations where the virus may play a role but where existing diagnostic tests are inadequate or require invasive procedures. PCR was used for the potential detection of B19 infection in immunocompromised patients in whom antibody responses are impaired and in anaemic malaria infected patients in whom hypergammaglobulinaemia may interfere with the detection of B19 specific antibodies. PCR was also used in the neonatal situation where little or no B19 IgM is produced and for the non-invasive diagnosis of fetal infection which currently requires fetal blood sampling. The PCR assay was also used to explore the role of B19 virus in patients with a variety of arthritic conditions. Finally, B19 PCR was applied to the detection of B19 DNA in clotting factor concentrates.

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CHAPTER 1

INTRODUCTION

1.1 PARVOVIRUSES

1.1.1 Classification

The *Parvoviridae* (*parvo* meaning small) are a family of very small DNA viruses. There are three genera within the family, Dependoviruses (previously called adeno-associated viruses or AAVs), Densoviruses and Parvoviruses. All three genera share a similar genome organisation (Berns 1990). Densoviruses infect insects while both Dependoviruses and Parvoviruses infect mammals, including humans. However, Dependoviruses have not been shown to cause disease in man.

The small amount of DNA contained in the parvoviruses does not carry sufficient genetic information to code for their replication requirements in host cells. Parvoviruses are able to replicate by using factor(s) contributed by a helper virus or the host cell. The Dependoviruses require a helper virus, such as an adenovirus or herpes virus (Buller *et al* 1981; Bauer & Monreal 1986). Viruses of the genus Parvovirus are autonomous and these are able to replicate because certain cellular helper functions are transiently provided by cells during the late S or early G-2 phase of replication (Tattersall 1972).

Since its discovery the human autonomous parvovirus has been variously known as human parvovirus-like agent, serum parvovirus-like virus and B19. The Study Group on Parvoviridae of the International Committee for the Taxonomy of Viruses has recommended the name B19 virus (Siegl *et al* 1985) and this name is now used

exclusively.

Parvovirus-like particles have also been observed by electron microscopy in faeces (Paver *et al* 1973). These particles have not been characterised and so their classification will await definitive molecular studies. The pathogenic potential of these parvovirus-like particles is uncertain.

1.1.2 History

B19 virus was discovered in 1974 by Cossart and colleagues (Cossart *et al* 1975) during an evaluation of tests for hepatitis B surface antigen (HBsAg). A small number of sera were reactive in a countercurrentimmunoelectrophoresis (CIE) test using polyclonal human sera but unreactive in a reverse passive haemagglutination assay using a monoclonal antiserum. The polyclonal antisera was shown to be detecting an antigen distinct from HBsAg. Electron microscopy revealed virus particles with a diameter of 23nm and morphology and buoyant density characteristic of parvoviruses.

1.1.3 Structure

The B19 virus is a non-enveloped, icosahedral virus with a range of particle diameters from 20-25nm, mean 23nm (Cossart *et al* 1975). The mean density in caesium chloride is 1.43 (Clewley 1984). The virus capsid compromises at least two structural proteins, VP-1 and VP-2, with molecular weights of 83 kd and 58 kd respectively (Cotmore *et al* 1986; Young & Ozawa 1988). VP-2 constitutes 96% of the virion mass. The virus is very hardy: viral infectivity is resistant to ether, chloroform,

DNase, and RNase treatment.

There is a single antigenic type of B19. Infection appears to be followed by lifelong immunity indicating a single neutralisable type although secondary B19 infection has been described in an immunocompromised child (Pillay *et al* 1991). Limited studies using an immunodiffusion assay have revealed antigenically identical viruses from different clinical situations (Serjeant *et al* 1981). Studies with mouse monoclonal antibodies have revealed minor variations in binding with different isolates (Cohen 1988).

1.1.4 Genome organisation & replication

The genome of B19 is positive single stranded DNA, 5.5 kilobases in length (Shade *et al* 1986). The 5' half of the genome encodes at least one non-structural protein, NS-1, and the 3' half encodes the structural proteins. Because of the small genome size, proteins are encoded in overlapping reading frames, VP-1 being identical to VP-2 except for an additional 227 amino acids at the amino terminus.

Both the 5' and 3' termini contain palindromic sequences (Astell & Blundell 1989) that fold into hairpin duplexes. The 5' sequences ensure the integrity of the DNA for DNA polymerase priming. B19 has several theoretically possible promotor sites although only that at p6 at the left end of the genome is functional (Shade *et al* 1986). The promotor is up regulated by NS-1 (Doerig *et al* 1989).

The autonomous parvoviruses are highly host and tissue specific. For viral replication to occur the cell must be in the S phase of mitosis. This limits the range of cells permissive for B19 replication. B19 has been shown to productively infect

erthryoid precursor cells, burst forming units(BFU) (Mortimer *et al* 1983a; Young *et al* 1984; Potter *et al* 1987). The NS protein appears to be lethal to BFU cells (Ozawa *et al* 1988). The virus may also infect fetal cardiac cells (Porter *et al* 1990).

B19 virus replicates in the nucleus of infected cells. B19 DNA synthesis is assumed to be similar to that of other automonous parvoviruses and proceeds by a single-strand displacement mechanism (Berns 1990). This involves the formation of double-stranded replicative intermediates. Progeny DNA is formed by strand displacement and is dependent on some function(s) found only in the late S phase of the cell cycle. Single positive and negative strands are synthesised in equal numbers and packaged separately within newly synthesised capsids.

1.1.4(a) Genomic variation

Restriction endonuclease analysis of many isolates collected between 1972 and 1984 indicates that the genome is relatively invariate (Morinet *et al* 1986; Mori *et al* 1987; Umene & Nunoue 1990). This is borne out by the observation that oligonucleotide sequences from all regions of the genome, (excluding the hairpin termini), selected for use in the polymerase chain reaction assay, amplify various isolates of B19 (Frickhofen & Young 1990). Such stability is a known property of DNA genomes.

In contrast with these observations, Koch & Adler(1990) were able to find variations in the ability of different oligonucleotide sequences to amplify B19 isolates from different geographic areas. Their results may be due to very minor sequence variations in the genome which have a major effect on the ability of the oligonucleotide primers that they selected, to anneal to the genome.

1.1.5 Latency

Dependoviruses readily produce latent infection (Hoggan *et al* 1972). AAV latent infection has been shown to occur naturally in primary African monkey cells and human embryonic kidney cells (Hoggan *et al* 1972) and following infection of continuous human cell lines (Berns *et al* 1975). Recently the site of AAV integration has been localised to chromosome 19q in a somatic human cell line (Kotin & Berns 1990). The viral DNA was integrated into cellular DNA *in vitro* as a tandem repeat of several copies, with the termini of the viral genome close to the junction with cellular sequences (Cheung *et al* 1980). The question of whether automonous parvoviruses can establish latent infection by integration has not been resolved. Autonomous parvovirus B19 has hairpin termini and a similar genomic organisation to the dependoviruses and the cloned duplex form of autonomous parvovirus DNA is infectious.

1.1.6 Clinical syndromes

1.1.6(a) Aplastic crisis

Aplastic crisis was the first clinical syndrome shown to be associated with B19 infection. It is characterised by an acute, self-limiting cessation of erthropoiesis following B19 virus infection in individuals with underlying haemolytic anaemias. Patients develop acute symptoms of severe anaemia with a critically low haemoglobin, reticulocytopenia, and occasionally leukopenia and thrombocytopenia. Bone marrow examination shows a complete absence of erythroid precursors. The anaemia is self-limiting, but blood transfusion is required until the bone marrow recovers. Reticulocytes then reappear in the peripheral blood and haemoglobin concentrations return to steady state values.

Initially the association between aplastic crisis and B19 infection was documented in patients with sickle cell anaemia attending a clinic in London, UK (Pattison *et al* 1981) and others attending a much larger clinic in Kingston, Jamaica (Serjeant *et al* 1981). Aplastic crisis due to B19 infection has been shown to occur in many different haemolytic anaemias. These include hereditary spherocytosis (Kelleher *et al* 1983; Green *et al* 1984), beta-thalassaemia intermedia (Rao *et al* 1983), pyruvate kinase deficiency (Duncan *et al* 1983), autoimmune haemolytic anaemia (Bertrand *et al* 1985; Chitnavis *et al* 1990), hereditary stomatocytosis (Mabin & Chowdhury 1990) and red cell pyrimidine-5'-nucleotidase deficiency (Rechavi *et al* 1989).

Any underlying chronic haemolytic anaemia seems to predispose to this complication of B19 infection since the sole requirement for the development of anaemia appears to be a reduced red cell survival. Thus individuals who have haemolytic anaemias and who have recently been transfused may escape the aplastic crisis complication of B19 virus infection.

1.1.6(b) B19 infection in malaria infected individuals

Interest has recently focused on the role of B19 virus in malaria infection. The pathophysiology of the anaemia of malaria is both complex and multifactorial (Weatherall *et al* 1983). Whatever the individual factors involved, the general mechanisms appear to be those of increased red cell destruction and decreased red cell production. The survival of heat treated cells when injected into patients with malaria is markedly shortened (Looareesuwan *et al* 1987) and there is evidence for increased splenic removal of red cells in malaria infections (Wiler *et al* 1981).

In a preliminary study in Niger, West Africa (Jones *et al* 1990), it was found that 13 of 24 (54%) young children under 10 years of age with severe anaemia had evidence of recent B19 infection. Thirteen (54%) of these children had a laboratory confirmed diagnosis of malaria. To determine whether there is a link between the severity of anaemia in malaria infected subjects and coinfection with B19 virus will require a much larger seroepidemiological study (see detection of B19 infection in patients with severe anaemia living in a malarial endemic area).

1.1.6(c) Erythema infectiosum

Erythema infectiosum, also known as Fifth disease, is the commonest clinical manifestation of parvovirus B19 infection. This association was initially shown during an outbreak of erythema infectiosum in London (Anderson *et al* 1984) and subsequently confirmed in other studies throughout the world (Chorba *et al* 1986; Okabe *et al* 1984; Plummer *et al* 1985).

The clinical symptoms of erythema infectiosum develop in a biphasic fashion. Some 7-8 days after infection a prodromal influenza-like illness develops, characterized by headache, malaise, chills, and pyrexia. Individuals are then asymptomatic for a week. The second phase of illness occurs 17-18 days after infection with the development of a mild febrile illness and a maculopapular rash. The first sign of illness, particularly in children, is a marked erythema of the cheeks ("slapped cheeks" appearance) followed by the appearance of rash on the trunk and limbs. Initially the rash has a discrete erythematous maculopapular appearance and then becomes reticular, disappearing in the subsequent one to three weeks. Erythema infectiosum often resembles the rash of rubella (Anderson *et al* 1985a). A rash illness does not always occur following B19 virus infection and the only manifestation of the second phase of

the illness may be a mild, influenza-like illness (Anderson *et al* 1985b; Potter *et al* 1987).

Transient lymphopenia (Anderson *et al* 1985b; Potter *et al* 1987), neutropenia (Doran & Teall 1988; Hanada *et al* 1989) and thrombocytopenia (Mortimer *et al* 1985a; Schwarz *et al* 1989a) are uncommon complications of B19 virus infection. They are rarely severe enough to cause problems.

1.1.6(d) B19 associated arthropathy

Joint involvement occurs in 80% of adult cases women and in 8% of children (Ager *et al* 1966; Anderson *et al* 1984). Women are much more commonly affected than men (Reid *et al* 1985). The commonest presentation is of an acute onset, symmetrical polyarthropathy involving the small joints of the hands, wrists, ankles, and knees (White *et al* 1985; Naides *et al* 1990). Recovery usually occurs within 2-4 weeks (Reid *et al* 1985) although persistence of symptoms for up to two years after infection has been described (Naides *et al* 1990). The pathogenesis of B19 associated arthropathy is not known although immune complexes have been demonstrated in experimentally infected volunteers (Anderson *et al* 1985b) and in 50% of B19 infected patients (Woolf 1990). B19 DNA has been detected in the synovial fluid of a woman with B19 associated arthropathy (Dijkmans *et al* 1988) although another study failed to demonstrate B19 DNA in synovial fluid, cells or membranes of B19 infected patients with persistent joint symptoms (Woolf 1990). The B19 arthropathy may also occur in the absence of the rash illness (White *et al* 1985).

Parvoviruses have been advanced as candidates in the aetiology of rheumatoid arthritis. A parvovirus-like agent, RA-1, has been isolated from rheumatoid synovial

cells (Simpson *et al* 1984) and when inoculated into a suckling mouse reproduced a form of limb arthritis. However, RA-1 is serologically distinct from B19 (Brown 1984).

Rheumatoid factor reactivity has been reported in B19 associated arthropathy (White *et al* 1985; Reid *et al* 1985; Luzzi *et al* 1985; Naides & Fields 1988) and three of 19 B19 infected individuals attending an early synovitis clinic fulfilled the American Rheumatism Association's diagnostic criteria for definite rheumatoid arthritis (White *et al* 1985).

Besides the rheumatoid factor activity accompanying some B19 infections autoimmune markers have also been detected in patients recovering from B19 infection (Solonika *et al* 1989). Sixty eight percent of subjects recovering from recent B19 infection had elevated levels of anti-double stranded and anti-single stranded DNA. A cytotoxic IgM anti-lymphocyte antibody was also detected in 88% of subjects. This autoimmune state resolved as infection receded. The role of subclinical autoimmune phenomena in individuals is unknown but B19 could be important as a trigger for or cause a flare of an underlying autoimmune disorder.

1.1.6(e) B19 infection in pregnancy

Twenty eight to 54% of women of reproductive age are susceptible to B19 infection (Mortimer *et al* 1985b; Koch & Adler 1989; Enders & Biber 1990) and infection has been reported in pregnant women in several community outbreaks of erythema infectiosum (Schwarz *et al* 1988a; Rodis *et al* 1988; Rodis *et al* 1990). Fetal death (Mortimer *et al* 1985b; Lefere *et al* 1986; Grey *et al* 1986) and non immune hydrops (Brown *et al* 1986; Bond *et al* 1986; Anand *et al* 1987) have been described in

pregnancies complicated by B19 infection. One report describes advanced liver disease in a premature neonate infected with B19 (Metzman *et al* 1989). In another case report a B19-infected woman who terminated her pregnancy at 11 weeks had an abortus with eye anomalies and histological evidence of damage to multiple tissues (Weiland *et al* 1987; Hartwig *et al* 1989). The percentage of fetal hydrops caused by B19 infection is not clear at present. One study has reviewed the prevalence of B19 infection in 50 non-immune hydropic fetuses by *in situ* hybridisation (Porter *et al* 1988). Evidence of B19 infection was found in 8% of the cases.

The fetal risk following maternal infection has been assessed in a number of studies. An early study of 42 infected pregnant women demonstrated fetal hydrops complicating 26% of pregnancies, fetal death occurred in 18% of the subjects (Scharz *et al* 1988a). Recently two prospective studies of B19 infection in pregnancy involving more than 100 subjects have been reported. In a study performed in Germany (Enders & Biber 1990) 114 B19 infected pregnant women were studied and in a UK study (Public Health Laboratory Service Working Party on "Fifth" disease [PHLS] 1990) 190 subjects were studied. Fetal death occurred in 8% of the German pregnancies while the estimated risk of fetal death due to B19 in the UK study was 9%. The UK study showed that excess fetal loss was more pronounced in second trimester infections. Intra-uterine blood transfusion may have altered the outcome in five hydropic cases in the German study and in one hydropic case in the UK study.

Fetal hydrops occurred in 9% of the German subjects and in 0.5% of the UK pregnancies. In both studies the pregnancies were followed up. The relatively common and minor congenital abnormality of hypospadias occurred in two newborns while all the other newborns were normal. Neither study was large enough to detect a rare teratogenic effect. Follow up of infants in the UK study one year after birth has not revealed any late congenital nor developmental problems. The maximum

transplacental transmission rate was estimated in the UK study to be 33%.

1.1.6(f) Infection in the immunocompromised

Persistent B19 infection has been described in both immunocompromised adults and children. The patients have been a child with Nezelof's syndrome (Kurtzman *et al* 1987), children with acute lymphocytic leukemia (four cases) (Van Horn *et al* 1986; Kurtzman *et al* 1988a; Coulombel *et al* 1989) and a child with T cell immunoficiency (Davidson *et al* 1989). Case reports of chronic B19 infection in immunocompromised adults include a patient with chronic myelomonocytic leukemia (CMML) (Malarme *et al* 1989), a bone marrow transplant recipient for acute myeloid leukemia (Weiland *et al* 1989) and patients with HIV infection (Mitchell *et al* 1990; Antunez *et al* 1990; Frickhofen *et al* 1990). Pure red cell aplasia of 10 years duration linked to persistent B19 infection has been described in two teenage brothers with humoral and cellular deficiences (Kurtzman *et al* 1989).

B19 infection in these subjects has been characterised by either persistent anaemia or a remitting and relapsing anaemia. B19 viraemia occurs and recurs in periods of anaemia, although the genome copy number observed in sera is often low, 10^5-10^{10}/ml, compared to 10^{10}-10^{14}/ml in transient aplastic crisis (Frickhofen & Young 1989). The bone marrow picture is typical of that seen in aplastic crisis complicating haemolytic anaemia.

Three patterns of immune response have been observed in the immunocompromised, no antibody response, B19 IgM but no B19 IgG and B19 IgM with only a weak B19 IgG response. Several patients have been given infusions of immunoglobulin preparations containing B19 antibodies. These transfusions have produced either

transient (Kurtzman *et al* 1988a) or permanent fall in virus titre (Kurtzman *et al* 1989), a reticulocytosis and recovery from the anaemia.

1.1.7 Pathogenesis

The diseases caused by B19 virus reflects its requirement for actively dividing erythroid precursor cells and depends on two interacting factors: the survival time of the circulating erythrocytes in the host and the immune response to the virus. The virus replicates in erythroid precursor cells, producing lysis of these susceptible cells and transient loss of red cell precursors from the bone marrow. In haematologically normal hosts this leads to a transient reticulocytopenia without a significant fall in the circulating erythrocyte number. In patients with haemolytic anaemia however, the life-span of red cells is much shorter than normal, so that the loss of erythroid precursors in the marrow leads to a rapid fall in the circulating erythrocyte population and to development of the aplastic crisis of B19 infection.

The pathogenesis of B19 infection has been elucidated by two volunteer studies (Anderson *et al* 1985b; Potter *et al* 1987). Six to seven days after acquisition of the virus an intense viraemia develops with up to 10^{12}viral particles/ml present in the peripheral circulation. Viraemia coincides with the prodromal influenza-like illness. The second phase of B19 disease depends on the immune response to the virus. Erythema infectiosum and arthralgia develop 17-18 days after acquisition of the virus and at the time of the appearance of specific IgM and IgM-virus immune complexes. The virus is no longer detectable by dot blot hybridisation, and the rash is thought to be due to the antibody response to the virus. This is supported by the observation that immunocompromised subjects, with chronic B19 infection, develop erythema infectiosum when given pooled human serum containing B19 antibodies (Kurtzman *et al* 1988a).

Fetal disease develops when the virus crosses the placenta and establishes infection in the fetal erythroid precursors and possibly myocardial cells. The precise pathogenic process(es) has not been determined but is likely to be multifactorial. The fetal erythrocyte life span is half that of the adult erythrocyte and the red cell volume is rapidly expanding during fetal development. This may render the fetus susceptible to marrow failure in a manner similar to subjects with haemolytic anaemia. The immaturity of the fetal immune response is also likely to play a role in pathogenesis, by allowing the B19 infection to persist. Erythroid aplasia and anaemia develop up to 11 weeks after the maternal infection.

Chronic B19 virus infection accompanied by chronic anaemia also occurs in immunodeficient patients. Although the individuals have normal red cell survival times, they fail to clear the virus and anaemia develops due to chronic failure of red cell production.

1.1.8 Host defences

B19-specific IgM is first detectable as the viraemia wanes 9-10 days after the onset of infection, and peak levels develop within one week (Anderson *et al* 1985b). Much of the early antibody is complexed with virus in immune complexes (Anderson *et al* 1985b). Specific IgG is not detectable until two weeks after the onset of infection, and peak levels develop more slowly over the following two weeks. The role of cell-mediated immunity following B19 infection is unknown. Several studies have shown that individuals with defective B cell immunity and chronic B19 infection subsequently clear the virus when passively immunised with normal pooled human immunoglobulin (Kurtzman *et al* 1988a; Kurtzman *et al* 1989). This would suggest

that the major mechanism for B19 clearance is antibody mediated.

1.1.9 Transmission

B19 infection is commonly spread by the respiratory route and can be detected in throat swabs for five days commencing one week after B19 inoculation (Anderson *et al* 1985b). It is not known whether the virus has a site of replication in the respiratory tract. Data from studies of outbreaks of erythema infectiosum suggest that the risk of infection among susceptible adults is approximately 20-50% following school exposure and 50% following a household exposure (Anderson *et al* 1990).

Blood borne transmission is another mode of B19 infection because an intense viraemia occurs in infected individuals. Blood borne spread has been shown to occur in recipients of whole blood and clotting factor concentrates (Mortimer *et al* 1983b; Corsi *et al* 1988) prepared from large pools of human plasma. Additional evidence comes from seroprevalence studies in which B19 seropositivity is much higher (89%) in children with haemophilia than in age-matched controls (38%) (Williams *et al* 1990).

Several procedures have been developed for the inactivation of HIV in clotting factor concentrates (Mannucci & Morfini 1988). These include a variety of chemical and/or physical processes including methods of heat treatment under different conditions (dry, steam, wet heat). Follow-up of cohorts of seronegative patients prior to and after infusion have shown that some of these treatments do not eliminate B19 infectivity (Corsi *et al* 1988; Lyon *et al* 1989) but may reduce it (Williams *et al* 1990). B19 infection acquired in this way is unlikely to be harmful to immunocompetent individuals, with a normal erythrocyte turnover, but it may pose

problems to the immunocompromised and pregnant patients.

1.1.10 Epidemiology

Due to the scarcity of B19 antigen available for diagnostic B19 serology (see laboratory diagnosis) only a few studies of B19 seroprevalence have been performed. B19 infection has been found in all countries in which B19 serological testing has been performed. This includes Europe, North & South America, the Caribbean, Scandinavia, Australia and Japan. Very limited epidemiological data has been collected from Africa.

B19 infection occurs throughout the year, although in temperate zones infection is most common in the late winter, spring and early summer months. In addition to the seasonality seen in temperate climates there are longer-term cycles of virus activity with peaks of activity occurring every 4-5 years in both the UK and Jamaica (Serjeant & Goldstein 1988). B19 infection is most commonly acquired between the ages of four and 10 years (Edwards *et al* 1981; Cohen & Buckley 1988) in temperate climates. In equatorial areas (Niger, Brazil) B19 infection is acquired earlier in life (Jones *et al* 1990; Nascimento *et al* 1990). Erythema infectiosum may occur in outbreaks and these have been described in schools (Anderson *et al* 1984; Grilli *et al* 1989) and hospitals (Bell *et al* 1989; Pillay *et al* 1991).

The seroprevalence of B19 infection has been assessed by measuring specific IgG; 60% of young adults are seropositive (Cohen *et al* 1983) and this seroprevalence rises to 87% in adults over 71 years of age (Cohen & Buckley 1988).

1.1.11 Control

There is no specific antiviral therapy nor a vaccine for B19 infection, and most individuals do not require symptomatic therapy. Cases of aplastic crisis require erythrocyte transfusion support until the bone marrow recovers. Administration of normal human immunoglobulins to immunodeficient, B19-infected patients may produce amelioration of viraemia and anaemia. Intra-uterine transfusions of B19 infected hydropic fetuses have been performed with good results. The fetuses recovered haematologically and no adverse congenital or developmental outcomes were observed (Gloning *et al* 1990; Enders & Biber 1990; PHLS 1990).

Most persons infected with B19 virus are asymptomatic when viral shedding is maximal, and therefore control of infection is difficult to achieve. Patients presenting with erythema infectiosum are no longer infectious and do not require isolation. Patients with aplastic crisis may be infectious at the time of presentation and should not be cared for near other at-risk haematology, immunodeficient, or pregnant patients.

1.1.12 Laboratory diagnosis

The laboratory diagnosis of B19 infection can be established by the direct detection of the virus or by detecting the antibody response to the virus. The temporal relationship between the onset of infection and the detection of the virus and the host response are shown in figure 1.

Aplastic crisis may develop during the viraemic phase of infection in patients with underlying haemolytic anaemia and thus detection of virus is a useful diagnostic test

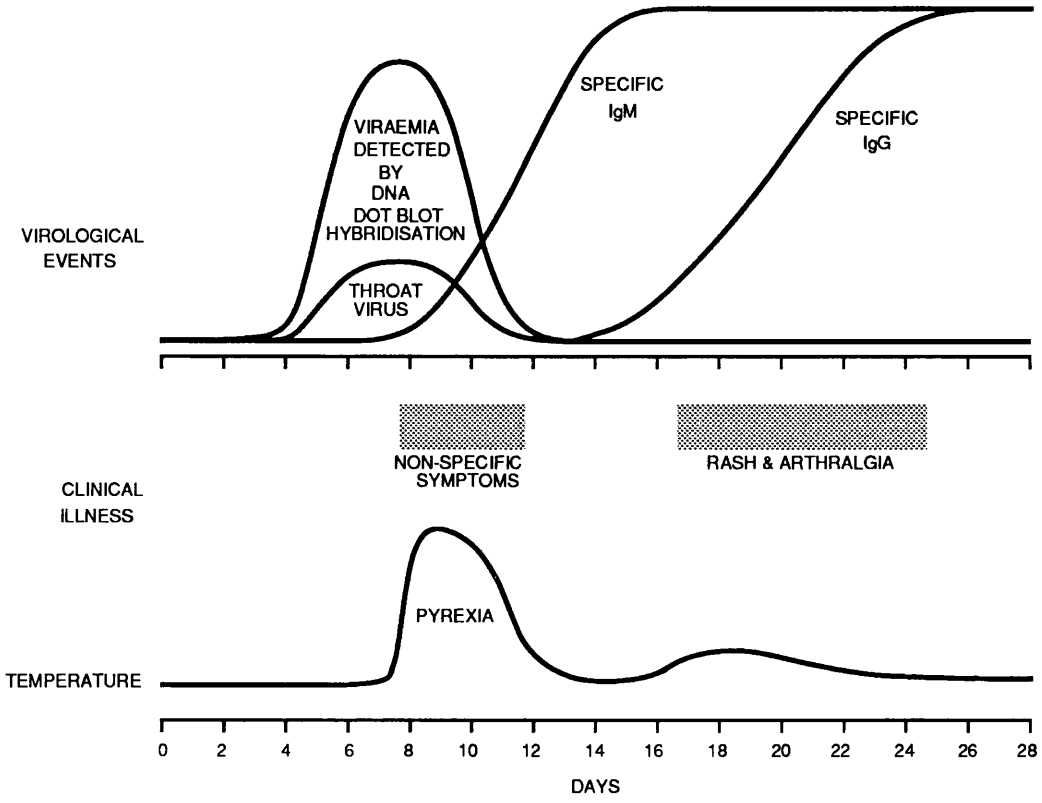

Figure 1: Serological and clinical profile of B19 infection

for this clinical group. However, in subjects who develop rash and arthralgia, the viraemia has already declined and B19 specific IgM antibody is present. In this clinical group diagnosis is established by the detection of the antibody response to the virus. The B19 infected immunocompromised patient with chronic anaemia is unable to make an appropriate antibody response and in such cases the diagnosis of B19 infection is established by the direct detection of virus or viral DNA.

Diagnosis of B19 infection in the fetus is problematic. The fetus fails to make a B19 specific IgM response to the virus and disease often develops after the decline of maternal B19 IgM, up to 11 weeks after the maternal illness (PHLS 1990). Thus to establish fetal infection, virus must be demonstrated in fetal serum or tissue. This requires invasive procedures to sample fetal blood or tissue (Anderson *et al* 1988). Virological confirmation of congenital infection is also difficult to establish in the neonate. B19 IgM detection is only 17% sensitive (PHLS 1990). Intrauterine infection can be implied by the demonstration of persisting B19 IgG at one year.

1.1.12(a) Antibody detection

At present viral antigens cannot be produced in high titre in tissue culture so that the antigen employed in antibody assays is obtained as viraemic serum from acutely infected individuals. As the viraemic phase of the disease is not usually accompanied by specific symptoms viraemic plasma is obtained by screening large numbers of

donated units of blood. Among the blood donor population in the UK the incidence of viraemia is of the order of 1 in 40,000 during epidemic periods (Anderson 1982). A number of recombinant B19 antigen expression systems are being developed to overcome the difficulties of obtaining B19 antigens. These include a transfected Chinese hamster ovary cell line (Kajigaya *et al* 1989) producing viral capsids, a baculovirus expression system (Brown *et al* 1990a) and an E. coli expression system (Rayment *et al* 1990; Eiffert *et al* 1990). Another approach has been to develop a 24 amino acid synthetic peptide constructed from the N-terminus of the VP2 structural protein (Fridell *et al* 1989).

B19 antibody may be detected by countercurrent immunoelectrophoresis (Serjeant *et al* 1981), immune electron microscopy, immunofluorescence (Brown *et al* 1990b) and antiglobulin ELISA (Anderson *et al* 1986; Schwarz *et al* 1988b) although the most widely used and sensitive tests are IgM and IgG capture radio- or enzyme-immunoassays (Cohen *et al* 1983).

B19 IgM is usually detectable within 1-2 days of the onset of rash or arthralgia and persists for 2-3 months (Cohen *et al* 1983); IgG is detectable for much longer and probably persists lifelong, although it may fall below the level of detection by currently available assays. Recent B19 infection may also be demonstrated by IgG seroconversion.

1.1.12(b) Virus detection

Direct detection of virus is required to establish the diagnosis of B19 infection in patients presenting in the early stage of infection, before the development of B19 specific antibody. Historically, attempts have been made to culture B19 in a variety

of routinely available tissue cultures and these have all failed. Due to the absence of an adequate tissue culture system, the early diagnosis of B19 infection may be established by (a) detection of B19 antigen in serum by CIE (Serjeant *et al* 1981), reverse capture radioimmunoassay (Cohen *et al* 1983) and EIA (Schwarz *et al* 1988b); (b) demonstration of viral particles by electron microscopy (Cossart *et al* 1975); or (c) by various hybridisation assays to detect B19 DNA (Anderson *et al* 1985c; Cunningham *et al* 1988).

B19 virus is not cultivatable in conventional cell culture because of the virus's specific cell tropism. Recently the virus has been propagated in bone marrow suspension cultures (Ozawa *et al* 1986; Ozawa *et al* 1987), in highly enriched haematopoietic progenitor cell cultures obtained from bone marrow (Srivastava & Lu 1988) and fetal liver culture (Yaegashi *et al* 1989). Infection is demonstrated by immunofluorescence or by dot blot hybridisation. The efficiency of B19 replication differs in the three systems, it is lowest in the bone marrow culture system, the fetal liver system is twice as efficent and the haematopoietic progenitor cell cultures 100 fold more efficient than the bone marrow culture. The yield of B19 virus (as measured by DNA copy number) produced by the most efficient cell culture system is 10^5 copies/cell. This is considerably lower than the peak viraemia detected in serum during *in vivo* infection.

CIE is a relatively insensitive test for B19 antigen, it was only able to detect B19 antigen in the original blood donor B19 described by Cossart *et al* (1975) to a dilution of 1 in 4 (Cohen *et al* 1983). In addition, when this technique was applied to sera obtained from patients with aplastic crisis, CIE was able to detect B19 antigen in only 36% of samples collected within one week of the clinical development of aplastic crisis (Anderson *et al* 1985c). All the positive samples were collected within three days of the onset of symptoms, indicating that CIE only detects antigen at the peak of

the viraemia.

The immunoassays are more sensitive tests than CIE. An RIA previously described by Cohen *et al* (1983) was able to detect B19 antigen in plasma obtained from blood donor B19 to a dilution of 1 in 10,000. Clewley (1985) was able to show that this assay was of comparable sensitivity to dot blot hybridisation, although Mori *et al* (1989) in a more extensive study demonstrated that only 63% of dot blot hybridisation positive samples were reactive in the RIA. Anderson *et al* (1985c) have suggested that the presence of B19 antibody in viraemic sera may interfere with the detection of B19 antigen in the RIA.

B19 virus may also be detected in serum during the viraemic phase of the illness by electron microscopy and by immune electron microscopy, as first observed by Cossart *et al* (1975). Assuming the dot blot hybridisation technique (described below) to be the "gold standard" for the detection of B19 virus, electron microscopy has been shown to be 88% sensitive (Mori *et al* 1989). Unfortunately this technique is labour intensive and dependent on the skill of the microscopist in identifying parvovirus particles. This limits its usefulness for routine diagnostic testing and for the screening of large numbers of samples. It is however a useful confirmatory test for the detection of viraemia.

Dot blot hybridisation using radiolabelled (Anderson *et al* 1985c), biotin labelled (Mori *et al* 1989) or digoxigenin labelled (Azzi *et al* 1990; Zerbini *et al* 1990) DNA or RNA probes (Cunningham *et al* 1988) can be applied to a variety of body fluids (serum, saliva, amniotic fluid) and tissues for the detection of B19 infection. DNA dot blot hybridisation is able to detect viral DNA in 53% of serum samples collected from aplastic patients within three days of the onset of symptoms (Anderson *et al* 1985c). The technique detects as few as 10,000 copies of B19 DNA and is 300 fold

more sensitive than RIA in terms of the number of viral particles detected. However, using even this highly sensitive method, viraemia can only be detected for six days or less in viraemic individuals from whom multiple sequential samples were obtained. False positive reactivities have been reported with dot blot hybridisation when applied to both tissues (personal communication, Dr M Anderson, London) and less commonly serum (Mori *et al* 1989). Southern blot hybridisation can be used to confirm dot blot reactivity. *In situ* hybridisation has been developed as a more specific test of B19 infection in tissues (Porter *et al* 1988). It has also been used to address the question of tissue tropisms of the virus (Porter *et al* 1990).

1.1.12(c) Disadvantages of the current methods of direct virus detection

There are many disadvantages to the techniques currently employed to directly detect B19 virus. Despite an intense viraemia, the virus is only detectable in serum for up to six days by current technologies. This limits the usefulness of viral detection for the diagnosis of infection to individuals who develop clinical symptoms at the time of the viraemia. This is almost always restricted to the patients presenting with aplastic crisis. Patients presenting with erythema infectiosum or arthritis are rarely viraemic as detected by the currently available tests. In B19 infected immunocompromised patients evidence is now emerging that chronic B19 viraemia undetectable by the current routine diagnostic assays may occur (Frickhofen & Young 1989). Finally, the current generation of assays have been unable to define the mechanism of the disappearance of virus from serum and whether partitioning of the virus into other body compartments occurs. The new and revolutionary technique of polymerase chain reaction amplification holds the promise of both extending our understanding of the biology of B19 infections and extending the diagnostic potential of direct DNA detection for patient diagnosis.

1.2 POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION OF DNA

1.2.1 History

The polymerase chain reaction (PCR) was invented by Mullis in 1984 (Mullis *et al* 1986; Mullis 1990). It is a method for the enzymatic amplification of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite DNA strands and flank the region in the target DNA to be synthesised. A repetitive cycle of temperature changes produce template denaturation, primer annealing and the extension of the annealed primers by a DNA polymerase. The primer extension products synthesised in one cycle can serve as a template in the next and so the number of target DNA copies approximately doubles at every cycle. This leads to the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers. Thus, 20 cycles of PCR theoretically yields a million fold (2^{20}) amplification. The specificity of the PCR products can be confirmed by a variety of techniques including Southern blot hybridisation, dot blot hybridisation, restriction enzyme cleavage and direct sequencing.

Initially, the Klenow fragment of *E.coli* DNA polymerase I was used in the assay to extend the annealed primers. This enzyme was inactivated by the high temperature required to separate the two DNA strands at the start of each PCR cycle. Fresh enzyme had to be added during each cycle. PCR was transformed into a self contained assay, that could be automated using a thermal cycling device, by the substitution of the Klenow DNA polymerase with *Taq* polymerase. This thermostable enzyme was isolated from a thermophilic bacterium *Thermus aquaticus* (*Taq*), discovered in a hot spring in Yellowstone National Park, USA, in 1969 (Brock &

Freeze 1969). It is able to withstand high temperatures, 50% activity is retained after 130 mins and 40 mins at 92.5°C and 95°C respectively (Gelfand 1989).

1.2.2 Applications

The PCR technique represents a landmark in the development of molecular biology. It has revolutionised the production of large quantities of clonal DNA leading to applications both in basic biology and medicine.

General applications for PCR in basic biology include amplification of DNA for direct sequencing, engineering of DNA by 5' addition of nucleotide sequences, site specific mutagenesis and *in vitro* DNA recombination. The technique can also be applied to amplifying specific sequences from a cDNA library and amplification of unknown DNA sequences. PCR can also be used to detect gene expression by reverse transcription of mRNA and then performing PCR.

There are numerous medical applications of PCR, indeed PCR was originally applied to the amplification of human beta-globulin DNA and to the prenatal diagnosis of sickle cell anaemia (Saiki *et al* 1985; Saiki *et al* 1986; Embury *et al* 1987). In addition to the detection of genetic diseases the technique has also been applied to the detection of HLA gene polymorphism and the analysis of forensic biological evidence. PCR has also transformed the detection of infectious diseases, particularly for viruses such as HIV (Simmonds *et al* 1990) and hepatitis C virus (HCV) (Garson *et al* 1990). PCR is both rapid and sensitive, detecting as little as a single copy of DNA and thus removing the need for time consuming and labour intensive culture systems. Direct sequencing of PCR products has also simplified the study of molecular diversity and evolution of viruses (Balfe *et al* 1990).

1.2.3 Problems

A consequence of the extreme sensitivity of the assay is the ability of PCR to amplify small amounts of target DNA contaminating the laboratory environment (Kitchin *et al* 1990). This can present a formidable problem, particularly if PCR is performed in the same laboratories used for cloning and propagation of the target DNA. A number of workers have described precautions to ensure contamination-free PCR (Kwok & Higuchi 1989; Cone *et al* 1990). These included aliquoting of reagents, the use of positive displacement pipettes, the physical separation into different laboratories of sample handling and PCR reagent preparation from the post-PCR steps such as setting up second round PCR, gel electrophoresis and hybridisation. Dedicated equipment should also be used in each of the laboratories.

1.2.4 Nested PCR

Nested PCR is a derivation of the original PCR technique, offering a number of advantages over the original method. This modification was also invented by Mullis (Mullis & Faloona 1987). The technique is very simple, PCR amplification is performed with a first set of primers and after this is completed the products are submitted to a second round of amplification using a primer pair "nested" within the first set of primers. Nested PCR offers a number of advantages over conventional single round PCR. The technique is more specific than single round PCR; requiring the target DNA to have a base sequence complementary to the oligonucleotide primers at four sites rather than at two sites. The technique is also more sensitive; allowing for saturation kinetics for the synthesis of target DNA, yielding the same quantity of PCR product despite the number of copies of target DNA in the starting

material. Finally nested PCR can be made semiquantitative. The first round PCR is less sensitive than the nested PCR so that the detection of a specific DNA product following first round PCR indicates the presence of a "large" quantity of target DNA in the starting sample, the detection of a product only after nested PCR indicates a "small" quantity of target DNA in the starting material.

A major disadvantage of the nested PCR technique is that the opportunity for contamination to occur is theoretically increased when transferring PCR products containing high titre target DNA from the first round PCR to the second round PCR.

1.3 AIMS OF THE INVESTIGATION

The first objective of the study was to develop a method for the detection of B19 DNA that was more sensitive than any of the technologies currently in use. The method to be developed for this purpose was nested PCR. It was hoped that this technique would enhance the sensitivity of B19 DNA detection by 10^4 so that single copies of DNA could be detected. The development of such a technique would necessitate the characterisation of the appearance and disappearance of such low levels of B19 DNA in serum. To determine this it was decided to apply the assay to sera submitted to the Department of Medical Microbiology for diagnostic B19 testing and serial sera collected from B19 infected volunteers. A further aim was to explore the relationship between the presence of B19 DNA in serum and the timing and magnitude of the humoral immune response to B19 infection. It was also attempted to determine whether host factors were associated with the presence, quantity and duration of B19 DNA in serum.

Another aim of the study was to apply the nested PCR assay to cases of unexplained rash illness, serologically B19 IgM negative/rubella IgM negative, to determine

whether this assay would extend the number of rash illnesses in which B19 virus could be implicated. The assay would also be used to determine whether B19 DNA, transiently detected in throat secretions by dot blot hybridisation, persists in infected individuals but below the level of detection by this technique. An additional aim was to examine the feasibility of using nested PCR as a diagnostic technique for the detection of B19 infection from throat swabs. Nested PCR was also to be applied to determine if B19 virus could be detected in peripheral blood mononuclear cells (PBMNC) of infected subjects. These cells could potentially eliminate virus from serum by phagocytosis and be the mechanism by which B19 viraemia is terminated.

It was planned to apply the nested PCR to a number of clinical situations in which it was postulated that parvovirus B19 may play a role but where existing diagnostic tests were considered inappropriate to test these hypotheses because of the predicted or known nature of the host response. The clinical groups envisaged included those unable to produce antibodies in response to infection (bone marrow transplant patients and neonates), individuals with potential immune complexed B19 antibody (arthritis patients), B19 infected pregnant subjects in whom the disease sequelae is known to develop often after the decline of the IgM antibody response and malaria infected subjects with a hypergammaglobulinaemia that could potentially interfere with the serological assays. Finally, the PCR assay was applied to the screening of blood factor concentrates to screen for B19 DNA below the level detectable by dot blot hybridisation.

CHAPTER 2

MATERIALS & METHODS

2.1 STORAGE OF SAMPLES AND REAGENTS

All sera, plasma, tissues and throat swabs were stored at -20°C, peripheral blood mononuclear cell fractions were stored at -70°C. Samples were only thawed immediately prior to use. All commercial reagents were stored as recommended by the manufacturers. DNA probes were stored at -20°C.

2.2 SEROLOGY

2.2.1 B19 IgM capture radio-immunoassay (MACRIA) & B19 IgG capture radio-immunoassay (GACRIA)

These assays have been modified from a published method (Cohen *et al* 1983). Briefly, polystyrene beads (Northumbria Biologicals, Cramlington, U.K.) were coated with anti-human IgM (Tago) by gentle agitation in anti-μ diluted 1/750 in carbonate/bicarbonate buffer pH 9.6 (0.2M anhydrous sodium carbonate, 0.2M sodium bicarbonate) at room temperature for 1 hour. IgG beads were prepared in a similar fashion with rabbit anti-human γ (Dako, Denmark) diluted 1/1000 in the same buffer. The beads were held in this solution at 4°C for at least 48 hours before use. Each bead was reacted in a plastic tray (Abbott, USA) with test serum, diluted 1/100 for the IgM test and 1/100 for the IgG test in phosphate-buffered saline (PBS) with 0.05% Tween 20 (Sigma)(PBST) and 10% fetal calf serum (FCS, Gibco, UK). The beads were incubated for 3 hours at 37°C. Following this, the beads were washed

three times in PBST. Plasma from a B19 viraemic subject SM (see appendix) was mixed with an equal volume of chloroform and then briefly spun at 12,000*g*. The supernatant was diluted 1/500 in PBST with 10% FCS. The beads were then incubated with 180µl of the mixture at room temperature overnight. The wash steps were repeated and then the beads were incubated with 180µl of a mouse monoclonal anti-B19 antibody diluted 1/5000 in PBST with 10% FCS at 37°C for 3 hours. The wash steps were again repeated and the beads incubated, for 90 minutes at 37°C, with radio-iodinated sheep anti-mouse antibody (Amersham,UK) diluted in PBST with 10% FCS and 5% B19 antibody negative serum at a dilution to produce 50,000 counts per minute in a 180µl volume. After a final wash the radioactivity bound to the beads was measured by counting for 5 minutes in an NE 1600 gamma counter (Nuclear Enterprises, UK). B19 IgM results were expressed as MACRIA arbitary units of B19 antibody when compared to dilutions of a control positive serum. Five or more MACRIA units were considered to indicate recent parvovirus infection. For the B19 IgG test sera binding more than twice as much label as the negative control were regarded as B19 IgG positive.

2.2.2 Detection of rubella specific IgM

Detection of rubella specific IgM was performed by MACRIA using a published method (Tedder *et al* 1982).

2.2.3 Absorption of rheumatoid factor activity from serum

Two hundred microlitres of Rheuma-Wellcotest latex suspension (Wellcome Diagnostics, UK) was centrifuged in an Eppendorf tube at 6,000*g* for 10 min. The

supernatant was discarded and 50-100µl test serum mixed with the latex particle deposit. This was incubated overnight at 4°C and the latex particles removed by centrifugation at 6,000*g* for 10 mins. Confirmation that the rheumatoid factor activity had been removed from the serum was determined by repeating the rheumatoid factor test.

2.3 B19 DNA DETECTION

Several different methods were used for the extraction of DNA from plasma, serum and tissues. The extraction methods used in the initial development of the nested PCR (see section - Development of nested PCR) were originally developed for dot blot and Southern blot hybridisation assays. Subsequently, for the experiments describing the application of nested PCR (see section - Application of nested PCR), samples were extracted using methods more appropriate to PCR. These extraction methods were generally simpler to perform on large numbers of samples and reduced the opportunity for sample to sample contamination.

2.3.1 Extraction methods employed in the development of nested PCR for the detection of parvovirus B19

2.3.1(a) Plasma

Total DNA was extracted from B19 viraemic or negative control plasma using the following protocol. Half of a millilitre of plasma was made up to 5mls with PBS and centrifuged at 250,000*g*. The pellet was resuspended in 100µl of a solution containing 0.5% sodium dodecyl sulphate (SDS), 20mM ethylenediaminetetra-acetic acid (EDTA) and 100µg/ml proteinase K and incubated for 30 min at 65°C. Proteins

were removed by a distilled phenol extraction, distilled phenol/chloroform extraction and one extraction with chloroform alone. The DNA was precipitated by addition of 1ml ice cold absolute ethanol. After overnight storage at -70°C, the DNA was pelleted in an Eppendorf centrifuge (10 min, 12,000*g*). The pellet was resuspended in 50µl of a solution containing 10mM Tris.Cl and 1mM EDTA (TE buffer pH 7.6). The concentration of DNA was determined by optical density measurements at 260nm and 280nm using a spectrophotometer (Philips PU 8625 UV/VIS).

2.3.1(b) Tissues

One gram of human placental tissue was macerated with a pestle and mortar. The macerated tissue was then treated as shown above (plasma method).

2.3.2 Techniques used in the evaluation of different serum DNA preparation methods for PCR

Three different methods of DNA extraction (A,B & C) are described for the detection of B19 in serum and plasma. These form part of a comparative evaluation of methods for use in PCR (described in section - Evaluation of different serum DNA preparation methods for PCR).

2.3.2(a) Method A (non-ionic detergent and proteinase K treatment)

This was adapted from a published method of DNA preparation for whole blood described by Higuchi (1989). Fifty microlitres of serum was added to a 100µl lysis solution containing 50mM potassium chloride, 10mM Tris-Cl pH 8.3, 2.5mM

magnesium chloride, 0.1mg/ml gelatin, 0.45% NP40 (nonidet P-40, Sigma), 0.45% Tween 20 (Sigma) and 0.006mg proteinase K (Sigma 4914). This was then incubated at 65°C for 1 hour and then at 95°C for 10 mins to inactivate the proteinase K. The precipitate formed was then centrifuged at 12,000*g* for 15 mins and the supernatant used in the PCR assay.

2.3.2(b) Method B

This was a modification of a previously published method for RNA extraction (Garson *et al* 1990). One hundred microlitres of serum or plasma was added to an equal volume of buffer containing 0.2M "Tris" - hydrochloric acid (HCl) pH 7.5, 25mM EDTA, 0.3M sodium chloride, 2% (weight/volume) SDS, and 200µg/ml proteinase K, mixed and incubated at 37°C for 40 mins. Proteins were removed by two extractions with distilled phenol/chloroform and one with chloroform alone. The DNA was precipitated by addition of 1ml of ice-cold absolute ethanol. After storage at -70°C overnight, the DNA was pelleted in an Eppendorf centrifuge (15 min, 12,000*g*, 4°C). The pellet was dissolved in 8µl sterile distilled water.

2.3.2(c) Method C

The method of Larzul *et al* (1987) was used. Briefly 100µl of serum was incubated at 65°C for 1 hour with 20µl of extraction buffer containing 2.5mg/ml proteinase K, 25mM sodium acetate, 2.5mM EDTA, and 0.5% SDS. The protein was phenol extracted and the DNA extracted with 2 volumes of diethyl ether. The diethyl ether was allowed to evaporate before adding the DNA to the PCR.

2.3.3 Extraction methods employed in the application of nested PCR for the detection of parvovirus B19

2.3.3(a) Serum and clotting factor concentrates

Serum was extracted using method A (non-ionic detergent and proteinase K treatment) described above. The clotting factor concentrates were resuspended according to the manufacturers' instructions and then extracted using method A as described for serum.

2.3.3(b) Throat swabs

The throat swabs, collected in viral transport medium (VTM; containing Hanks lactalbumin, BSA, benzyl penicillin, ciprofloxacin, amphotericin B & bicarbonate), were briefly vortexed to remove the DNA from the swab into the VTM. The VTM was transferred into ultracentrifugation tubes (Beckman, USA) and made up to 5mls with sterile distilled water. The tubes were centrifuged at 285,000*g* for 2 hours at 4°C. The pellet was resuspended in 0.5ml of a buffer containing 0.5% SDS, 100µg/ml proteinase K, 10mM Tris-Cl pH8, 10mM EDTA and 10mM sodium chloride. This was then incubated at 37°C for 12 hours and the proteins removed by one extraction with distilled phenol/chloroform and one extraction with chloroform alone. The DNA was precipitated by the addition of 1ml ice-cold absolute ethanol and 50µl of 3M sodium acetate. After storage at -70°C overnight the DNA was pelleted in an Eppendorf centrifuge (30 min, 12,000*g*, 4°C). The DNA pellet was then resuspended in 30µl sterile distilled water and the concentration of DNA determined by optical density measurements at 260nm and 280nm using a spectrophotometer (Philips PU 8625 UV/VIS). Three different quantities of DNA were assayed by PCR, 0.05µg, 0.5µg and 1µg.

2.3.3(c) *In vitro* isolation of peripheral blood mononuclear cells (PBMNC)and extraction of PBMNC DNA

Fifteen mls of heparinised (preservative-free) whole blood was centrifuged for 10 mins at 400*g*. The cell pellet was resuspended with sterile phosphate buffered saline (PBS) to a final volume of 35ml. The suspension was layered onto 15ml Ficoll-Paque (Pharmacia, Sweden) and centrifuged for 30 mins at 400*g*. The upper layer was discarded and the cell layer removed with a pipette. The cell suspension was washed twice (400*g*, 10 mins) with RPMI 1640 (Gibco, UK) medium containing 2mM glutamine, 10,000units/ml benzyl penecillin (Glaxo), 10mg/ml streptomycin (Glaxo) and 0.25mg/ml amphotericin B (Squibb). The cells were finally resuspended in 1ml of the RPM1 1640 solution containing glutamine and antibiotics with 10% DMSO and 30% fetal calf serum. The cell suspensions were aliquoted into cryotubes and stored at -70°C.

A third of a millilitre of cryopreserved PBMNCs were thawed and washed with PBS, the cells were pelleted by centrifugation at 12,000*g* for 1 min. One hundred microlitres of lysis solution (see method A - non-ionic detergent and proteinase K treatment) was added to the cell deposit, mixed and incubated at 65°C for 1 hour. The buffer was then inactivated by heating to 95°C for 10 mins. Five microlitres of this reaction mix was then added to the PCR assay.

2.3.3(d) Tissues

One gram of tissue was macerated using a pestle and mortar and incubated for 12 hours with buffer containing 0.5% SDS, 100µg/ml proteinase K, 10mM Tris pH8,

10mM EDTA pH8 and 10mM sodium chloride at 37°C. The proteins were removed by extraction with distilled phenol/chloroform and then with chloroform alone. The DNA was precipitated by addition of 1ml of ice-cold absolute ethanol and 50µl of 3M sodium acetate and stored overnight at -70°C. The DNA was resuspended in 30µl of sterile distilled water and the DNA concentration determined by spectrophotometry. Half of a microgram of DNA was used in the PCR assay.

2.3.4 DNA transfer

2.3.4(a) Alkali transfer of DNA

The DNA was run into a 2% agarose gel by electrophoresis. The DNA was denatured by treating the gel with 0.2M HCl for 10 mins. The gel was washed in sterile distilled water and the DNA transferred to a nylon membrane by capillary transfer with 0.4M sodium hydroxide overnight (Reed & Mann 1985). The membranes were floated on 0.4M sodium hydroxide for 2 mins and then washed in 0.3M sodium chloride and 0.03M sodium citrate (2xSSC) for 2 minutes to remove any agarose from the surface. The membranes were baked for two hours at 80°C under vacuum. Hybridisation was then performed.

2.3.4(b) Saline sodium citrate(SSC) transfer of DNA

The DNA was run into a 2% agarose gel by electrophoresis. The DNA was denatured by treating the gel with a solution containing 0.2M sodium hydroxide and 0.6M sodium chloride for 30 min. The gel was then washed in 3 changes of 1.5M sodium chloride and 0.15M sodium citrate (10xSSC) for 1 hour. The DNA was transferred to a nitrocellulose filter by capillary action overnight using 10xSSC. The membranes

were floated on 0.4M sodium hydroxide for 2 mins and then washed in 2xSSC for 2 minutes to remove any agarose from the surface. The membranes were baked for two hours at 80°C under vacuum and were then ready for hybridisation.

2.3.5 DNA dot blotting

The method described by Anderson *et al* (1985c) was used. Five microlitre volumes of each serum or extracted DNA were spotted onto nitrocellulose sheets (Schleicher and Schull, Germany) and allowed to dry. The DNA was denatured by floating the filter on a solution of 0.1M sodium hydroxide. Filters were neutralised in a buffer containing 0.1M Tris and 1M sodium chloride adjusted to pH 7.4. The filters were then washed in 2xSSC for 20 mins, blotted dry and baked for 2 hours for 80°C under vacuum. Hybridisation was then performed.

2.3.6 Random primer labelling of probe DNA

A modification of the random priming method developed by Feinberg and Vogelstein (1983, 1984) was used in which a mixture of random hexanucleotides was used to prime DNA synthesis from linear double-stranded template DNA. 1.5 ng/ml of probe DNA dissolved in sterile distilled water was denatured at 100°C for 10 mins and immediately cooled to 0°C in an ice bath. Reaction mix containing 10µl oligo-labelling buffer (OLB buffer; see below), 2µl of (10mg/ml) bovine serum albumin, 2.5µl of ^{32}P dCTP (Amersham, UK 300-4000 Ci/mM, 10 mCi/ml), 2 units of Klenow fragment of *E.coli* DNA polymerase I (BRL, UK) was added to 25ng boiled probe DNA and made up to a total volume of 50µl with sterile distilled H_2O. The reaction was incubated for at least 4 hours at room temperature. The radiolabelled probe was

separated from the unincorporated dNTPs by chromatography on a "Nick" column (Pharmacia, Sweden). The column was pre-rinsed with TE buffer pH 7.5, the reaction mix was then added to the column and flushed with 800µl of TE buffer. The first 400µl of eluate containing the unincorporated dNTPs was discarded and the second 400µl of eluate containing the radiolabelled probe was collected. The specific activity of the probe was determined using a scintillation counter.

The OLB was made from four component solutions. Solution O contained 1.25M Tris-Cl and 0.125M magnesium chloride at pH8. Solution A was made up of 1µl solution O with 18µl 2-mercaptoethanol, 5µl dATP, 5µl dTTP and 5µl dGTP, each triphosphate previously dissolved in TE pH7. Solution B contained 2M of Hepes, titrated to pH6.6 with 4M sodium hydroxide. Solution C consisted of hexadeoxyribonucleotides (P-L No.2166) suspended in TE at 90 OD units/ml. The solutions A :B :C were mixed in a ratio of 100: 50: 150 to make OLB.

2.3.7 DNA hybridisation

The membranes were incubated with 2xSSC, 0.5% SDS, 0.1mg/ml calf thymus DNA and 2x Denhardt's solution (0.04% each of bovine serum albumin, Ficoll 400 and polyvinyl pyrrolidone) at 65°C for 12 hours in a shaking waterbath. The prehydridisation mixture was replaced with 10ml/100cm^2 10xSSC, 3x Denhardt's, 0.5% SDS, 0.1µg/ml calf thymus DNA, 10% dextran sulphate, and 1-40 mCi heat-denatured ^{32}P-labelled probe DNA, pVTM-1. The membranes were incubated at 65°C for a further 12 hours.

The probe used, pVTM-1, has been described previously (Anderson *et al* 1985c). The 700bp parvovirus DNA insert (corresponding to nucleotides 3141 - 3856) was excised

with the restriction enzyme *Pst*I and subcloned into the *Pst*I digested and phosphatased vector pAT 153.

Following this hybridisation incubation, membranes were washed at 65°C successively in buffers containing 7xSSC, 1x Denhardt's, 0.5% SDS, 0.01mg/ml calf thymus DNA (2 washes of 20 mins); 1xSSC, 0.5% SDS (2 washes of 20 mins); and finally in 0.1xSSC, 0.5% SDS (1 wash of 10 mins). The membranes were blotted, air dried and exposed to Kodak X-Omat S film with an image - intensifying screen at -70°C.

2.4 NESTED POLYMERASE CHAIN REACTION (PCR) METHODS

2.4.1 Oligonucleotide synthesis

Oligonucleotides were prepared on an Applied Biosystems 381A DNA synthesiser according to the manufacturer's instructions.

2.4.2 Nested B19 polymerase chain reaction (PCR) assay

The PCR methods used in the development of the nested PCR are described in the section - Development of nested polymerase chain reaction. The method subsequently used in the application of nested PCR to clinical samples (Application of nested PCR) is described here. Five microlitres of extracted serum was inoculated into a 50µl PCR reaction volume containing 10mM Tris-Cl pH 8.3, 50mM potassium chloride, 1.5 mM magnesium chloride, 0.01%(weight/volume) gelatin, 1 unit recombinant *Taq* DNA polymerase (Perkin Elmer Cetus), 200 µM of each dNTP and

300ng of each oligonucleotide primer, Parpat-1 & Parpat 3AS. Following 1st round PCR amplification 1 µl of 1st round PCR product was transferred into a second 50µl PCR reaction mix. The 2nd round reaction mix, contained the same constituents as the 1st round mix, but substituted the 1st round primers for 300ng of each 2nd round primer, B19-1 & B19-2.

The oligonucleotides primers used are shown in table 1. The 1st round of amplification yielded a 1112bp product. Second round PCR was performed using sequences previously published by Salimans *et al* (1989a), this produced a 104bp product. Thirty five cycles of both 1st and 2nd round amplification were performed using the following conditions, 95°C for 1 min, 55°C for 1.5 min and 72°C for 1 min (Techne PHC-1 automated thermal cycler, Cambridge,UK). Control sera containing 10 and 1 copies of B19 DNA were included in each PCR assay.

Fifteen microlitres samples of the 1st and 2nd round PCR products were then analysed by electrophoresis on a 2% agarose gel. Bands were visualised by ethidium bromide staining.

2.5 COMBINED BIOTIN *IN SITU* HYBRIDISATION AND IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed using the APAAP (alkaline phosphate anti-alkaline phosphatase) method of Cordell *et al* (1984). Five µm paraffin wax embedded sections were cut onto 1% 3'-aminotriethoxysilane coated slides and air-dried at room temperature for at least 12 hours. The slides were baked at 80°C for 20

Round	Primer	Sequence (5'→ 3')	Position
1	Parpat 1	CTT TAG GTA TAG CCA ACT GG	2912-2931
1	Parpat 3AS	ACA CTG AGT TTA CTA GTG GG	4016-3997
2	B19-1	CAA AAG CAT GTG GAG TGA GG	3187-3206
2	B19-2	CCT TAT AAT GGT GCT CTG GG	3290-3271

Table 1: Oligonucleotide primer sequences for nested PCR

mins and dewaxed in 3 x 5 mins washes in xylene. The slides were then washed 3 x 5 mins washes in industrial methylated spirit and rinsed in deionized autoclaved water.

Cells cytospun onto 1% 3'-aminotriethoxysilane slides were air dried and fixed in acetone:methanol 1:1 for 3 mins and then rinsed in a solution containing 0.15M sodium chloride and 0.05M Tris pH7.6 (TBS).

Primary mouse monoclonal anti-cell type specific antibodies were applied to the sections for 60 mins at room temperature. Sections were rinsed in TBS for 2 mins and the secondary rabbit anti-mouse antibody (Dako Z259) diluted 1:30 in TBS was applied to the sections for 30 mins at room temperature and removed with a 2 min rinse with TBS. An APAAP solution containing 8mg/ml calf alkaline phosphatase (AP) (Sigma Type I P3877) in mouse monoclonal anti-AP tissue culture supernatant (kindly supplied by Dr D Masons, Oxford) was added to the slides for 30 mins at room temperature; the slides were then rinsed in TBS for 5 mins. The bound conjugate was visualised with Fast Red solution containing 10mg Napthol AS-MX phosphate (Sigma) added to 1ml dimethylformamide (DMF, Aldrich), 49mls 0.1M Tris pH8.2 and 50 µl of 1M levamisole, Fast Red (Sigma) was added at a concentration of 1mg/ml just before use. This mixture was passed through a 0.2 µm millipore filter directly onto the sections. When the colour had developed the reaction was stopped by rinsing the tissues in deionized autoclaved water.

Proteolytic digestion of the tissue sections was performed prior to *in situ* hybridisation. The sections were immersed in 1% Protease XIV (Sigma) in TBS for 20-30 mins at room temperature, then rinsed in deionized autoclaved water and TBS for 5 mins. Cytospun cells were not subjected to proteolytic digest at any stage. *In situ* hybridisation was then performed using a modification of the method of Porter *et*

al (1990). The DNA in sections and cytospun cells was denatured by placing the slides in deionised autoclaved water pre-heated to 95°C for 10-15 mins for the tissue sections and 3 mins for the cytospun cells. The slides were then placed on an ice block for 3 mins, then air dried.

The probe mixture consisted of 0.005% SDS, 0.05% polyvinyl-pyrrolidone, 10% dextran sulphate (Sigma), 50% deionized formamide (Aldrich) in a solution containing 0.3M sodium chloride, 0.06M Tris pH7, 4mM EDTA (2xSET). This solution was aliquoted and stored at -20°C. Sheared herring sperm DNA (Sigma) at a concentration of 500 μg/ml was added prior to use. The probe was then added to the mix at a final concentration of 1μg/ml. The probe was denatured in the hybridisation mix at 95°C for 20 mins and then snap frozen and stored on ice prior to use.

The probes used included a B19 probe, pYT 104 (gift of Dr P Tattersall, Yale), a Y chromosome repeat sequence probe pHY2.1 (gift of Dr H Cooke, Edinburgh) and a negative control probe, plasmid pBR 322 (without insert). All probes were labelled by the nick translation technique using biotin - II - dUTP (Sigma). The mean fragment length was approximately 200 bp, as determined by agarose gel electrophoresis.

The sections were covered with 6.5 μl probe solution and sealed with a cover slip and silicon grease. Hybridisation was performed at 37°C overnight. The cover slips were removed and the sections washed 3 x 5 mins in TBS with 0.5% Triton X100. Three high stringency rinses in 0.5xTBS at 65°C for 3 mins each were then performed. Non specific protein binding was blocked by the addition of 15% skimmed milk ("Marvel") in a solution containing 0.1M Tris, 0.1M sodium chloride, 2mM $MgCl_2$ pH7.5 (AP7.5) and 0.5% Triton for 20 mins at 37°C. The slides were then rinsed with AP7.5. Hybrids were detected using an avidin-alkaline phosphatase (Dako) conjugate

at a dilution of 1 in 250 in 2% bovine serum albumin (Sigma)/0.5% Triton/AP7.5 for 30 mins at 37°C. The excess conjugate was removed with 3 x 5 mins rinses in 0.5% Triton/AP7.5, 3 x 5 mins rinses in AP7.5 and 3 x 5 min rinses in a solution containing 0.1M Tris, 0.1M sodium chloride and 0.1M $MgCl_2$ pH9 (AP9).

Substrate was prepared by adding 40 µl of 75mg/ml of nitroblue tetrazolium (Sigma, dissolved in 100% N, N dimethyl formamide [Aldrich]) and 120 µl of 50mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma, dissolved in 70% N, N dimethyl formamide) to 40mls of AP9 prewarmed to 37°C. Substrate was filtered through a 0.4µm millipore membrane prior to use. The reaction was allowed to continue until the positive control tissues demonstrated strong specific hybridisation signal, evident as a purplish brown precipitate within the nucleii. The sections were rinsed in deionized autoclaved water to stop the reaction. The sections were mounted with Kaiser's glycerol jelly.

2.6 METHOD FOR CALCULATING GENOME COPY NUMBER IN SAMPLES

The method used was based on the mean molecular weight of a nucleotide base pair being 660 daltons. The copy number was calculated by multiplying the mass of DNA in grams by Avogadro's number and then dividing this result by the product of the number of nucleotide base pairs multiplied by 660.

2.7 STATISTICAL ANALYSIS, THE *F* TEST

The *F* test developed by Fisher tests the null hypothesis that the groups of samples being compared are drawn at random from a single global population (Altman 1991). If this is true then there are two consequences of this: 1) the group means for the individual groups will, within stochastic error, be the same, and: 2) the variances of the groups being analysed will also be largely homogeneous. The *F* statistic is a test of the first of these two statements.

Departures from the hypothesis will lead to a significant *F* test result due to non-equality of the group means (departure from 1. above). The second part of the null hypothesis is not tested and has to be assumed true (if it is not then the *F* test is weakened, but even so is usually adequate). A significant *F* test hence indicates that the group means are not the same (N.B. it does not indicate why they differ, nor does it follow that the result is significant because of any single group).

CHAPTER 3

DEVELOPMENT OF NESTED POLYMERASE CHAIN REACTION

3.1 INTRODUCTION

The development and validation of nested PCR for the detection of B19 virus is described. Initially oligonucleotides B19-1 & 2, were selected and optimised from a previously published method (Salimans *et al* 1989a) to form the inner set of primers for second round PCR. These were found to have sequences unique to B19 virus by comparative analysis using the Los Alamos gene bank. An outer set of primers were then selected (see below-Selection of the outer primers) and characterised to form the first round PCR. The sequences of these are shown in table 1. Finally the two assays were linked and optimised for the detection of B19 DNA in a semi-quantitative fashion.

3.2 OPTIMISATION OF THE INNER PRIMERS, B19-1 & B19-2

3.2.1 Evaluation of Salimans PCR reaction conditions

PCR was performed using the primers and reaction conditions (B19-1 & B19-2, table 1, figure 2) described by Salimans *et al* (1989a) to yield a 104bp product. The optimal cycling conditions were a 95°C melt temperature for 1 minute, a 55°C anneal temperature for 1.5 minutes and 72°C extension time for 2.5 minutes (personal communication, Dr M Salimans, Leiden). Two ngs of pYT-103 (pYT103 is a DNA probe complimentary to B19 DNA nucleotides 2 - 5108) and 1ng of pAT-153 (*E.coli*

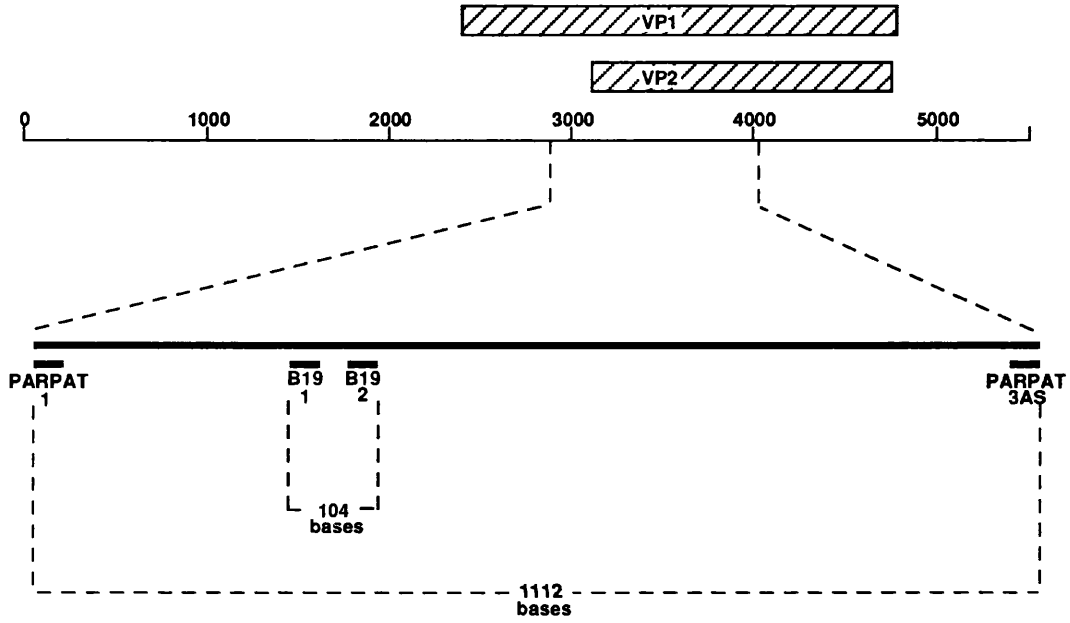

Figure 2: The positions of the oligonucleotide primer sequences in the B19 genome

The positions of the 1st round primers (parpat-1 & parpat-3AS) and 2nd round primers (B19-1 & B19-2) are shown. VP1 & VP2 indicate the regions of the genome coding for the viral structural proteins.

plasmid vector without insert) were used as positive and negative controls respectively. The total reaction volume was 50 µls. Ten microlitres of the PCR reaction mix were removed after every 10 cycles of amplification between 10 and 50 cycles and analysed by electrophoresis on a 2% agarose gel. Specific products were not visible after 10 to 30 cycles, 104bp products were only weakly visible after 40 and 50 cycles. The specificity of these products was confirmed by alkali transfer to Genescreen (Nen Research) filters and hybridisation with pVTM-1 (pVTM-1 is a DNA probe complimentary to B19 DNA nucleotides 3141 - 3856). Only a weak signal was obtained (data not shown).

3.2.2 Comparison of Salimans PCR reaction conditions with Saiki reaction conditions

The Salimans method utilises a PCR reaction mixture that differs in several ways from the more generally used method described by Saiki (1989). The buffer of the former method contains 50mM NaCl in Tris buffered to pH 9.6 as opposed to 50mM KCl in Tris-Cl buffered to pH 8.3 and 0.2mg/ml BSA in the Saiki buffer. Similarly there is 10mM $MgCl_2$ in the former as opposed to 0.001% gelatin w/v and 15mM $MgCl_2$ in the latter. The method described by Salimans *et al* (1989a) uses 1µg of each primer diluted in Tris/EDTA/NaCl contrasting with 300ng diluted in water used in the Saiki method.

The controls were assayed using the Saiki reaction mix while maintaining the same cycling times and temperatures as used in the method of Salimans and colleagues. Ten microlitres of product were removed after every 10 cycles between 10 and 50 cycles as before, analysed on a gel, alkali transferred and hybridised. 104bp products were visible after 20 and 50 cycles of amplification. Non-specific DNA products

began to appear on the gel following 40 cycles of amplification. The 104bp product, but not the non specific products, hybridised with pVTM-1 following alkali transfer to a Genescreen filter (data not shown). A PCR assay was subsequently performed using 35 cycles of amplification, this did not produce the non-specific DNA products encountered with 40 cycles of amplification. As a result of this experiment the Saiki reaction mix was adopted and 35 cycles of amplification performed in all subsequent experiments.

3.2.3 Determination of the optimal magnesium chloride concentration for B19-1 & B19-2 primers

The PCR reaction is dependent on the magnesium concentration for the annealing of the primers to their templates (Britten & Davidson 1985) and for the activity of *Taq* polymerase (Gelfand 1989). Therefore it was important to determine the optimal $MgCl_2$ concentration for the primers being used. The reaction mix described by Saiki (1989) contains less $MgCl_2$ (0.5mM/ml) than in the reaction mix described by Salimans *et al* (1989a). The effect of changing the $MgCl_2$ concentration over the range 5-30mM range in 5mM increments was investigated. Little change in the amount of B19 specific product (as determined by the 104bp band intensity in the gel) was observed over the range, but the amount of product was maximal at 15mM concentration (data not shown). This concentration of magnesium was used in all subsequent experiments with this set of primers.

3.2.4 Determination of the optimal primer concentration for B19-1 & B19-2

The optimal primer concentration was determined by titration of B19-1 & B19-2 from 60ng-600ng (6-60 picomoles) of each primer per 50µl reaction volume. This experiment was repeated on a number of occasions with newly prepared batches of primers and various low copy number B19 DNA controls. No difference was observed in the intensity of the B19 specific product detectable on gel electrophoresis in the 300-600ng primer range. Smaller quantities of primer produced less B19 specific 104bp product and these results were less reproducible with different primer batches. Three hundred nanograms of each primer was therefore used in subsequent PCR assays for reasons of economy.

3.2.5 Variation of extension time

The 104bp products observed in the gel with the 2.5 minute extension time at 72°C used in the previous experiments were indistinct (plate 1). *Taq* polymerase extends along the DNA template at approximately 1000 bases/minute (Innis *et al* 1988) so that the extension time utilised in the assay was unnecessarily long for the size of product to be generated. In an attempt both to increase the efficiency of the reaction and to enhance the sharpness of the product bands observed in a 2% agarose gel following electrophoresis, the extension time was shortened to 1 minute. The 104bp product seen in the gel appeared much more well delineated with the 1 minute extension time (plate 2).

Plates 1 & 2

Effect of altering the DNA extension time on PCR performance

The effect of altering the DNA extension time from 2.5mins (plate 1) to 1 min (plate 2) in single round PCR using primers B19-1 & B19-2 is shown. PCR products were electrophoresed in a 2% agarose gel and stained with ethidium bromide. Key; A = B19 viraemic plasma A1; B = pAT 153 (negative control); C = pVTM-1. One ng of each DNA sample was used in the PCR assay. MW = molecular weight marker, DRIgest III (Pharmacia, Sweden).

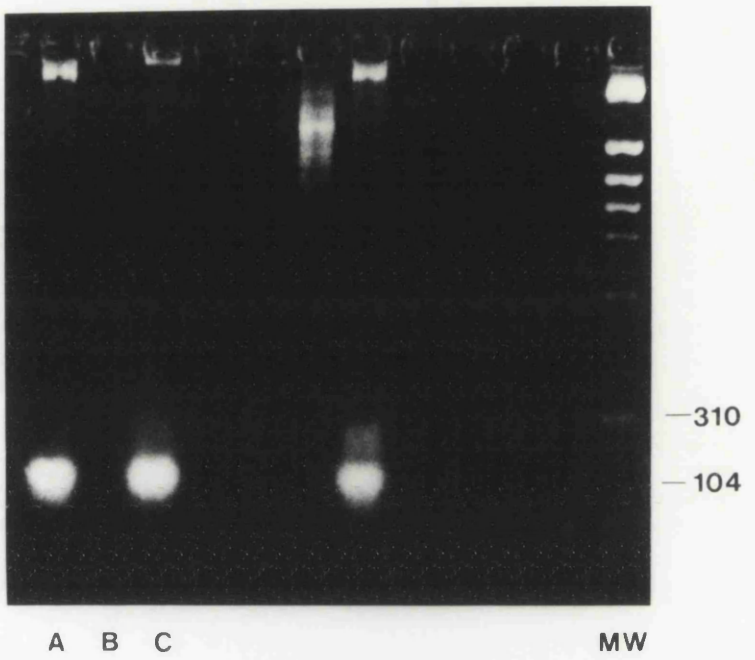

Plate 1

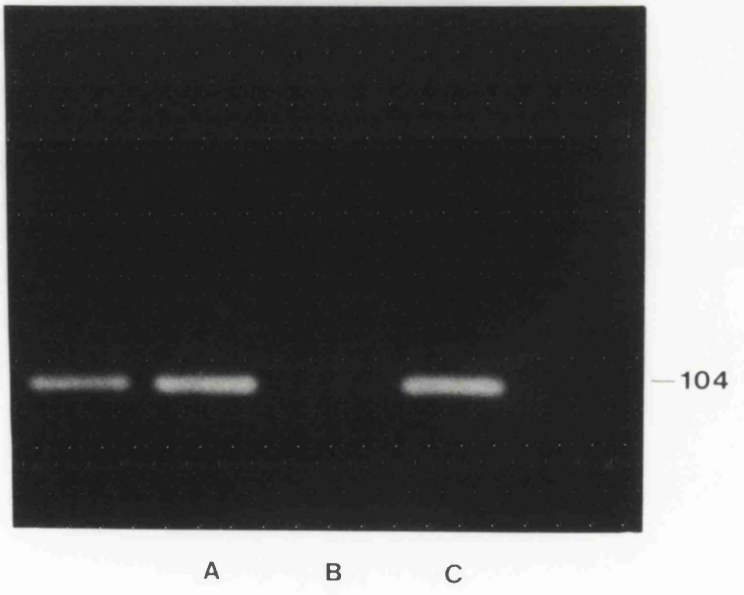

Plate 2

3.2.6 Comparison of different blotting methods for confirmatory hybridisation of the PCR reaction product

One nanogram of pVTM-1 was amplified by PCR for 35 cycles, loaded into four wells of a 2% agarose gel and electrophoresis performed. The gel was divided into four parts and the DNA transferred by either alkali transfer or saline sodium citrate (SSC) transfer to either Zeta probe (Biorad) or Genescreen filters. The filters were hybridised with pVTM-1 and autoradiographed (plate 3). Alkali transfer to Zeta probe gave the greatest signal as determined by autoradiography. All subsequent hybridisations of PCR products were performed by this method.

3.2.7 Summary

The optimum PCR conditions using primers B19-1 and B19-2 utilised the reaction mixture described by Saiki (1989) with a 15mM $MgCl_2$ concentration and 300ng of each primer in a 50 µl reaction volume. The cycling conditions used were 95°C for 1 minute, 55°C for 1.5 minutes and 72°C for 1 minute. Optimum hybridisation of the PCR reaction product was obtained following alkali transfer onto Zeta probe filters.

3.3 DEVELOPMENT OF AN OUTER SET OF PRIMERS

3.3.1 Selection of the outer primers

A second set of primers was selected, homologous to regions flanking B19-1 & B19-2, for the purpose of developing nested PCR (table 1, figure 2). At this time a contamination problem developed, thought to be due to pVTM-1 DNA; the

Plate 3

Comparison of different blotting methods for confirmatory hybridisation of the PCR reaction products

Single round PCR was performed using primers B19-1 & B19-2. DNA products were electrophoresed into a 2% agarose gel and transferred by alkali transfer (i,iii) or by saline sodium citrate (SSC) transfer (ii,iv) to either Genescreen (i,ii) or Zeta probe (iii,iv). The membranes were hybridised with ^{32}P labelled pVTM-1 and autoradiographed with Kodak X-Omat S film for 3 hours at -70°C. Key; A = no DNA added (negative control), B = 1 ng of pVTM-1.

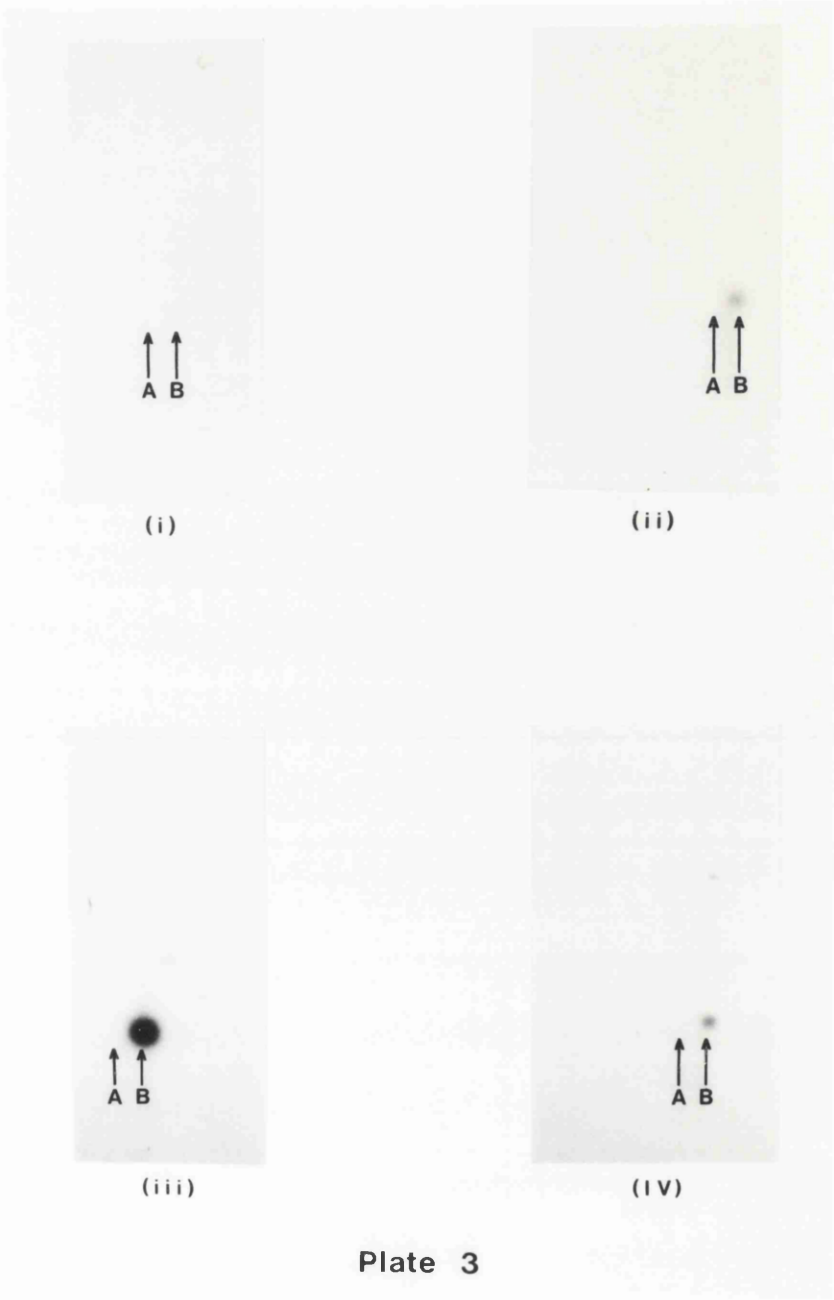

Plate 3

oligonucleotide sequences were therefore selected to fall outside the nucleotide positions 3141-3856, corresponding to the sequence of pVTM-1. Additional criteria for the selection of the primers were the uniqueness of the sequences to B19 virus (as determined by Genebank search), the avoidance of nucleotide repeats, a G-C ratio of approximately 50%, the lack of complimentarity between the primers or with B19-1 & B19-2 and a size of 20 bases per primer (Saiki 1989).

Upstream of pVTM-1, nucleotides 2912-2931 were found to meet these criteria. This primer was designated Parpat-1. For up to 400 bases downstream of PVTM-1 there were no sequences unique to B19 virus. However since the efficiency of PCR amplification decreases with increasing size of the DNA sequence to be amplified, a compromise was made to limit the size of the PCR product. The oligonucleotide antisense sequence from nucleotides 3997-4016 was selected. It was found to share 80% homology with the mouse beta 2 microglobulin gene and 40% homology with mouse interferon-induced MX2mRNA. This primer was designated Parpat-3AS.

These primers fall within the structural coding region of the genome (figure 2).

3.3.2 Optimisation and specificity of PCR using Parpat-1 and Parpat-3AS

The calculated T_m anneal temperature (4°C for each G/C and 2°C for each A/T ([Thein & Wallace 1986]) for these primers was 60°C. An anneal temperature 5°C below the calculated T_m was selected for the PCR assay, this temperature was identical to that used in the PCR assay with B19-1 & B19-2 primers. Initially therefore the PCR was performed using the same conditions as those found to be optimal for the B19-1 and 2 primers. Three hundred nanograms of each primer was

used per reaction. The positive control used was 1ng of DNA extracted from viraemic plasma SM (see appendix), the negative control was 1ng of DNA extracted from normal human placenta and found to be B19 DNA negative by dot blot hybridisation. A DNA fragment was detectable on gel electrophoresis corresponding to the predicted 1112bp product in the gel lane containing the positive control reaction product (data not shown). DNA products were not observed in the gel lanes bearing the negative control reaction products. Hybridisation with pVTM-1 following alkali transfer confirmed the specificity of this 1112bp product.

3.3.3 Determination of the optimal primer concentration for Parpat-1 & Parpat-3AS

The primers were titrated from 120ng-600ng (12-60 picomoles) of each primer per reaction volume with 1 ng of SM DNA. There was no difference in the concentration of B19 specific product (as determined by the intensity of the ethidium bromide staining of the reaction product run in a 2% agarose gel) observed between 300-600ng although at 120ng less product was present (data not shown). Non-specific DNA amplification did not occur over this primer concentration range. For reasons of economy and consistency with the inner reaction a 300ng concentration was selected for subsequent PCR assays.

3.3.4 Summary

Two primers, designated Parpat-1 & Parpat-3AS, were selected for PCR in sequences flanking the position of pVTM-1 in the B19 virus genome (table 1, figure 2). These were used in reaction conditions identical to those used for primers B19-1 & B19-2 and found to produce a B19-specific product with DNA extracted from plasma SM

but not with human placental DNA. The optimal primer concentration was 300ng per reaction. No further optimisation of the PCR reaction was undertaken.

3.4 EVALUATION OF NESTED PCR USING PARPAT-1, PARPAT-3AS, B19-1 & B19-2 PRIMERS

3.4.1 Comparison of nested with single round PCR

Nested PCR was performed using the sets of primers previously characterised. Plasma SM titrated in B19 negative serum was assayed. B19 negative serum was defined as serum testing negative for B19 DNA by DNA dot blot hybridisation and negative for B19 IgM and IgG by MACRIA and GACRIA respectively. A modification of a sample addition method previously described by Koch and Adler (1990) was used, in which no pre-extraction of sample DNA was performed. One microlitre of the dilution series was added to a 50µl PCR reaction mix containing Parpat-1 & Parpat-3AS but no *Taq* polymerase and heated for 6 minutes at 95°C. *Taq* polymerase was then added and 35 cycles of amplification performed (1st round PCR). One microlitre of PCR product were then transferred to a second 50µl PCR reaction mix containing B19-1 & B19-2 and a further 35 cycles of amplification performed (2nd round PCR). First and 2nd round products were analysed following gel electrophoresis by ethidium bromide staining (plates 4,5) and by alkali transfer and hybridisation (plate 6). First round PCR was able to detect B19 DNA diluted 10^{-2} in plasma SM, second round PCR increased this sensitivity to 10^{-8} (as detected by ethidium bromide staining of the gel). No additional sensitivity was produced by alkali transfer and hybridisation of the 2nd round PCR products of further dilutions of SM. The dilution series of plasma SM was also submitted to PCR amplification using just the primers B19-1 & B19-2, B19 DNA was detected to a dilution of 10^{-5} by gel electrophoresis and ethidium bromide staining (plate 7). Nested PCR was able to

Plate 4

Titration of B19 viraemic plasma in single round PCR with primers Parpat-1 & Parpat-3AS

B19 viraemic plasma SM was titrated in B19 negative serum and subjected to single round PCR using primers Parpat-1 & Parpat-3AS to produce a 1112bp product. The plasma was added directly to the PCR reaction mix and heated to 95°C for 6 minutes prior to addition of the *Taq* polymerase and thermal cycling. PCR products are shown following electrophoresis in an ethidium bromide stained 2% agarose gel. Key; neg = no DNA added; lane 1 = product from undiluted viraemic plasma; lane 2 = plasma at a dilution of 10^{-1}; lane 3 = plasma at a dilution of 10^{-2}; MW = molecular weight marker, 1 kb DNA ladder (BRL, USA).

Plate 5

Titration of B19 viraemic plasma in nested PCR

B19 viraemic plasma SM was titrated in B19 negative serum and subjected to nested PCR using primers Parpat-1 & Parpat-3AS for the first round amplification and B19-1 & B19-2 for the second round amplification to produce a 104bp product. The serum was added directly to the first round PCR reaction mix and heated to 95°C for 6 minutes prior to addition of the *Taq* polymerase and thermal cycling. The PCR products following second round amplification are shown in an ethidium bromide stained 2% agarose gel. Key; neg = no DNA added; lanes 1-9 contain products of the titration of viraemic plasma from 10^{-1} (lane 1) to 10^{-9} (lane 9); MW = molecular weight marker, 1 kb DNA ladder (BRL, USA).

Plate 4

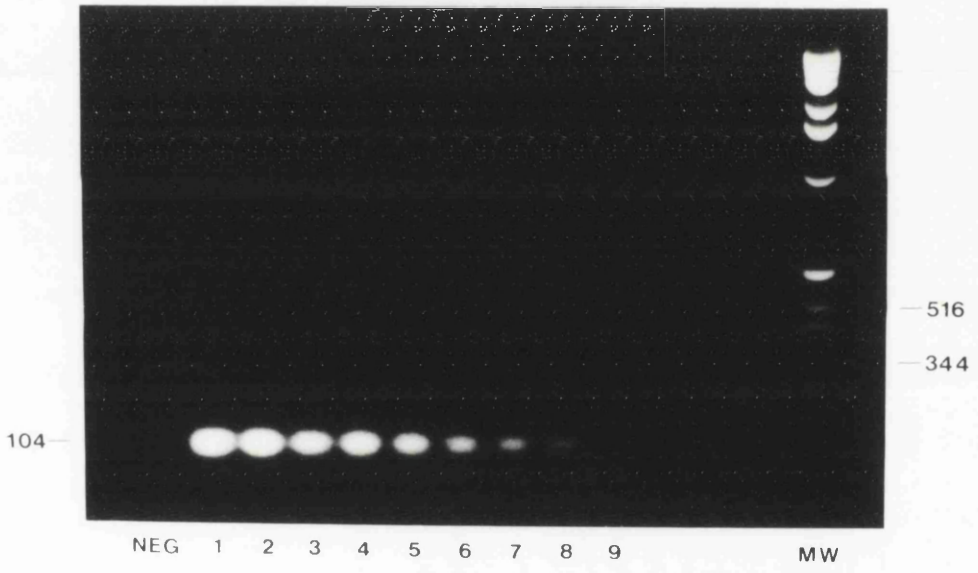

Plate 5

Plate 6

Southern blot hybridisation of B19 viraemic plasma titration following nested PCR

B19 viraemic plasma SM was titrated in B19 negative serum and subjected to nested PCR (see plates 4,5). DNA products were alkali transferred to Zeta Probe membrane and probed with ^{32}P labelled pVTM-1 and autoradiographed with Kodak X-Omat S film for 24 hours at -70°C. Rounds 1 & 2 indicate products of first and second round amplification. Key; neg = no DNA added; lanes 1-8 contain PCR products of the titration of viraemic plasma from 10^{-1} (lane 1) to 10^{-8} (lane 8).

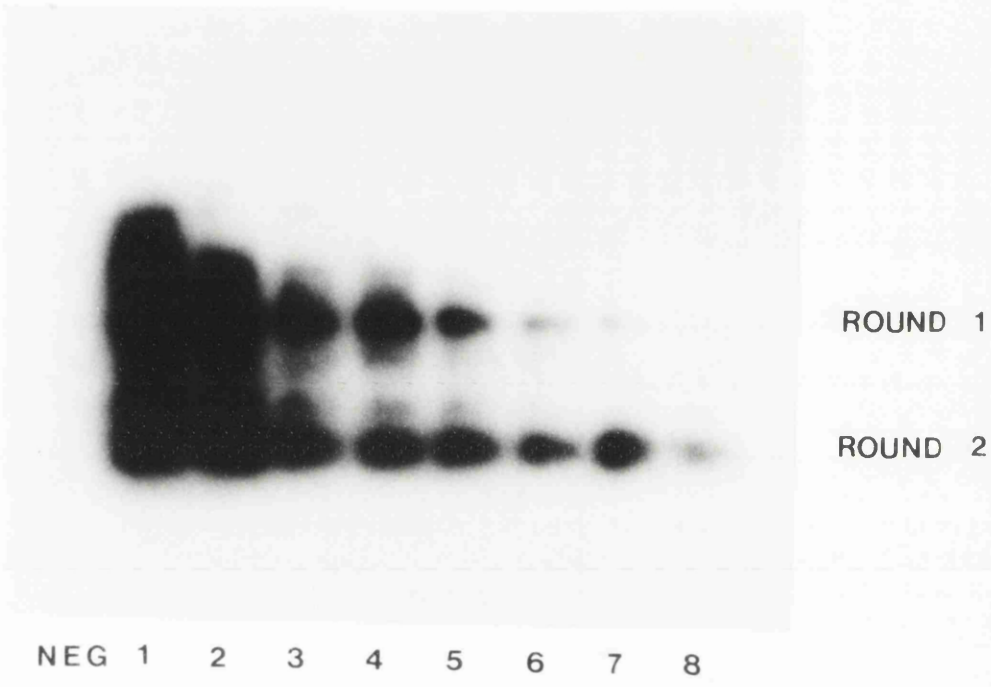

Plate 6

Plate 7

Titration of B19 viraemic plasma in single round PCR with primers B19-1 & B19-2

B19 viraemic plasma SM was titrated in B19 negative serum and subjected to single round PCR using primers B19-1 & B19-2. The serum was added directly to the PCR reaction mix and heated to 95°C for 6 minutes prior to addition of the *Taq* polymerase and thermal cycling. PCR products are shown following electrophoresis in an ethidium bromide stained 2% agarose gel. Key; neg = no DNA sample; lanes 1-5 contain PCR products of the titration of viraemic plasma from 10^{-1} (lane 1) to 10^{-5} (lane 5); MW = molecular weight marker, 1 kb DNA ladder (BRL, USA).

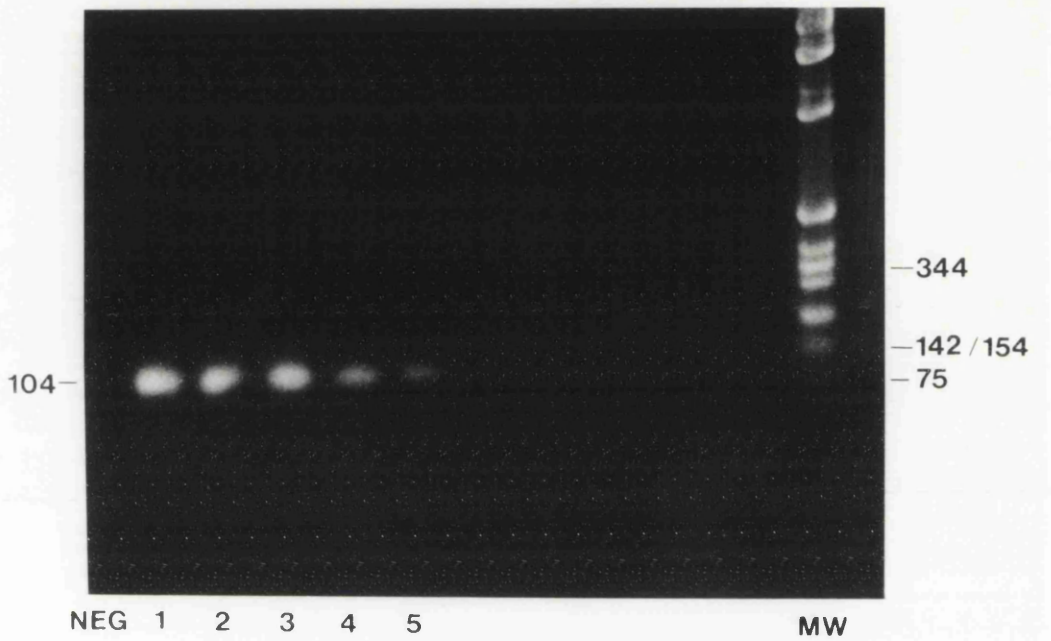

Plate 7

enhance the sensitivity of B19 DNA detection by 10^3 compared with single round PCR amplification.

3.4.2 Determination of the sensitivity of nested PCR

The sensitivity of the nested PCR was determined in two ways. Firstly plasma A1, known to contain $7x10^{10}$ B19 particles/ml as determined by electron microscopy (kindly provided by Dr C.Potter, Oxford), was titrated in B19 negative serum and assayed. One microlitre of sample was added to the PCR reaction mix and heated to 95°C for 6 minutes. The samples were then submitted to nested PCR amplification. In the first nested PCR experiment second round PCR product was detected to a dilution of 10^{-10} and in a second experiment to a dilution of 10^{-8}. The performance of both these PCR assays was assessed by the inclusion of cut-off controls (see methods - Nested B19 PCR assay) and found to be similar. The explanation for the difference in the titration end points could be due either to pipetting errors in the dilution of very low copy numbers of virus or the reaching of the Poisson distribution endpoint where there is a diminishing statistical probability of any one µl sample containing a copy of B19 DNA. Nested PCR was calculated to detect a single copy of B19 DNA/microlitre in A1 when using the 10^{-8} dilution endpoint, while the 10^{-10} dilution endpoint would suggest that although $7x10^{10}$ B19 particles/ml were visualised by electron microscopy in the plasma, there was degeneration of some particles with preservation of their DNA.

The second method employed to quantify the nested PCR was by determining the amount of B19 DNA in plasma SM by dot blot hybridisation and comparing this result with the SM titration endpoint in PCR previously described. Plasma SM was titrated in TE buffer, 5 µl of sample were dot blotted and hybridised with pVTM-1.

B19 DNA was detectable in plasma SM to a dilution of 10^{-4} (plate 8) in the dot blot hybridisation assay. The limit of sensitivity of B19 dot blot hybridisation was shown to be 5 x 10^4 copies (0.35pg) of pVTM-1 DNA and 5 x 10^5copies (35pg) of pYT103 DNA. This indicates that plasma SM contains between 10^8 and 10^9 copies of B19 DNA/microlitre and would suggest that the 10^{-8} dilution of SM, in which B19-specific product was detectable by nested PCR, contained 1-10 copies of B19 DNA.

3.4.3 Summary

Nested PCR was developed using Parpat-1 & Parspat-3AS in 1st round PCR and B19-1 & B19-2 in 2nd round PCR. Nested PCR was 10^6 times more sensitive than first round PCR and 10^3 more sensitive than 2nd round PCR. The sensitivity of the assay was determined by titration of plasma A1, of known B19 particle copy number, and by comparison of a plasma SM titration in PCR with quantified dot blot hybridisation. Both methods indicate that the assay was able to detect between 1-10 copies of B19 DNA in plasma without prior DNA extraction.

3.5 EVALUATION OF NESTED PCR WITH CLINICAL SERUM SAMPLES WITHOUT PRIOR DNA EXTRACTION

Nested PCR was performed on 11 B19 IgM positive/dot blot negative sera, one fetal serum known to be dot blot positive for B19 DNA, and sequential sera collected from five B19 virus inoculated volunteers (Anderson *et al* 1985b; Potter *et al* 1987). The B19 IgM positive samples had been submitted to the diagnostic laboratory for B19 investigation. Three of the patients had a rash illness, four had rash with arthralgia and the other four had arthralgia alone. The sera were collected between 1 and 60 days (median 7 days) after the onset of symptoms. The samples contained between 7

Plate 8

DNA dot blot hybridisation of a titration of B19 viraemic plasma using pVTM-1 and pYT 103

Samples were titrated in TE buffer and dotted onto a nitrocellulose sheet (Schleicher & Schull). Samples were probed with ^{32}P labelled pVTM-1 and autoradiographed with Kodak X-Omat S film for 48 hours at -70°C. Key; row A shows 10-fold dilutions of B19 viraemic plasma SM from undiluted (position 1) to 10^{-9} (position 10); row B shows 10-fold dilutions of pYT 103 from 5 x 10^8 copies (35ng) (position 1) to 0 copies (position 10); row C shows 10-fold dilutions of pVTM-1 from 5 x 10^9 copies (35ng) (position 1) to 5 copies (0.035fg) (position 10). The autoradiograph shows positive signal in position 4 of row B and in positions 5 and 6 of row C. These have failed to photograph.

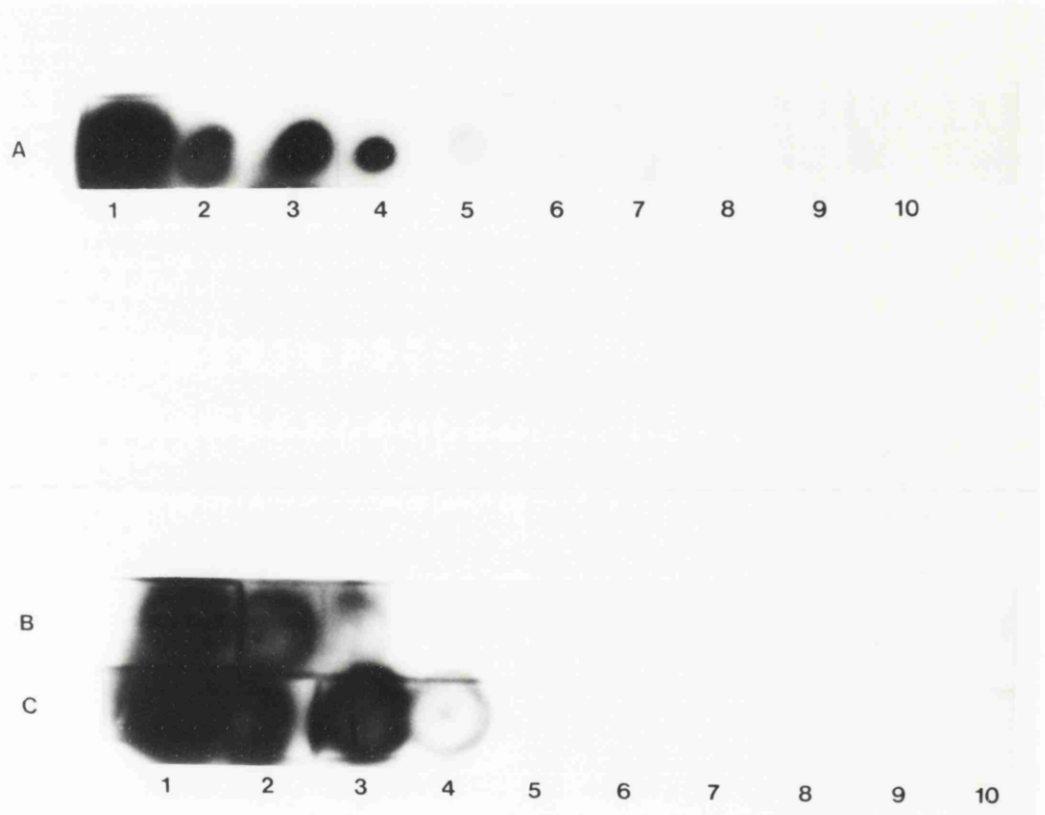

Plate 8

to greater than 100 MACRIA arbitary units of B19-specific IgM. The fetus had been found to be hydropic on ultrasound examination and serum was collected at 24 weeks gestation.

Four of the five B19 infected volunteers were inoculated in 1984 with the purpose of defining the clinical and serological course of B19 infection and have been previously described (Anderson *et al* 1985b; Potter *et al* 1987). The fifth was inoculated in 1989 (see appendix). The subjects were inoculated with B19 virus obtained as plasma from a unit of blood donated to the National Blood Transfusion Service. The volunteers were inoculated intranasally with plasma diluted in Hank's buffered saline containing 0.2% bovine serum albumin. Serum was collected two days before inoculation and up to 3 months post inoculation. Dot blot hybridisation, B19 IgM and IgG serology were performed on all the sera. None of the sera tested were visibly haemolysed

One microlitre of serum was added directly to a 50µl volume of 1st round reaction mix and heated to 95°C for 6 minutes. This was followed by 35 cycles of amplification, 1µl of product was transferred into 50µl of 2nd round reaction mix and subjected to a further 35 cycles of amplification. Fifteen µicrolitres of both 1st and 2nd round product were analysed in a 2% agarose gel following electrophoresis and ethidium bromide staining.

Only one of the 11 B19-IgM containing sera was found to contain B19 DNA by nested PCR and this was only detectable after 2 rounds of PCR amplification. The patient had presented with rash and arthralgia with a date of onset of symptoms 2 days previously and her serum was found to contain 14 units of B19 IgM. The fetal sample was found to contain B19 DNA after both 1st and 2nd round amplification.

The profile of DNA detection and serological results for the volunteers are shown in

figure 3. Viraemia was detectable by dot blot hybridisation in the serum of three of the volunteers from day 6 to day 9/10, between days 8 and day 10 in volunteer C and between days 5 and 11 in volunteer D. The 1st round PCR yielded detectable DNA only in those sera that were dot blot positive. The single exception to this was a serum collected at day 3 from volunteer D. In each case B19 DNA became undetectable by nested PCR between 2 days before to 9 days after the appearance of B19 IgM.

3.6 EVALUATION OF DIFFERENT SERUM DNA PREPARATION METHODS FOR PCR

Three different methods, method A, method B and method C (see method section), were evaluated for the extraction of B19 DNA from plasma SM and compared in PCR using B19-1 & B19-2 primers. Method A was my own modification of a previously published method (Higuchi 1989). One hundred microlitres of undiluted plasma SM and SM at a dilution of 1/100 in B19 negative serum were extracted using each method. The extracted DNA was added to a 50µl PCR reaction mix and subjected to 35 cycles of amplification with B19-1 & B19-2. The results are shown in plate 9.

B19 specific PCR product was detectable in undiluted plasma SM by all 3 methods and in diluted SM by methods A and C following gel electrophoresis. The quantities of B19 specific product generated by PCR following the different DNA extraction methods were estimated by determining the ethidium bromide staining intensities of the 104bp products observed after gel electrophoresis. Only a small amount of B19 specific DNA could be detected in the reaction product of diluted SM sample extracted by method B. Method A produced the greatest quantity of B19 specific

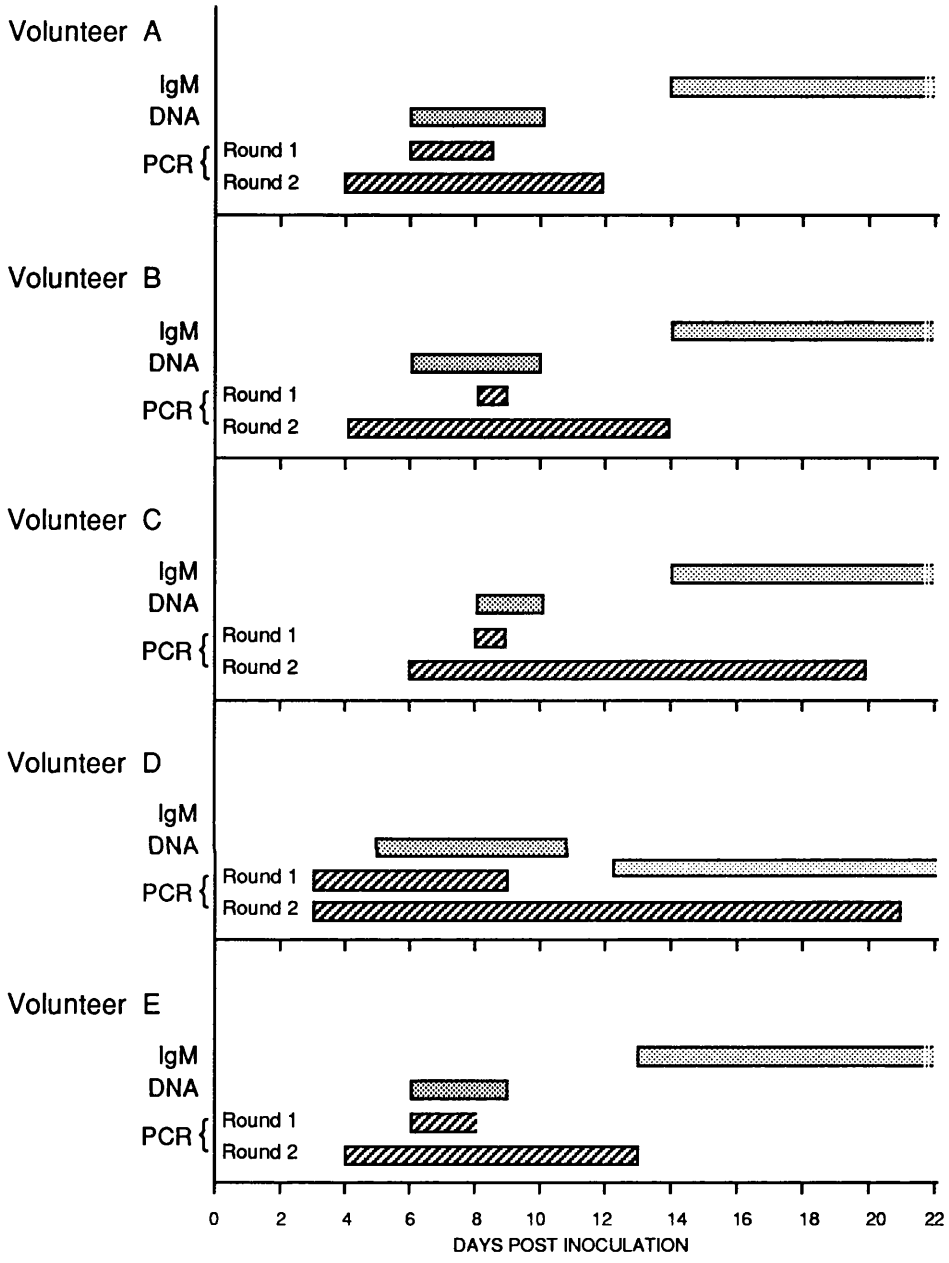

Figure 3: Profiles of B19 markers in five B19 infected volunteers

Serial sera obtained from 5 B19 infected individuals were tested for B19 IgM by MACRIA and for B19 DNA by dot blot hybridisation and by nested PCR. Serum was added directly to the PCR (direct serum addition method). Horizontal bars indicate a positive reaction in the test and all samples were tested for each method indicated

Plate 9

Comparison of different preparation methods for the extraction of DNA from serum for B19 PCR

DNA was extracted from undiluted (i) and 1/100 dilution (ii) of B19 viraemic plasma SM titrated in B19 negative serum by 3 methods; A, B & C (see methods section). Single round PCR using primers B19-1 & B19-2 was performed on the extracted DNA. PCR products are shown following electrophoresis in an ethidium bromide stained 2% agarose gel. Key; + = positive control, 1ng of pVTM-1; - = negative control, no DNA added; MW = molecular weight marker, 1 kb DNA ladder (BRL, USA).

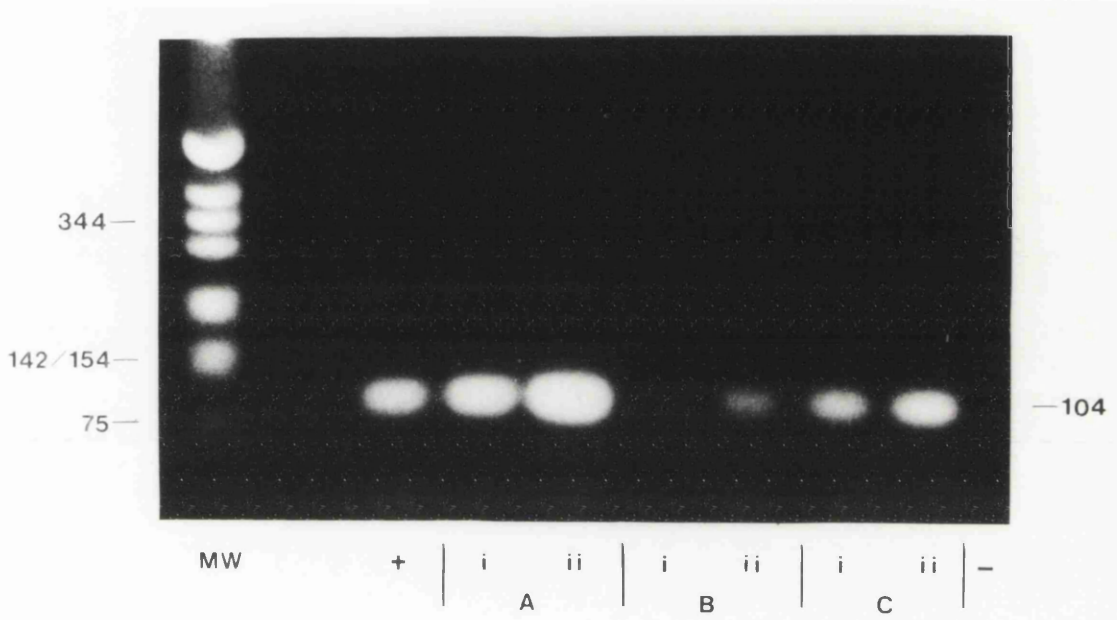

Plate 9

product, method C produced less B19 specific product and method B generated the least amount of B19 specific product. Therefore method A appeared to be the optimal of the three methods.

3.7 EVALUATION OF NESTED PCR WITH CLINICAL SERUM SAMPLES FOLLOWING DNA PREPARATION USING METHOD A, NON-IONIC DETERGENT AND PROTEINASE K TREATMENT

The 11 B19 IgM positive sera and B19 infected volunteer samples, previously assayed by direct addition of serum to the PCR reaction mix, were reassayed following DNA extraction using method A, non-ionic detergent and proteinase K treatment. Five microlitres of extract was added to 50µl of reaction mix and submitted to 1st and 2nd round PCR. Nine of the 11 B19 IgM positive sera contained B19 DNA detectable on gel electrophoresis, after 2 rounds of PCR amplification, a further sample produced only a small amount of 104bp product, the specificity of which was confirmed by hybridisation with pVTM-1. The sample previously shown to contain B19 DNA after 2 rounds of amplification following direct serum addition to the PCR, contained B19 specific product detectable by gel electrophoresis after both 1st and 2nd round PCR. The serum that did not contain detectable B19 DNA had been collected 2 months after the onset of an arthritic illness and contained only 7 MACRIA arbitary units of B19 IgM.

Pre-B19 inoculation sera from 2 of the volunteers contained B19 DNA after nested PCR and therefore could have been contaminated with extraneous B19 DNA. B19 DNA was detectable after 1st round PCR from days 4,5 and 6 to days 20,25 and 20 respectively in the other 3 volunteers. B19 DNA was detected after 2 rounds of amplification on the day of inoculation in two of these volunteers. A day 0 serum

was not collected from the 3rd volunteer. Two of the volunteers had detectable B19 DNA 46 days post inoculation by nested PCR , their last serum collection. Serum from the 3rd volunteer contained B19 DNA detectable by nested PCR up to 36 days post inoculation. The results obtained with this volunteer are shown in figure 4.

First round PCR was able to detect B19 DNA during the phase of B19 infection when no other diagnostic markers are present, the period between the disappearance of viraemia (detected by dot blot hybridisation) and the appearance of B19-specific IgM.

The yield of specific PCR product, as determined by the intensity of the 104bp product following gel electrophoresis and ethidium bromide staining, was similar for each of the detergent treated sera. Indeed a titration of the positive control plasma SM produced similar quantities of B19 specific PCR product at each dilution, even in the dilution of plasma one log above the dilution producing no B19 specific product (plate 10). This would suggest that the nested PCR had reached saturation kinetics. This contrasts with the direct serum addition experiment where the amount of B19 specific reaction product generated in the nested PCR assay decreased with increasing dilutions of target DNA. Method A for DNA extraction from serum was used in all the subsequent experiments described.

3.8 DETECTION OF B19 DNA BY NESTED PCR IN SERA COLLECTED FROM DIVERSE GEOGRAPHIC LOCATIONS AND ARCHIVAL SPECIMENS

PCR amplification is dependent on the specificity of primer binding to complementary sequences on the target DNA. Nucleotide sequence differences found in different viral strains (Morinet *et al* 1986; Mori *et al* 1987; Umene & Nunoue 1990) may lead to failure of the primers to bind to the target DNA with consequent

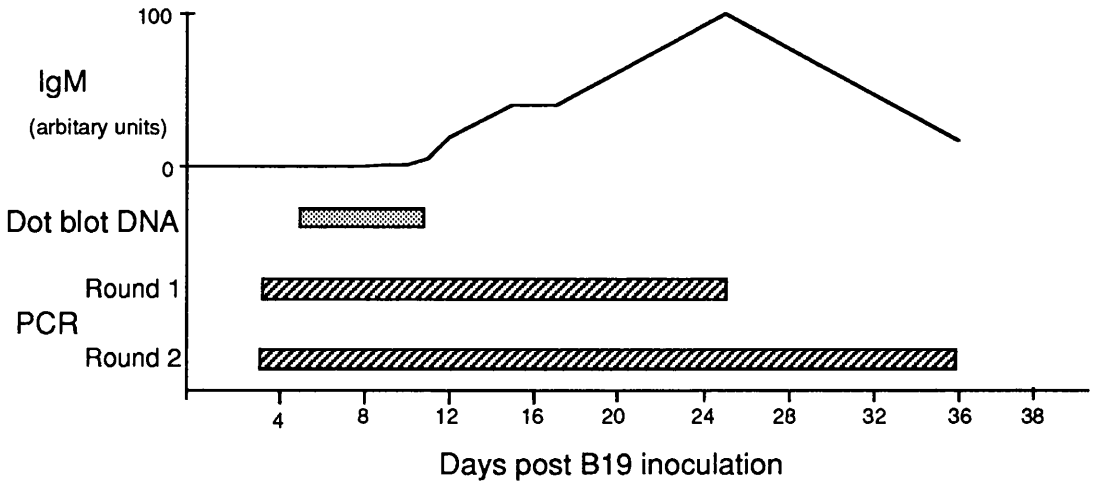

Figure 4: Pattern of B19 markers in an infected volunteer following DNA extraction by method A

Serial sera obtained from a B19 infected volunteer were tested for B19 IgM by MACRIA and for B19 DNA by dot blot hybridisation and by nested PCR. DNA was extracted for PCR using method A - proteinase K/detergent treatment (see methods section). Horizontal bars indicate a positive reaction in the test and all samples were tested for each method indicated. The magnitude of the IgM response is indicated by the line.

Plate 10

Nested PCR of a titration of B19 DNA extracted from viraemic plasma by method A

DNA was extracted from B19 viraemic plasma SM, titrated in B19 negative serum by method A (see method section). Nested PCR was performed, PCR products are shown following electrophoresis in an ethidium bromide stained 2% agarose gel. Key; A = B19 viraemic plasma containing 10 copies of B19 DNA; B = B19 viraemic plasma containing 1 copy of B19 DNA; C = dilution of B19 viraemic plasma containing 0 copies of B19 DNA; - = B19 negative serum. MW = molecular weight marker, 1 kb DNA ladder (BRL, USA).

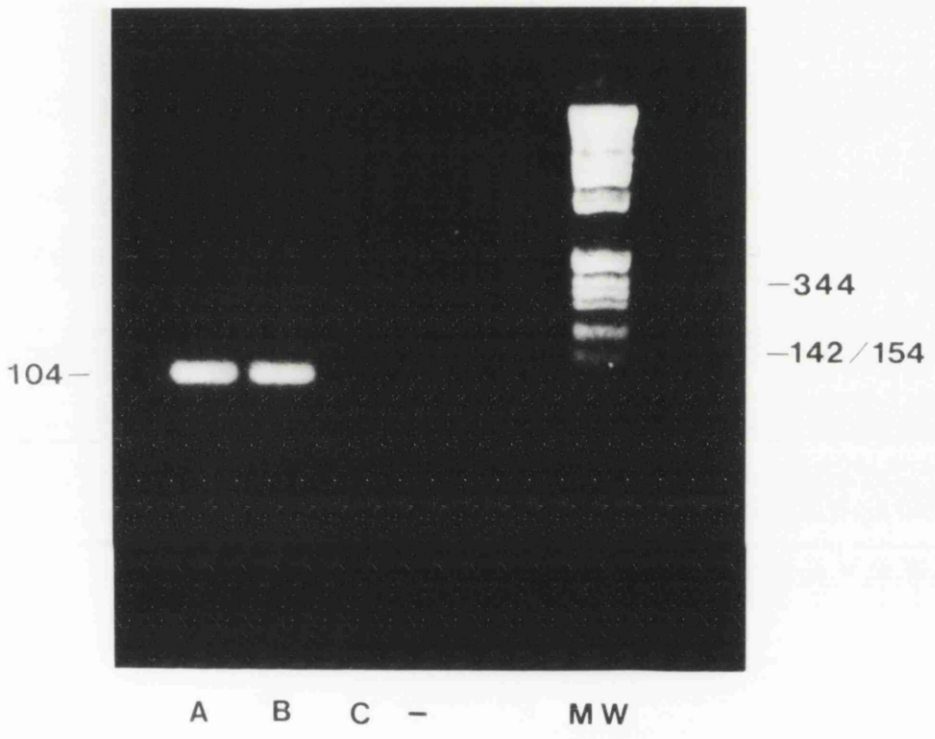

Plate 10

failure of amplification. The ability of the nested PCR assay to detect B19 DNA in potentially divergent strains of B19 virus was assessed by assaying dot blot positive sera from diverse geographical locations and archival material. Twelve samples were collected between 1980 & 1987.

All sera were assayed following detergent and proteinase K extraction (method A). First round and 2nd round B19-specific products were detected in all the samples tested (table 2). These results suggest that the primers used in the nested PCR are from a conserved region of the B19 genome.

Location	Year	Diagnosis	PCR 1st round	PCR 2nd round
Chicago, USA	1980	Aplastic crisis	+	+
Winnipeg, Canada	1980	Rash	+	+
Chicago, USA	1982	Aplastic crisis	+	+
Chicago, USA	1984	Aplastic crisis	+	+
Oxford, UK	1984	" "	+	+
London, UK	1984	" "	+	+
Jamaica	1985	Aplastic crisis	+	+
Preston, UK	1985	" "	+	+
Jamaica	1986	Aplastic crisis	+	+
Iceland	1986	Rash	+	+
Oxford, UK	1987	Rash	+	+
London, UK	1987	Aplastic crisis	+	+

Table 2: Archival B19 DNA dot blot positive sera from diverse geographic locations

CHAPTER 4

APPLICATION OF NESTED POLYMERASE CHAIN REACTION

4.1 SERA SUBMITTED TO THE DIAGNOSTIC B19 LABORATORY

One hundred and forty nine sera, collected from 127 patients up to 150 days (median 10 days) after the onset of symptoms, submitted to the diagnostic laboratory for B19 serological testing, were assayed for the presence of B19 DNA by nested PCR. All the sera were tested for evidence of recent B19 infection by DNA dot blot hybridisation and by MACRIA and GACRIA for B19 specific IgM and IgG respectively. Sera were considered to have a laboratory confirmed B19 diagnosis if B19 DNA (detected by DNA dot blot hybridisation) or B19 IgM were detectable. Eighty sera collected from 58 patients fulfilled these criteria, 79 sera contained B19 IgM and the other serum was DNA dot blot positive. An additional convalescent serum, B19 IgM negative, but collected from a patient previously shown to be IgM positive was also included in this diagnostic group. A further 68 sera collected from 68 subjects did not contain markers of recent B19 infection. The diagnoses of these patients are shown in table 3. All B19 IgM negative sera collected from patients with a rash illness were tested for rubella IgM, all were negative.

Nested PCR was performed on these sera and the products examined for the presence of B19 specific DNA by gel electrophoresis and ethidium bromide staining. Seventy percent of the sera, with a laboratory confirmed diagnosis of B19 infection, contained B19 DNA, 16% of these exhibited B19 specific product after both 1st & 2nd round PCR while the other 84% contained detectable B19 DNA only after both rounds of amplification. B19 DNA was detected after both 1st & 2nd round PCR amplification in

	* Confirmed B19 Diagnosis	Negative Controls
Rash	18	39
Rash and arthralgia	24	10
Arthralgia	12	10
Pyrexia	1	3
Asymptomatic	3	-
Other	1	6
	59	68

* DNA dot blot positive or B19 IgM positive

Table 3: Diagnosis of 127 patients submitted to nested PCR for the detection of B19 DNA

the DNA dot blot positive sample. B19 DNA was not detected in any of the sera not fulfilling the criteria of a laboratory confirmed B19 infection (negative controls). Forty nine of these sera were collected from patients with a rash illness, suggesting that it is unlikely that the existing B19 IgM test fails to detect cases of B19 associated rash illness.

4.1.1 Nested PCR of PCR negative sera using an annealing temperature of 45°C

The nested PCR was modified to determine whether any of the sera, with a laboratory confirmed B19 diagnosis but from which B19 DNA could not be detected by PCR, contained B19 DNA with base mismatches to the complimentary primer sequences. DNA from such variant strains would consequently fail to amplify. The nominal T_m of the primers was 60°C. The stringency of the PCR conditions was reduced by decreasing the annealing temperature from 55°C to 45°C, so that the samples were cycled at 95°C for 1 min, 45°C for 1.5 mins and 72°C for 1 min for both rounds of amplification. Sera collected from 4 patients with a recent onset of symptoms (≤ 21 days) but not found to contain B19 DNA by nested PCR using a 55°C annealing temperature, were reassayed by the modified nested PCR. Non specific DNA amplification occurred, manifesting as multiple ethidium bromide stained bands in the agarose gel following electrophoresis (plate 11). Confirmation of the specificity of the reaction products was sought by alkali transfer and hybridisation with pVTM-1 (plate 12). Only one of the sample products contained B19 specific DNA. This sample is included in the subsequent analysis of B19 PCR positive and negative sera. The development of multiple banding using a 45°C anneal temperature makes the use of this reduced stringency PCR unsatisfactory for assaying sera without confirming specificity with hybridisation. No further testing was performed using this modified assay.

Plate 11

Nested PCR using a 45°C anneal temperature

Nested PCR was performed on sera collected from 4 B19 infected patients (lanes B-E) whose sera were not found to contain B19 DNA by nested PCR using a 55°C anneal temperature. PCR products are shown following electrophoresis in an ethidium bromide stained 2% agarose gel. Key; MW = molecular weight marker, 1 kb DNA ladder (BRL, USA). - = B19 negative serum, A = B19 viraemic serum; B-E = test sera; F = B19 viraemic plasma containing 10 copies of B19 DNA; G = B19 viraemic plasma containing 1 copy of B19 DNA.

Plate 12

Southern blot hybridisation of PCR products using a 45°C anneal temperature

Southern blot hybridisation of nested PCR products was performed on sera collected from 4 B19 infected patients (lanes B-E) whose sera were not found to contain B19 DNA by nested PCR using a 55°C anneal temperature. DNA products were alkali transferred to Zeta Probe membrane and probed with ^{32}P labelled pVTM-1 and autoradiographed with Kodak X-Omat S film for 24 hours at -70°C. Key; - = B19 negative serum, A = B19 viraemic serum; F = B19 viraemic plasma containing 10 copies of B19 DNA; G = B19 viraemic plasma containing 1 copy of B19 DNA.

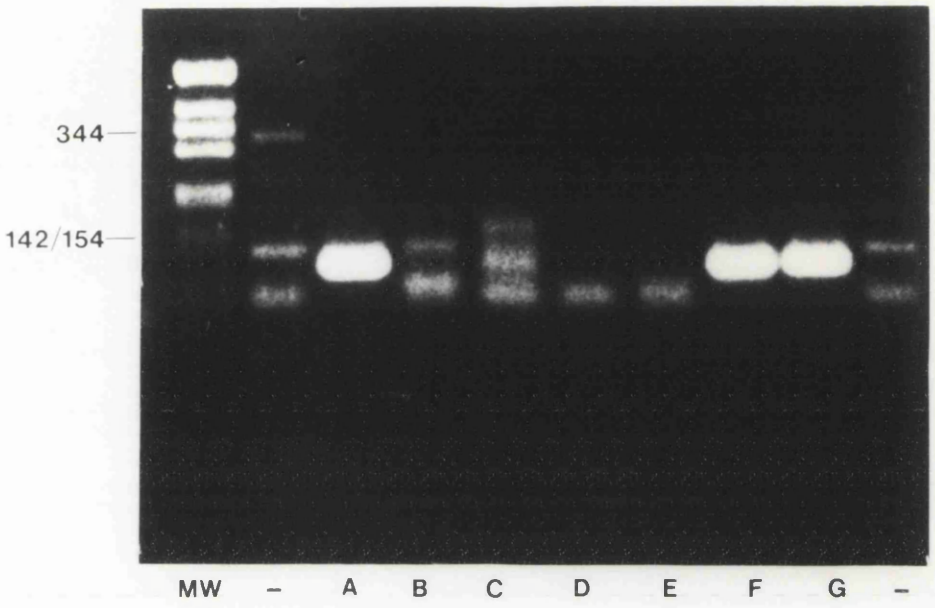

Plate 11

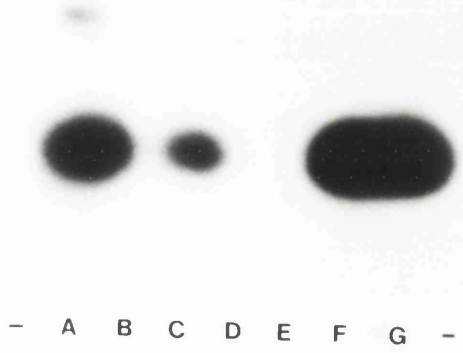

Plate 12

4.1.2 Analysis of the PCR patterns obtained from sera with a laboratory confirmed B19 diagnosis

Three patterns of PCR results (PCR 1st & 2nd round positive; PCR 1st round negative & 2nd round positive; and PCR negative) were obtained from the 81 sera with a laboratory confirmed diagnosis of B19 infection. The PCR patterns were analysed in terms of the B19 IgM and IgG content of the sera (figure 5,6). A statistically significant variation (*F* test) for B19 IgM ($p<0.005$) and IgG ($p<0.05$) values was detectable among the three PCR groups. B19 IgM values were lower in the PCR negative group than in the PCR positive groups. B19 IgG values were lowest in the PCR 1st & 2nd round positive group, higher in the 1st round negative/2nd round positive group and highest in the PCR negative group.

Sets of two or more sequential sera were available from 22 patients and the PCR results of 16 of these sets are shown in figure 7. There was a trend for patients to lose B19 DNA from their serum over time although there were insufficient time points from individual patients to draw any conclusions about the rate of this loss. However, it can be seen that all three PCR patterns were observed in patients up to 15 days after the onset of symptoms, suggesting considerable patient variability in the timing of the loss of B19 DNA from serum. This B19 DNA decay was also confirmed by observations on the interval between the onset of symptoms and collection of serum from patients from whom only a single serum was collected. This interval, known in 33 of the B19 infected patients, was longest in those who were PCR negative (mean 39 days) and much shorter in those who were PCR 2nd round positive (mean 10 days) or PCR 1st & 2nd round positive (mean 3 days)(figure 8). This trend was statistically significant (*F* test, $p<0.005$). There was no significant difference in the ages of the patients in the three PCR groups (figure 9). Patients were also analysed by B19 symptom type, unfortunately the

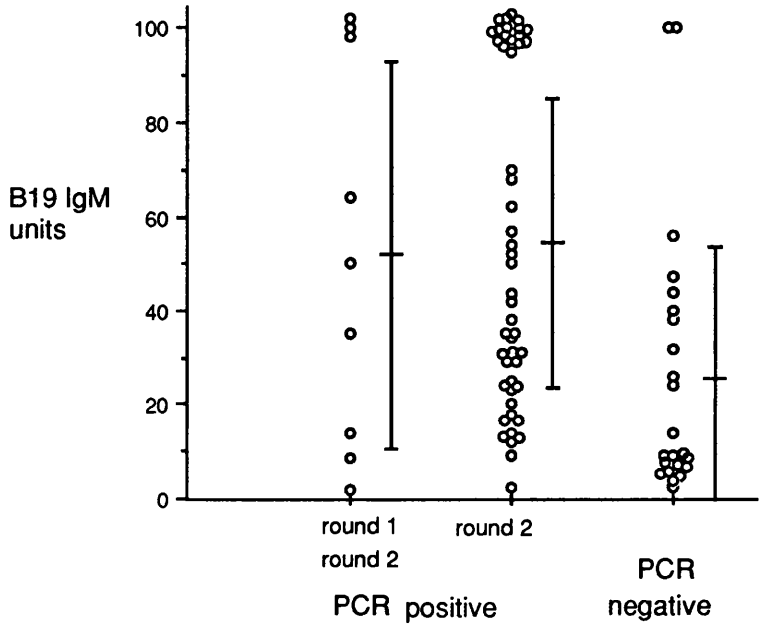

Figure 5: B19 IgM values of 81 B19 PCR positive & negative sera from patients with a laboratory confirmed diagnosis of B19 infection (*F* test, p<0.005)

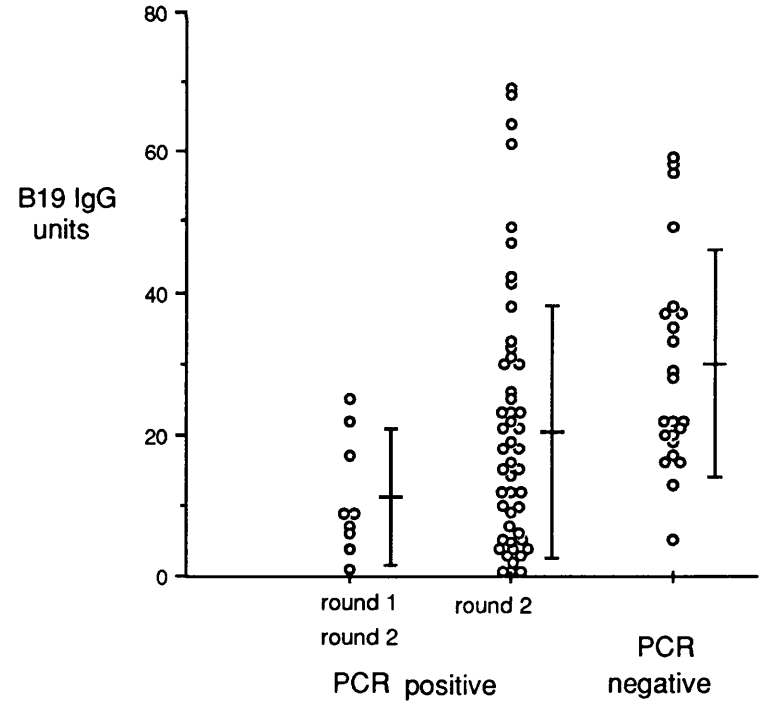

Figure 6: B19 IgG values of 81 B19 PCR positive & negative sera from patients with a laboratory confirmed diagnosis of B19 infection (*F* test, p<0.05)

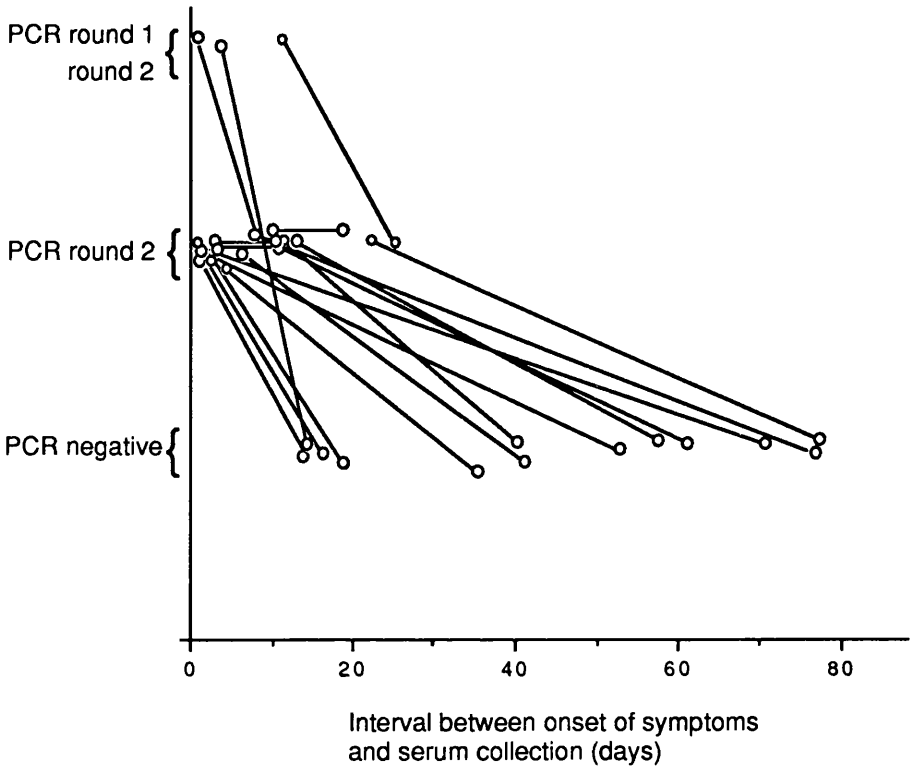

Figure 7: PCR profiles of 16 sets of sera from patients with recent B19 infection

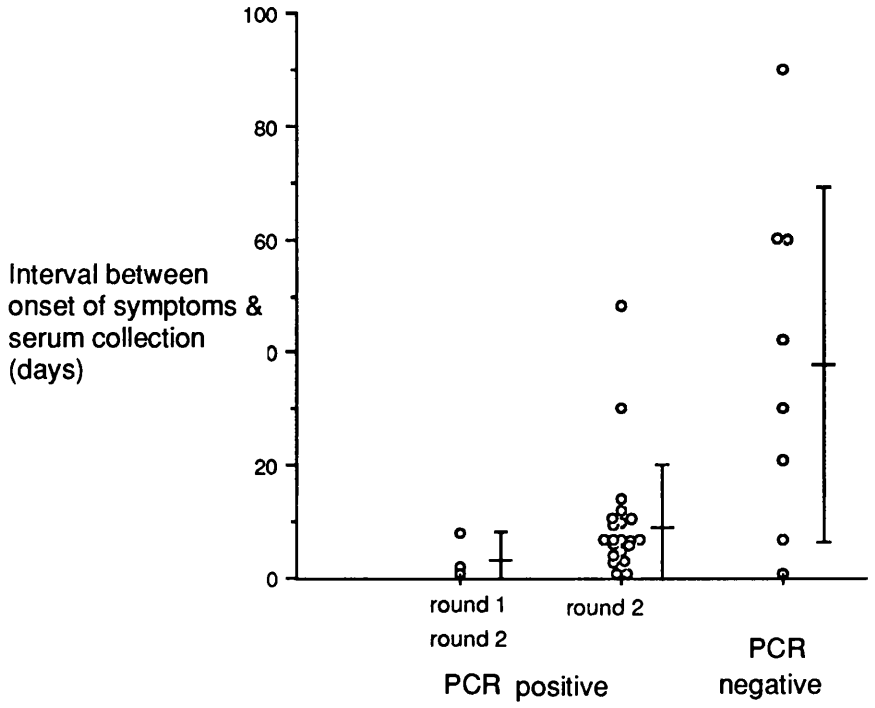

Figure 8: Interval between onset of symptoms and serum collection in 33 B19 PCR positive and negative patients with a confirmed diagnosis of B19 infection (*F* test, $p<0.005$)

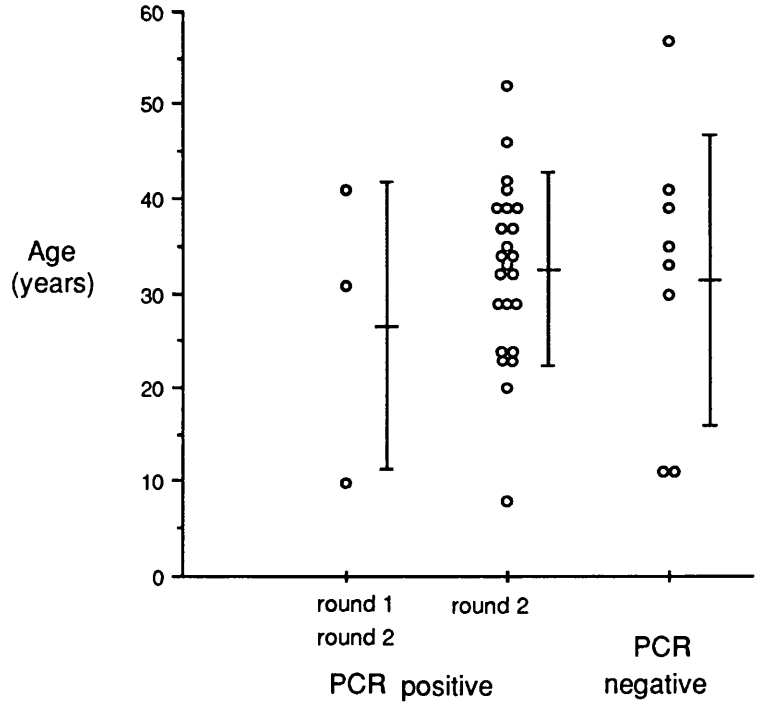

Figure 9: Age distribution of 35 B19 PCR positive and negative patients with a laboratory confirmed diagnosis of B19 infection (*F* test, n.s.)

number in each diagnostic group was too small to draw any statistically meaningful conclusions (table 4).

4.2 LONGITUDINAL COLLECTION OF SERA, THROAT SWABS AND PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMNC)

A nosocomial outbreak of B19 infection occurred in North London in 1990 involving the staff and patients of a paediatric oncology ward. A total of 11 staff, two patients and a parent of one of the patients were infected over a four week period. Baseline sera were collected from all the staff and patients on the ward following the detection of B19 IgM from the first case. The sera were submitted to dot blot hybridisation, B19 IgM and IgG testing. Seronegative individuals and subjects developing a clinical illness compatible with B19 infection were followed up serologically for the development of B19 infection. Ten B19 infected subjects were studied intensively, six had presented with rash and arthralgia, three presented with rash alone and one presented with arthralgia alone. All 10 individuals were found to be B19 IgM positive and had a known date of onset of symptoms. They were followed up with the collection of throat swabs, PBMNC and further serum samples up to 6 months post-onset of symptoms. A number of these subjects were lost to follow up so that only 4 were followed to the end of the study. Negative control PBMNC were collected from five B19 IgG seropositive and two B19 IgG seronegative individuals. A negative control throat swab was collected from an uninfected subject.

DNA was extracted from the throat swabs and nested PCR performed with 0.5μg of DNA, alkali transferred and hybridised with pVTM-1. Dot blot hybridisation was also performed, using 1μg of DNA. Samples not found to contain B19 specific DNA were diluted to a 0.05μg DNA concentration and retested because of the potential presence of PCR inhibitors in saliva (personal communication, Ms J Fox, London). They were also

	PCR Positive		PCR Negative (%)
	1st + 2nd round (%)	2nd round (%)	
Rash	1 (25)	8 (32)	2 (25)
Rash + arthralgia	2 (50)	10 (40)	2 (25)
Arthralgia	-	4 (16)	4 (50)
Pyrexia	1 (25)	-	-
Asymptomatic	-	2 (8)	-
Other	-	1 (4)	-

Table 4: Diagnoses of patients* with laboratory confirmed B19 infection, stratified by PCR result

* Patients from whom a single serum was collected.

retested at a 1 μg DNA concentration to assay for the presence of B19 DNA in low copy numbers. DNA extracted from PBMNC was submitted to dot blot hybridisation and nested PCR followed by alkali transfer and hybridisation with pVTM-1. Nested PCR, dot blot hybridisation and B19 IgM & IgG tests were carried out on the sera.

None of the samples contained detectable B19 DNA by dot blot hybridisation. B19 IgM was detectable in all the subjects at 1-2 weeks and was undetectable by 4 months (figure 10). B19 DNA was detectable by PCR in all the sera tested 1-2 weeks after the onset of symptoms; It was detectable in two sera following 1st & 2nd round amplification, while in the other eight sera B19 DNA was detected only after both rounds of amplification. At subsequent time points, when B19 DNA was detectable in serum, it was only detected following both rounds of amplification. In general B19 DNA was only present in sera containing B19 specific IgM, however at 4 months, B19 DNA was detected in one serum that contained only 1 au of IgM. Too few sera were collected to draw firm conclusions about the temporal relationship between B19 DNA and IgM detection.

B19 DNA was detectable from all the throat swabs at 1 week following two rounds of PCR amplification and was undetectable in all subjects by 6 months (figure 10). Two of 19 throat swabs were inhibitory to the PCR so that B19 DNA was only detectable when diluted to 0.05μg. B19 DNA was also detectable in the PBMNCs after two rounds of amplification, and remained present in three of four subjects to the end of the study. Alkali transfer and hybridisation with pVTM-1 confirmed the specificity of the PCR reaction products observed following agarose gel electrophoresis. One sample, a throat swab, contained B19 DNA only detectable following alkali transfer and hybridisation. B19 DNA was not detected in any of the PBMNC or throat swab controls.

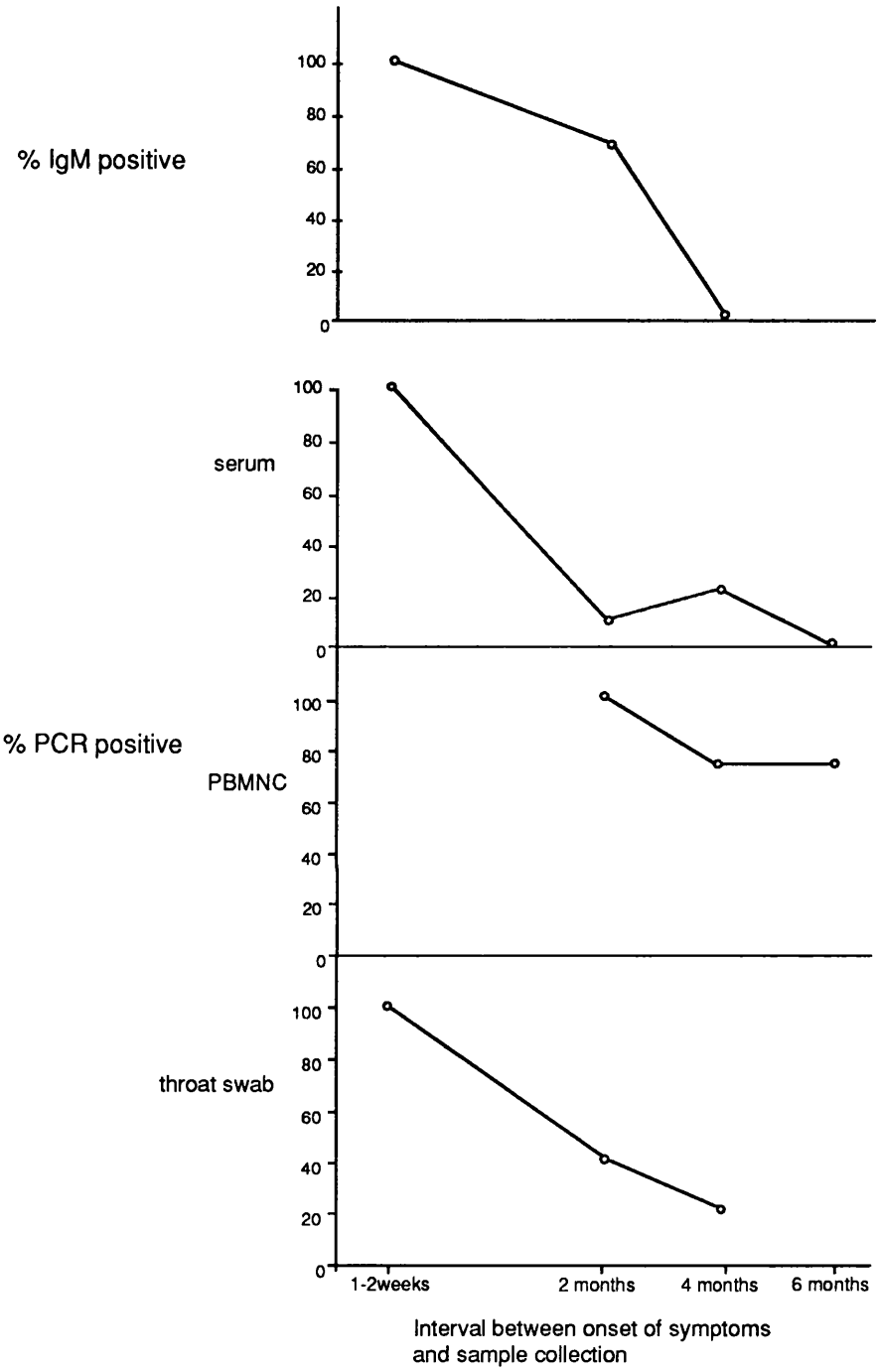

Figure 10: Detection of B19 DNA in serum, PBMNC & throat swabs by PCR & serum IgM by MACRIA collected from subjects during a nosocomial B19 outbreak

Thus it appears that following infection, B19 DNA can be detected in the PBMNC fraction and persists at this site after the disappearance of B19 DNA and IgM from serum.

4.2.1 Titration of throat swab and PBMNC DNA

To further characterise the presence and persistence of B19 DNA in throat swabs and PBMNC the quantity of B19 DNA in these samples was determined by PCR titration. DNA was extracted from these samples, titrated with water in $\log_{10}$ dilutions and nested PCR performed. The mean copy number of B19 DNA detected in the PBMNC containing B19 DNA was 220/ml of whole blood at 2 months and 4 copies/ml at 4 and 6 months. A mean of 50 copies of B19 DNA was detected in the throat swabs at 1-2 weeks. This had declined to a mean of 1 copy by 2 months in those throat swabs still found to contain B19 DNA.

4.2.2 Characterisation of the B19 DNA-containing cell type in PBMNC by immunolabelling and *in situ* hybridisation

Immunolabelling and *in situ* hybridisation was performed on the PBMNC fraction to attempt to define the cell type containing B19 DNA. The PBMNC collected at 2 months, from four subjects, one male and three females, containing between 3.5×10^5 and 6.2×10^6 cells/ml were centrifuged onto slides and immunolabelled with monoclonal antibodies to either glycophorin A (JC159, Dako), an erythrocyte lineage marker, or CD45 (LCA, Dako), a marker found on most leucocytes. A monoclonal antibody to desmin (Der11, Dako), a smooth muscle antigen was used as a negative control. The slides were then treated with biotinylated probes. The probe used for the detection of B19 virus was pYT 104 (gift of Dr P Tattersall, Yale). A positive control Y repeat probe pHY 2.1 (gift of Dr H Cooke, Edinburgh) and a negative control plasmid pBR 322

(without insert) were also used.

The Y repeat probe labelled the female PBMNC (female cells are known to contain 200 homologous sequences/cell of the Y repeat sequence on autosomes [Cooke *et al* 1982]) (plate 13) and male PBMNC (plate 14). Thus, the *in situ* hybridisation was shown to detect as few as 200 copies of DNA. The results of glycophorin A immunolabelling and B19 *in situ* hybridisation of a positive control B19 infected bone marrow and the PBMNC are shown in plates 15 and 16 respectively. Specific hybridisation and immunolabelling of the bone marrow are seen in plate 15. B19 DNA was not detected in any of the PBMNC preparations examined. However, the immunolabelling demonstrated that although the vast majority of the cells in the PBMNC fraction were of the leucocyte lineage, there were a few scattered nucleated cells of the erythrocyte lineage within the PBMNC fraction (plate 16).

4.3 DETECTION OF FETAL B19 INFECTION

4.3.1 Maternal and fetal sera

Twenty pairs of maternal and fetal sera were submitted to the department between 1987-1990 for the investigation of potential B19 disease in the fetus. Six of the patients were investigated during the 2nd trimester of pregnancy and twelve patients were investigated in the 3rd trimester. The gestational age of the fetuses were not given for the other two subjects. The diagnoses for these fetuses are shown in table 5. Nested PCR, dot blot hybridisation and B19 IgM and IgG testing were performed on the sera.

B19 infection was demonstrable in five hydropic fetuses by dot blot hybridisation and PCR and in three mothers by the B19 IgM test (table 6). In two of these cases the B19

Plate 13

In situ hybridisation of female PBMNC using probe 2.1, homologous to "Y" repeat sequences

In situ hybridisation was performed on PBMNC collected from a B19 infected female using "Y" repeat probe 2.1. There are 200 "Y" repeat sequences scattered on autosomes. Arrow indicates positive hybridisation signal. Original magnification x40.

Plate 14

In situ hybridisation of male PBMNC using probe 2.1, homologous to "Y" repeat sequences

In situ hybridisation was performed on PBMNC collected from a B19 infected male using "Y" repeat probe 2.1. There are 2000 "Y" repeat sequences scattered on the Y chromosome and 200 homologous sequences on the autosomes. The arrow indicates positive hybridisation signal. Original magnification x40.

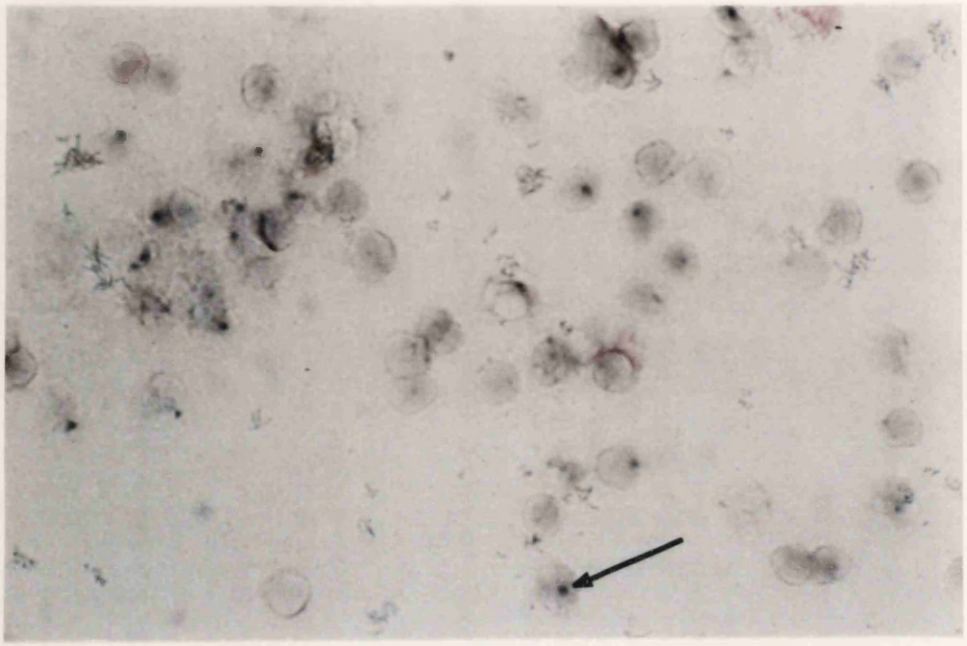

Plate 13

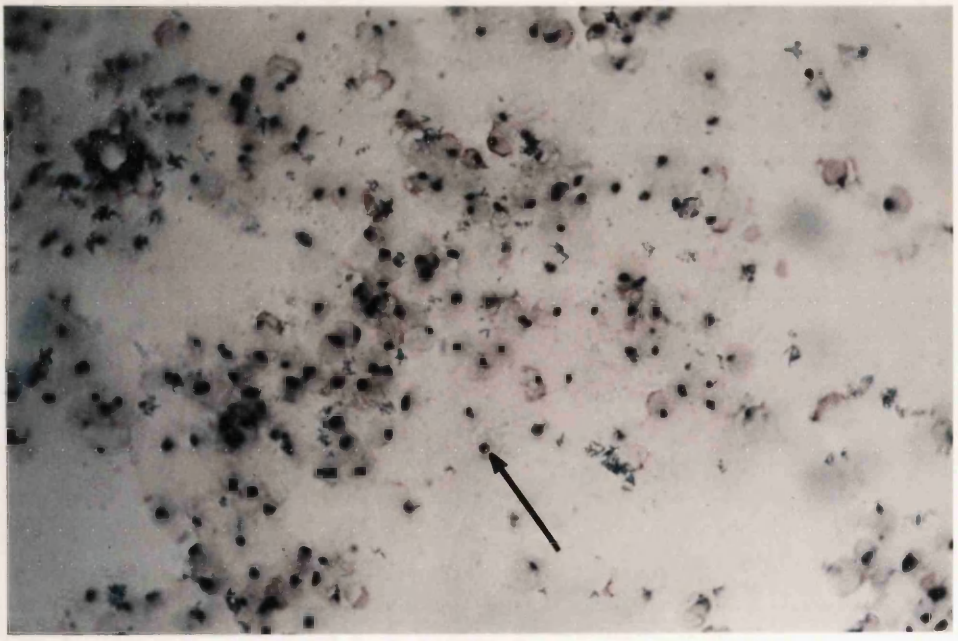

Plate 14

Plate 15

Combined *in situ* hybridisation and immunolabelling of B19 infected bone marrow

In situ hybridisation using a probe to B19 DNA, pYT-104 and immunolabelling using a glycophorin A (JC159, Dako) antibody were performed on B19 infected bone marrow. The arrow indicates positive hybridisation signal. Positive immunolabelling appears as turquoise staining. Original magnification x40.

Plate 16

Combined *in situ* hybridisation and immunolabelling of PBMNC obtained from a B19 infected subject

In situ hybridisation using a probe to B19 DNA, pYT-104 and immunolabelling using a glycophorin A (JC159, Dako) antibody were performed on PBMNC obtained from a B19 infected subject. The arrow indicates positive immunolabelling. Original magnification x40.

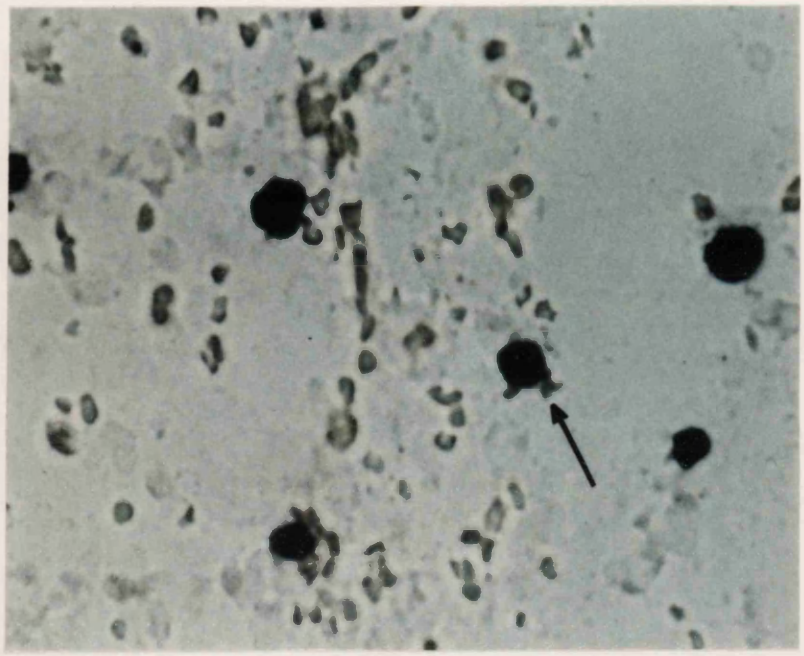

Plate 15

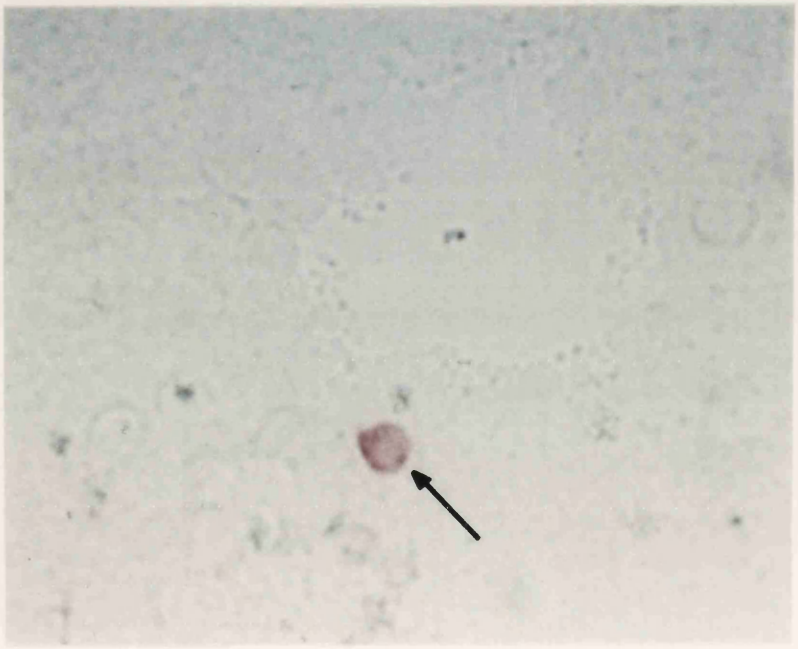

Plate 16

8 Fetal hydrops
3 Ventriculomegaly
3 Intra-uterine growth retardation
1 Hydrocephalus
1 Oligohydramnios
1 Polyhydramnios
1 Fallots tetralogy
2 Not known

Table 5: Diagnoses of 20 pregnant patients investigated for fetal B19 infection by nested PCR

Subject	Diagnosis	Gestation (weeks)	Maternal			Fetal		
			IgM	PCR		Dot blot	PCR	
				1st round	2nd round		1st round	2nd round
1	Fetal hydrops	24	2.8	-	-	+	+	+
2	Fetal hydrops	21	2.5	-	+	+	+	+
3	Fetal hydrops	26	6.6	-	-	+	+	+
4	Fetal hydrops	20	18.5	-	-	+	+	+
5	Fetal hydrops	21	5.2	-	-	+	+	+

Table 6: Serological, dot blot hybridisation and PCR results obtained from five B19 infected pregnancies

IgM detected was near the cut off for the test (5au). A maternal clinical illness was recorded for only one patient and this occurred 7 weeks before the development of fetal hydrops (subject 5).

Nested PCR detected B19 DNA in dot blot positive fetal sera but not in any of the 15 other fetal sera tested. B19 DNA was detected by nested PCR in only one maternal serum (subject 2).

4.3.2 Fetal tissues

Abortuses were obtained from two women found to have hydropic fetuses in the second trimester of pregnancy. Both patients, investigated serologically and found to be B19 IgM positive, opted for an elective termination of pregnancy. DNA was extracted from the placenta and lungs of one of the fetuses and from the placenta of the other fetus by proteinase K/SDS treatment and phenol/chloroform extraction. The tissues were probed for the presence of B19 DNA by dot blot hybridisation using pVTM-1 and by nested PCR. The placental tissues from one of the abortuses was also probed by *in situ* hybridisation using biotin labelled pYT104. Post mortum salivary gland tissue obtained from a 70 year old patient who died of a myocardial infarct was used as a negative control.

B19 DNA was detected in all the abortus tissues examined by dot blot, nested PCR and *in situ* hybridisation (plate 17).

Plate 17

In situ hybridisation of placental tissue obtained from a hydropic fetus.

In situ hybridisation using a probe to B19 DNA, pYT-104 was performed on placental tissue obtained from a B19 infected hydropic fetus. The arrow indicates positive hybridisation signal. Immunolabelling (red staining) can also be seen, using an antibody, EBM 11 (Dako) that detects macrophages. Original magnification x40.

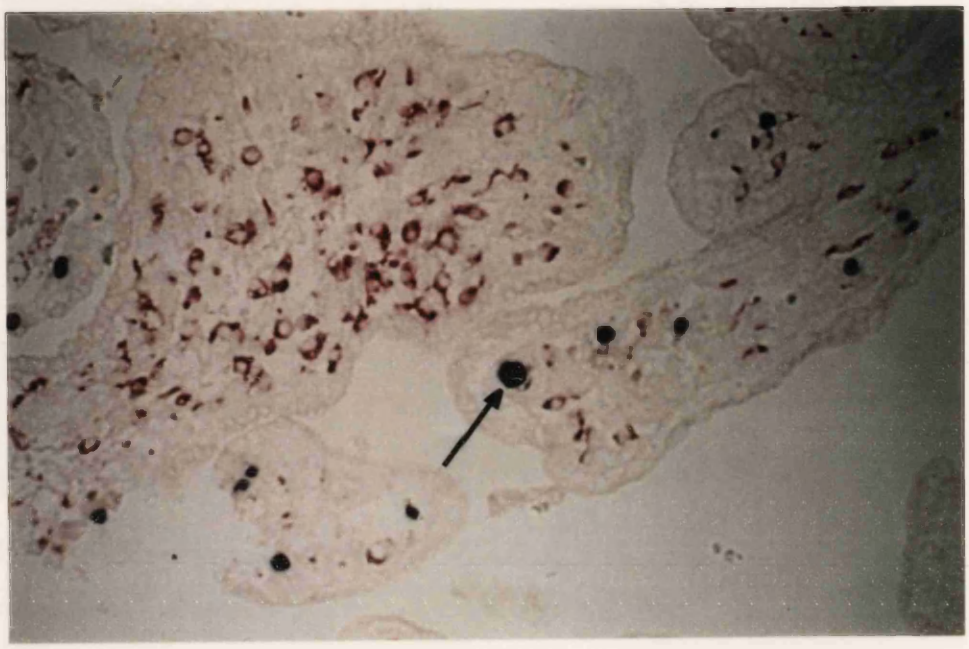

Plate 17

4.4 DETECTION OF INTRAUTERINE B19 INFECTION IN THE NEONATE

Twenty seven B19 infected pregnant women were enrolled in this study. Sera were collected from the women at the antenatal booking visit and following delivery. Placental cord blood was collected from the neonates at delivery. The samples were collected between 1986-1989. The mothers were shown to have had a B19 infection in pregnancy by the demonstration of a seroconversion in 23 subjects and by the presence of B19 IgM at booking in the 4 other individuals.

The study was performed anonymously, the only clinical information available was the date of the first day of the last menstrual period (LMP), the estimated date of delivery (EDD) and the actual date of delivery. No information was available on the course of the pregnancy or whether the offspring were healthy. Three of the four B19 IgM positive sera were collected during the 1st trimester of pregnancy and the other sample during the 3rd trimester. Of the 23 patients who seroconverted, seven booked during the 1st trimester, fourteen booked in the 2nd trimester, one booked in the 3rd trimester and the trimester at the booking visit of the other seroconvertor was not provided. This would suggest that most of the infections occurred in the 2nd and/or 3rd trimesters of pregnancy. None of the infants was born prematurely.

Dot blot hybridisation, B19 IgM and IgG testing and nested PCR were performed on the placental cord sera. All the sera contained B19 IgG but none contained B19 IgM. B19 DNA was not detectable in any of the samples by either dot blot hybridisation or nested PCR.

4.5 DETECTION OF B19 INFECTION IN THE IMMUNOCOMPROMISED

4.5.1 Allogeneic bone marrow transplant donors and recipients

In this retrospective study, sera collected from 10 adult allogeneic bone marrow transplant recipients and donors were investigated for evidence of recent B19 infection/reinfection. Four of the patients had acute myeloid leukemia, three had acute lymphocytic leukemia, two had chronic granulocytic leukemia and one had chronic myeloid leukemia. All the recipients were conditioned with total body irradiation and received a variety of immunosuppressive cytotoxic drugs. They received T cell depleted bone marrow from HLA matched donors. Blood and platelet transfusions were given throughout treatment.

Sera were collected from the recipients before transplantation and then weekly for 4 weeks and at 2 months after transplantation. Serum was also collected from the donors prior to transplantation. All the samples were tested for B19 DNA by dot blot hybridisation and nested PCR. Sera collected immediately pre-transplantation from donors and recipients and those collected 1 month after transplantation from the recipients were also tested for B19 IgM and IgG. It was therefore possible to determine whether recipients were potentially at risk of acquiring primary B19 infection, contracted from their donor bone marrows (donor seropositive/recipient seronegative), or of developing a recurrence of B19 infection (donor seropositive/recipient seropositive or donor seronegative/recipient seropositive). Donor/recipient pairs, where both subjects were seronegative were used as controls since these recipients were only at risk from community or blood transfusion acquired primary infection.

Three donor/recipient pairs were found to be donor seropositive/recipient seronegative; two pairs were donor B19 seropositive/recipient seropositive; four pairs were donor B19

seronegative/recipient seropositive and one pair were tested where both donor and recipient were seronegative. B19 DNA was not detected in any of the sera tested by dot blot hybridisation or nested PCR. Four of the seronegative recipients had demonstrable B19 IgG at 4 weeks post transplantation. Three of these had received bone marrow from a seropositive donor, while these individuals and the other seronegative recipient had received blood transfusions over that period. Since bone marrow recipients develop their donor's immunological memory, B19 infection cannot be inferred from these seroconversions. In addition the possibility of passive acquisition of B19 IgG from a seropositive blood transfusion cannot be excluded. None of the samples tested contained B19 IgM.

This study failed to demonstrate either primary or recurrent B19 infection in 10 allogeneic bone marrow transplant patients followed up for 2 months post transplantation.

4.5.2 Renal transplant recipient

During the nosocomial outbreak of B19 infection previously described, B19 viraemia was detected in a renal transplant recipient by dot blot hybridisation. The 12 year old boy had undergone a renal transplant for cystinosis 27 days before the detection of B19 viraemia. Three pre-transplant sera had been stored from this patient and following transplantation three further sera were collected. Dot blot hybridisation, nested PCR and B19 IgM and IgG testing were performed on all the sera tested (table 7).

In the serum collected 19 months before transplantation, no B19 antibodies were detected. Four months later the patient suffered a febrile illness and serum collected at that time contained both B19 IgM and IgG. B19 DNA was detected following both 1st and 2nd rounds of PCR amplification, although not by dot blot hybridisation. A small

	Pre transplant			Post transplant		
Months	-19	-15	0	1	2	3
DNA (PCR)	-	+	-	+	-	-
DNA (Dot blot)	-	-	-	+	-	-
IgM	-	+	-	-	+	-
IgG	-	+	+	-	+	+
Hb (g/dl)	10.0	9.2	9.7	10.4	10.4	11.4
		↑ Pyrexial illness				

Table 7: B19 serological profile in a renal transplant recipient

drop in haemoglobin value was noted at that time. A serum collected on the day prior to transplantation showed the presence of B19 IgG but not IgM. DNA was not detectable by either dot blot hybridisation or PCR.

At renal transplant the patient received two units of blood, stored aliquots from which tested negative for B19 DNA and IgM. His post transplant immunosuppression consisted of azathioprine, cyclosporin and prednisolone. On day 17 post transplant, his mother developed a rash and arthralgia and this was confirmed to be due to B19 virus by the detection of B19 IgM in her serum. Prophylactic normal pooled human immunoglobulin was adminstered to the patient intramuscularly. However, 10 days later, B19 DNA was detected in his serum by dot blot hybridisation and following both 1st & 2nd round PCR; B19 specific antibodies were not detectable. He subsequently developed a brisk B19 IgG response and a low B19 IgM response, by which time B19 DNA was no longer detectable. The IgM response was not detectable one month later. Over this period of viraemia he had no clinical symptoms and his haemoglobin level, leucocyte count and platelet count remained stable.

PCR was able to provide confirmatory evidence, in addition to the serology, that the patient had suffered 2 episodes of B19 infection.

4.6 DETECTION OF B19 INFECTION IN PATIENTS WITH SEVERE ANAEMIA LIVING IN A MALARIAL ENDEMIC AREA

4.6.1 Patients & methods

The study was carried out at the Kenya Medical Research Institute (KEMRI) Coastal Unit, a malaria paediatric hospital in Kilifi District, Kenya. Killifi district covers a coastal strip of Kenya approximately 140kms long and 90kms wide. The hospital serves

an estimated population of 60,300 with 10,300 children aged 6 months to 7 years. The vast majority of the population of Coast Province live in a rural setting. Malaria infection in this area is hyperholoendemic with year round transmission. Samples were collected from April 1989 until November 1989. Subjects were recruited into the study if they fulfilled the case definition for severe malaria; this was defined as malaria infection presenting with a haemoglobin concentration of less than 5 g/dl, or presentation with coma, fits or greater than 20% parasitaemia. Non severe malaria was defined as malaria infection not fulfilling the criteria for severe infection. Patients were recruited where possible with an age matched control randomly picked from the local community.

A total of 340 subjects were studied. Sixty four patients fulfilled the critera for severe malaria and 117 patients fulfilled the criteria for non-severe malaria. Twenty one of the patients with severe malaria presented with severe anaemia and the remaining 43 presented with the other diagnostic criteria for severe malaria. The age distribution of the population are shown in figure 11.

Plasma was collected from each subject at the time of clinical presentation. Evidence of B19 infection was sought by DNA dot blot hybridization, MACRIA and GACRIA. The plasmas were all coded so that the clinical groups were not known at the time of testing. Nested PCR was subsequently performed on a subgroup of patients with anaemia. Full blood counts, haemoglobin electrophoresis and quantitation of the degree of parasitaemia were also performed.

4.6.2 DNA detection

B19 viraemia was not detectable by dot blot hybridization in any of the plasmas tested. Twenty samples, collected from patients with severe malaria and anaemia, were tested

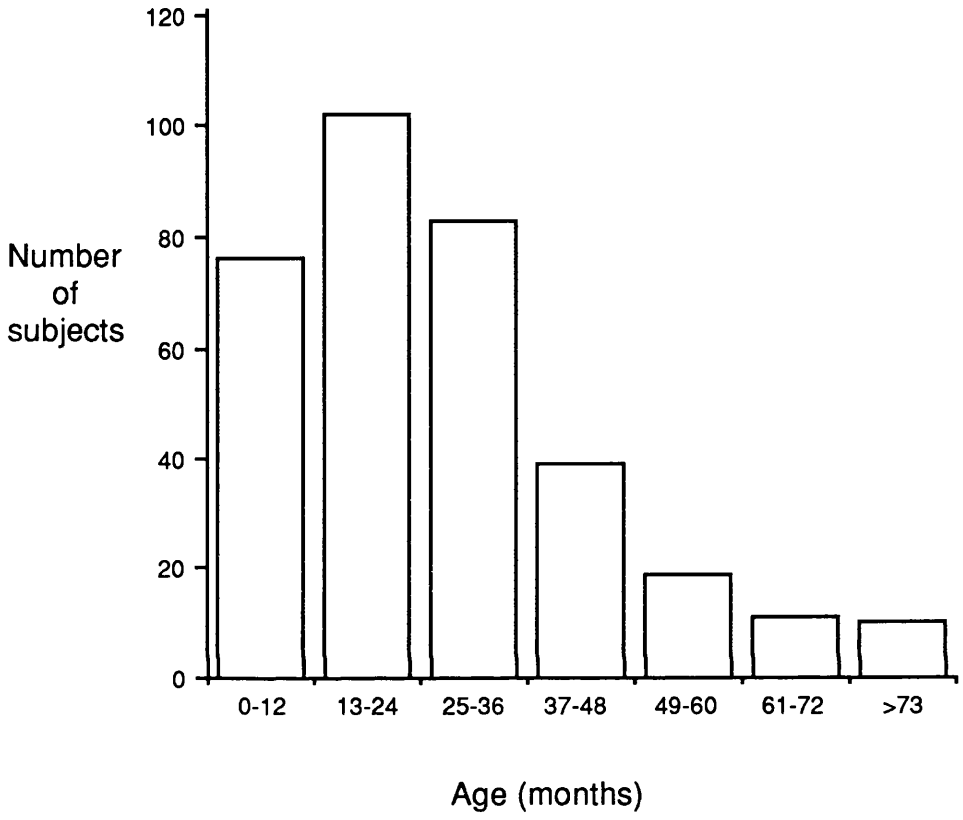

Figure 11: Age distribution of 340 malaria infected patients and community controls investigated for evidence of B19 infection

for the presence of B19 DNA by nested PCR. Additionally, 5μg of *Plasmodiumfalciparum* DNA (gift of Dr C.Newbold, Oxford) was also tested in the assay to determine whether this DNA could produce false positive results in the nested PCR.

PCR products corresponding to 1112bp and/or 104 bp B19 specific products were not detected in the *Plasmodium falciparum* DNA PCR reaction, confirming the specificity of nested PCR for B19 DNA in plasma collected from malaria infected patients. B19 specific DNA was detected in only one test sample following nested PCR; the specificity of this product was confirmed by alkali transfer and hybridization with pVTM-1. This sample was collected from a 21 month old patient with severe malaria and anaemia (haemoglobin concentration 2.3g/dl, normal haemoglobin electrophoresis pattern). The PCR was repeated using primers B19-1 & 2 alone to determine whether B19 DNA, with nucleotide sequence differences in the regions of the B19 genome complimentary to primers Parpat 1 & 3AS, was present in the samples. None of the 20 samples retested by single round PCR using B19-1 & 2 were found to contain a B19 specific product following gel electrophoresis, alkali transfer and hybridisation with pVTM-1.

4.6.3 IgG and IgM detection

The performance of plasmas collected from malaria infected patients in the MACRIA and GACRIA tests was evaluated to test the hypothesis that such plasmas would be inhibitory in these serological assays. Four plasmas, B19 IgG and IgM negative by GACRIA and MACRIA, collected from malaria infected children in the Gambia (gift of Dr A.Hill, Oxford) were selected. B19 IgG and IgM control sera containing 100 au of B19 IgG and IgM respectively were titrated in each of these plasmas and in a B19 negative plasma collected at the North London Blood Transfusion Service Centre (BTS). The titrations were then assayed for B19 IgG (figure 12) and IgM by GACRIA and

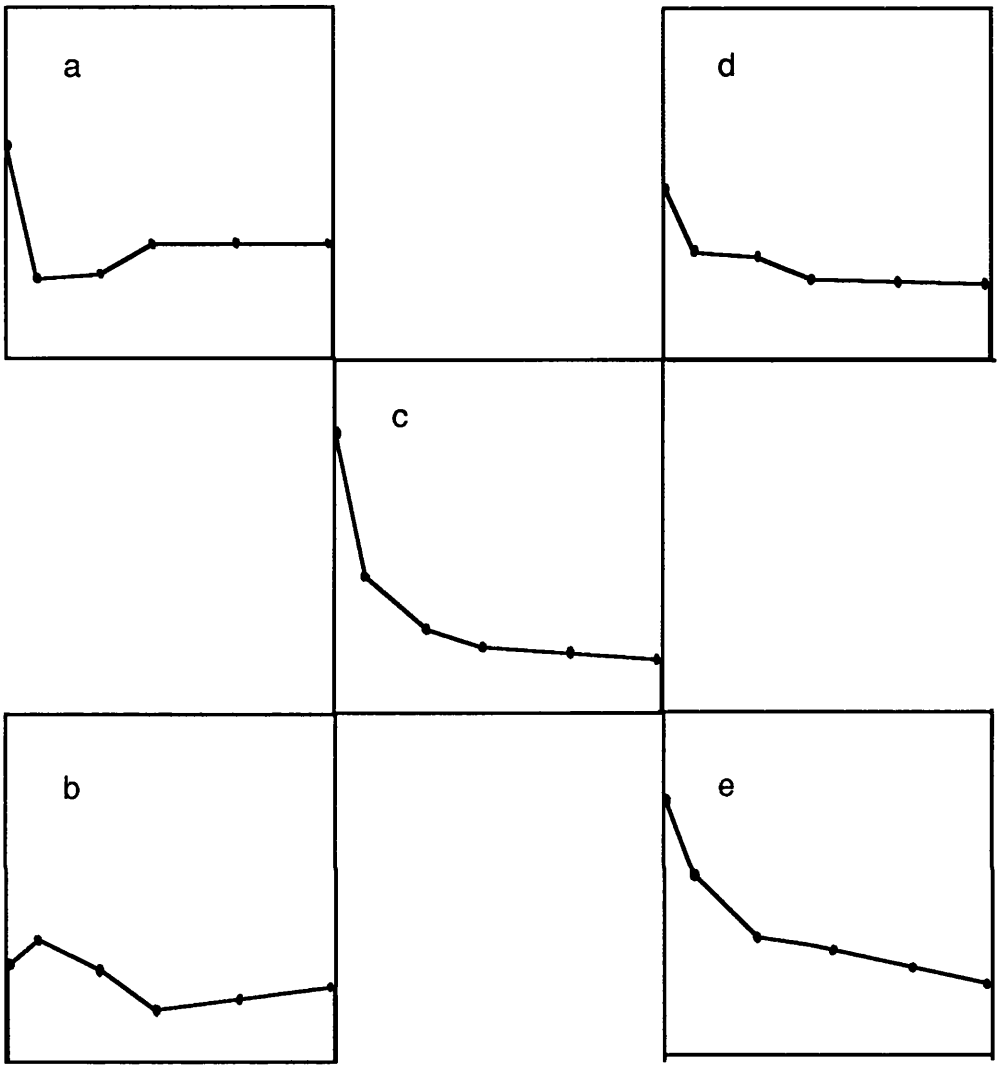

Figure 12: GACRIA profiles of a B19 IgG positive control serum (100au) titrated in B19 negative serum and plasma from patients with severe and mild malaria infection

Horizontal axis shows dilution of B19 IgG control serum from 50au to 4.4au (left to right side of graph). Vertical axis shows test/negative ratio obtained in GACRIA test from 0 to 8 (bottom to top of graph). a,b = plasma from patients with severe malaria. c = plasma from BTS donor. d,e = plasma from patients with mild malaria.

MACRIA respectively.

The B19 IgM controls were not inhibited when diluted in the Gambian plasmas (data not shown). The 100 au IgG control gave lower T/N ratios when titrated in three of the four Gambian plasmas than when titrated in the BTS plasma (figure 12). A prozone effect was observed in one of the malaria infected plasma titration series (graph b, figure 12). These results support the hypothesis that the malarial infected plasmas are inhibitory to the GACRIA but not the MACRIA assay.

There was sufficient sample volume to test 333 plasmas for B19 IgM and 242 plasmas for B19 IgG. B19 IgG was only detectable in eight subjects. One of these seropositive subjects was the individual with severe anaemia and severe malaria whose plasma contained B19 DNA detected by PCR. B19 IgM was detected in two plasmas, one collected from a 2 1/2 year old B19 IgG negative child with mild malaria (haemoglobin concentration 7.1g/dl, normal haemoglobin electrophoresis pattern) and the other from a B19 IgG seropositive 10 month old control subject with a microcytic anaemia (haemoglobin of 5.3g/dl, normal haemoglobin electrophoresis pattern). There was sufficient sample volume to perform PCR on the plasma collected from the B19 IgM positive control patient. This was found to contain B19 DNA by nested PCR.

4.7 DETECTION OF B19 INFECTION IN PATIENTS WITH ARTHRITIS

4.7.1 Patients and methods

Sera were collected from 214 consecutive patients attending rheumatology outpatient clinics at two district general hospitals in Hertfordshire between 1986 and 1988. The patients were classified into 5 clinical diagnostic groups (table 8). These were early

Diagnostic Group	B19 IgG			B19 IgM		
	+	-	NT	+	-	NT
Early rheumatoid arthritis	23	27		2	46	2*
Established rheumatoid arthritis	29	28		3	54	
Inflammatory joint disease	15	11		1	25	
Non-inflammatory joint disease	20	17		0	37	
Undifferentiated arthritis or synovitis	18	24	2	5	37	2
	105	107	2	11	199	4

+ = present
- = absent
NT = not tested
* Screening B19 IgM positive, insufficient sample for confirmatory testing

Table 8: B19 IgG and IgM seroprevalence among 214 patients attending two rheumatology clinics between 1986 and 1988

rheumatoid arthritis (less than one year of symptoms) (50 cases), established rheumatoid arthritis (greater than one year of symptoms) (57 cases), inflammatory joint diseases (26cases), non-inflammatory joint disease (37 cases) and undiffentiated arthritis/synovitis (44 cases). The inflammatory joint disease group contained all the non-rheumatoid connective tissue disease and the non-inflammatory arthritis group encompassed osteoarthritis and trauma related joint disease. No firm diagnosis could be made for the patients in the undiffentiated arthritis or synovitis group. The clinical symptoms and signs of the patients were recorded along with details of their medication. The diagnoses of the patients were reassessed six months after the initial visit.

The sera were all coded so that the clinical groups were not known at the time of B19 testing. All the sera were screened for B19 IgM and IgG. Samples found to contain B19 specific IgM on screening were absorbed with latex coated with human IgM to remove potential rheumatoid factor activity and then retested for B19 IgM. Only those samples found to contain B19 specific IgM after this absorption step were considered to demonstrate evidence of recent B19 infection.

Ninety six unselected sera and 11 additional sera found to be B19 IgM positive on initial testing were also tested for the presence of B19 DNA by nested PCR.

4.7.2 Results

There did not appear to be any real difference in the IgG seroprevalence of B19 infection between the clinical groups (table 8). Seventeen sera were reactive in the B19 IgM test. There was sufficient of fifteen of the sera to perform rheumatoid factor absorptions and then retest the absorbed sera in the MACRIA assay. Following rheumatoid factor absorption eleven sera remained B19 IgM positive and these were considered to show

evidence of recent B19 infection (table 8). None of these patients reported a recent rash illness preceding the onset of joint symptoms. B19 DNA was detected in six of these by nested PCR and confirmed by alkali transfer and hybridisation with pVTM-1. This confirmed that there was evidence of recent B19 activity in these patients. B19 DNA was not detectable in the other five sera confirmed B19 IgM positive.

The symptoms of two of the B19 infected patients in the undiffentiated arthritis/synovitis group had resolved prior to the six month follow up visit. The diagnoses of the other B19 infected patients remained unchanged at six months follow up. The natural course of arthritic diseases is very variable; so that it was not possible to determine whether those patients with evidence of recent B19 infection had an altered disease course when compared with uninfected controls from the same diagnostic group. However, no striking differences were noted.

4.8 DETECTION OF B19 DNA IN CLOTTING FACTOR CONCENTRATES

A total of 25 clotting factor concentrates prepared by a variety of techniques were tested for the presence of B19 DNA. These included untreated factor VIII and factor VIII concentrates subjected to chloroform treatment, dry heat treatment, steam heat treatment, pasteurisation, tri-n-butyl phosphate (TNBP) treatment and monoclonal antibody purified factor VIII. More than one batch of some of the products were tested so that a total of 34 samples were examined.

Total DNA extracted from the concentrates was examined for the presence of B19 DNA B19 by dot blot hybridisation, Southern blot hybridisation and by PCR. The specificity of PCR products containing B19 specific DNA was confirmed by dot blot hybridisation

with pB 1906, a plasmid containing a probe homologous to a 2,200bp fragment of B19 DNA, (performed by Ms K Zakrzewska, Florence).

The results are shown in table 9. Only one concentrate (protromplex, steam heated) contained sufficient B19 DNA to be detectable by all three assays used. Untreated Kryobulin contained B19 DNA detectable by Southern blot hybridisation and nested PCR. In five other concentrates (Koate, batch A untreated concentrate, Thromb-hibin, dry heated concentrate, Emoclot Octa VI, batches A and B, Concentré de Facteur IX Humain monoclate) B19 DNA was detectable only by nested PCR.

There were two batches of five of the factor VIII concentrates (Koate, Kryobulin VI, Factor VIII:C HS (coagulant hepatitis safe, Uman Complex IX VI, Concentre de Factuer VIII humain). Concordant negative results were obtained for all the batches of four of the products. The 5th product, Koate untreated, gave discordant results with one batch containing B19 DNA detectable by PCR while the other batch did not contain B19 DNA. Three batches of two products were also tested (Kryobulin TIM3 (thermo-inactivated material), Emoclot Octa VI), one product gave concordant B19 negative results while two batches of Emoclot Octa VI contained B19 DNA while the third did not.

It was possible to demonstrate the presence of B19 DNA in clotting factor concentrates by each of the DNA assays used. Only two samples contained sufficient B19 DNA to be detectable by either dot blot or Southern blot hybridisation while seven out of twenty five concentrates were shown to contain B19 DNA after nested PCR. B19 DNA was detected in two of three untreated concentrates as well as in four of twenty treated concentrates.

Table 9: B19 DNA detection in 25 clotting factor concentrate preparations

Products	Hybridisation assays dot blot	Hybridisation assays Southern blot	PCR round 1st	PCR round 2nd
Untreated				
1 Kryobulin, Immuno	-	+	-	+
2 Koate, Cutter (a)	-	-	-	+
" " (b)	-	-	-	-
3 Uman Cry VIII, Biagini	-	-	-	-
Treated				
Chloroform treated				
4 Kryobulin VI, Immuno (a,b)	-	-	-	-
Dry heated				
5 Koate, Cutter	-	-	-	-
6 Hemofil T, Hyland	-	-	-	-
Steam treated				
7 Protromplex, Immuno (f.II,IX,X)	+	+	-	+
8 Kryobulin TIM*2, Immuno	-	-	-	-
9 Kryobulin TIM*3, Immuno (a,b,c)	-	-	-	-
10 Thromb-Hibin, Immuno	-	-	-	+
Pasteurisation				
11 Factor VIII Hepatitis Safe, Behringwerke	-	-	-	-
12 Factor VIII:Coagulant Pasteurised, Behringwerke	-	-	-	-
13 Factor VIII:Coagulant Hepatitis Safe, Behringwerke (a,b)	-	-	-	-
14 Haemate Hepatitis Safe, Behringwerke	-	-	-	-
15 Haemate Pasteurised, Behringwerke	-	-	-	-
16 Koate Hepatitis Safe, Bayer Cutter	-	-	-	-
17 Factor VIII:C, Armour	-	-	-	-
Tri (n-Butyl) Phosphate (TNBP) treated				
18 Octonativ, Kabi Biopharma (and dry heated)	-	-	-	-
19 Emoclot, AIMA	-	-	-	-
20 Emoclot Octa VI, AIMA (a,b)	-	-	-	+
" " " (c) (and ion exchange chromatography)	-	-	-	-
21 Uman complex IX VI, Biagini (a,b)	-	-	-	-
22 Concentré de facteur VIII humain, Biotransfusion (a,b)	-	-	-	-
23 Concentré de facteur IX humain, Biotransfusion (and monoclonal purified)	-	-	-	+
Monoclonal purified				
24 Monoclate, Armour (dry heated)				
25 Hemofil M, Baxter (TNBP treated)	-	-	-	-

(a), (b), (c) = different batches

*TIM = Thermoinactivated material

CHAPTER 5

DISCUSSION

5.1 DEVELOPMENT OF NESTED PCR

The development of the first published nested PCR technique for the detection of parvovirus B19 DNA is described (Patou & Ayliffe, in press). The inner set of primers, used for second round amplification, were those previously described by Salimans *et al* (1989a). The outer pair of primers, used in the first round amplification, were a novel set developed specifically for this assay. The reaction conditions for the assay were optimised for cycle number, cycling times, cycling temperatures, the type of reaction buffer used, primer concentration and magnesium concentration.

The assay was developed so that the first round reaction produced relatively little amplification of B19 DNA. First round PCR product could be detected in a gel following ethidium bromide staining when 10^7-10^8 copies of B19 DNA were present in the starting material. The second round PCR was able to boost this amplification so that 1-10 copies of B19 DNA in the starting material would produce a visible product in an ethidium bromide stained agarose gel.

The explanation for the poor amplification produced by the first round PCR was not investigated but there are a number of possible explanations for this finding. The one minute PCR extension time was relatively short for a 1112bp product because *Taq* polymerase synthesizes DNA at approximately 1000 bases per minute (Innis *et al* 1988). Another possible explanation is that the 1112bp target forms a secondary

structure that is relatively inaccessible to the enzyme. Secondary structure formation has been shown to inhibit the PCR reaction (Innis 1989). A further possibility is that the viral DNA may have remained complexed with protein despite the extraction processes used, such proteins could block the extension of the primers by the *Taq* polymerase.

The sensitivity of the PCR assay was determined using target DNA that would accurately reflect the "state" of B19 DNA in the test samples subsequently assayed. For this reason viraemic plasma rather than cloned DNA was selected as the target. The quantity of B19 DNA in two plasmas was determined by limiting-dilution, dot blot hybridisation or by viral particle counting by electron microscopy. Both plasmas produced similar results, indicating that the assay could detect 1-10 copies of B19 DNA (by direct visualisation of PCR products in an ethidium bromide stained agarose gel). The specificity of the reaction products was confirmed by Southern blot hybridisation with a complimentary B19 probe, pVTM-1. Southern blot hybridisation did not enhance the sensitivity of the nested PCR, consistent with the observation that the sensitivity of the assay had been optimised. Southern blot hybridisation did however enhance the detection of B19 specific product following first round PCR (see plate 6) to within 10-100 fold of the sensitivity achieved with nested PCR.

The sensitivity of the assay compares favourably with previously published PCR methods for B19 DNA detection. Salimans *et al* (1989a) could detect 10^3-10^4 copies of B19 DNA by gel electrophoresis and ethidium bromide staining and 100 copies by subsequent hybridisation. Clewley (1989) was able to detect 10-100 copies of B19 DNA by PCR and hybridisation. Koch and Adler (1990) were able to detect 10^3 DNA copies by PCR and a single copy by subsequent hybridisation. Frickhofen and Young (1990) describe a PCR assay that was able to detect 30 copies of B19 DNA using dot blot hybridisation to detect B19 specific products.

There were two direct consequences of the relative differences in the efficiency of first and second round amplification. Firstly, since the first round of amplification was so inefficient, the opportunity for DNA contamination when carrying over products to the second round PCR was low. The second consequence was that the nested PCR was semi-quantitative, distinguishing large "quantities" of B19 DNA from "small" quantities depending on whether reaction product was detectable after first or second round PCR.

To confirm the specificity of the PCR products obtained in the subsequent nested PCR experiments Southern blot hybridisation was performed. Four different combinations of DNA transfer methods and membranes were compared for their ability to detect the low molecular weight 104bp DNA product of the second round PCR. Alkali transfer to Zeta Probe membrane was the best of the combinations tested. This method was used in the subsequent experiments.

Initially the nested PCR was evaluated for the detection of B19 DNA in serum by directly adding the serum to the PCR reaction mix. After a 6 minute denaturation step at 95°C PCR was performed. This was a modification of the method used by Koch and Adler (1990). They added sera to a reaction mix (excluding *Taq* polymerase), denatured the DNA for 3 minutes at 94°C, added *Taq* polymerase and then performed PCR. A modification of this method was developed so that the PCR tubes would not have to be reopened to add *Taq* polymerase, thereby introducing a further step that could potentially result in DNA contamination. In an experiment comparing this modification with the Koch and Adler method no difference in sensitivity could be detected (data not shown).

It was surprising that such a sensitive PCR assay, when applied to 11 B19 IgM

containing sera, could only detect B19 DNA in one sample. Clewley had previously shown that B19 DNA was detectable in 60 of 95 (63%) of B19 IgM positive sera. B19 DNA was detectable in sera collected 1-3 days after the onset of symptoms in most cases, but in two cases sera collected 84 days after the onset of symptoms contained B19 DNA. In addition, when the technique was applied to a longitudinal collection of sera from B19 infected volunteers PCR was not able to detect B19 DNA for more than 9 days into the B19 IgM positive phase of the illness. The results obtained from the longitudinal sera are in accord with those independently obtained by Koch and Adler (personal communication).

There are a number of possible explanations for the contrasting results between the PCR experiment utilising direct sample addition and Clewley's study. These include differences in the primer pairs used and the method of sample preparation. Clewley extracted DNA from the sera by proteinase K/SDS treatment followed by phenol/chloroform extraction and ethanol precipitation prior to PCR amplification.

To explore whether differences in the method of sample preparation prior to PCR amplification could account for the differences observed between the two studies, three different methods of DNA extraction from serum for PCR were compared. The optimal technique was an extraction method based on a modification of an existing technique (Higuchi 1989) using detergents and proteinase K treatment. The serum panel used in the initial evaluation of the direct serum addition method were retested using this technique. Very different results were obtained, B19 DNA was detectable in 10 of 11 of the B19 IgM containing sera. It was also detectable both earlier in the course of infection and for a longer period in the sequential volunteer sera. The explanation for the observed difference in results obtained with the direct serum addition method and the extracted DNA technique is unclear. It is known that whole blood contains inhibitor(s) of PCR. Haem has been shown to be one such inhibitor

(Higuchi 1989). Although none of the sera tested were visibly haemolysed it seems likely that inhibitor(s) were present. It is tempting to speculate that at least one of these inhibitors is B19 IgM. This is based on the observed temporal relationship between the presence of B19 IgM and PCR positivity (figure 3). A putative IgM-virus complex may be coagulated or precipitated by the 95°C melt temperature of the PCR. B19 IgM mediated inhibition would not explain the observed differences in B19 DNA detection by PCR at the beginning of infection.

The optimised DNA extraction method was quick (one and half hours preparation time) and simple to perform. It did not require the multiple decanting steps required of phenol/chloroform extractions and so the potential for cross contamination of sample DNA during preparation was low. This method was used in the subsequent experiments for serum and PBMNC DNA extraction.

The nested PCR method was shown to be capable of detecting B19 DNA from widely varying geographic locations and archival sources. This suggests that the primers anneal to a relatively conserved region of the genome. However one of four B19 IgM containing serum that tested negative for B19 DNA by nested PCR using a 55°C anneal temperature was shown to contain B19 DNA when the anneal temperature was reduced to 45°C (plates 11,12). Unfortunately non specific products were also generated at this annealing temperature.

There is very limited data on the diversity of the B19 genome and conflicting evidence on the relative abilities of primers selected from different areas of the genome to amplify B19 DNA. Koch and Adler (1990) found that primers selected from the VP1 region were able to amplify isolates of B19 not detected by primers from the VP2 and NS1 regions. However, Frickhofen and Young (1990) were unable to demonstrate a difference in detection rates using six primer pairs selected from the

VP1 and NS1 regions. Eight other primer pairs selected from the hairpin and VP2 regions gave less consistent results. Clewley used two primer pairs selected from the NS1 and VP1 regions respectively to assay placental specimens collected from women with proven B19 infections. He found that the NS1 primers could detect B19 DNA in 70% of the samples while the VP1 primers could only detect B19 DNA in 45% of the placentas. Thirty one percent were positive with both sets of primers.

5.1.1 Contamination problems

Contamination is a potential problem with all PCR assays. There are however, problems specifically associated with PCR for the detection of B19 DNA. The virus contains single-stranded DNA that forms a ready template for PCR without pre-DNA extraction. B19 viraemia during the acute phase of infection is particularly intense, with up to 10^{12} viral particles/ml detectable (Anderson *et al* 1985b), so that correct sample handling to ensure no cross contamination of samples is particularly important. The virus is also particularly hardy, viral infectivity is retained after prolonged periods at room temperature or after heating, ensuring the integrity of the DNA. In addition, all the serological assays used for the detection of B19 antibodies employ viraemic plasma as the source of antigen.

The precautions described by Kwok & Higuchi (1989) were taken to ensure that the PCR assay remained free from DNA contamination. In addition dedicated gowns, gloves and overshoes were worn in the post-PCR handling laboratory. No equipment or reagent products were ever moved from the post-PCR handling area to the other laboratories. Spillages were swabbed with 1M HCl. All samples referred to the Department for B19 testing were aliquoted in a class 3 cabinet for potential PCR testing, prior to any diagnostic testing. The positive controls used in experiments

assaying clinical samples by PCR were selected to contain only 1-10 copies of detectable B19 DNA. Potential contamination of the PCR assay was continually monitored for by the routine inclusion of negative control samples (a maximum of every 10th sample assayed).

DNA contamination was seen in the PCR assay prior to the instigation of these measures but never afterwards. It was usually difficult to determine the source of the contamination. However, on one occasion the contamination could be traced to a primer batch recently ethanol precipitated and dried with a flow of nitrogen gas. The nozzle used to direct the gas flow had been used previously to dry an ethanol precipitated preparation of pVTM-1. During another episode of contamination a member of staff working in the reagent preparation area had developed an acute B19 infection. PCR products were the likely source of a further episode of contamination when the PCR product laboratory and the reagent preparation area were cleaned on the same day with the same mop. On this occasion contamination was seen simultaneously in the B19 PCR assay and in PCR assays for other viruses. Contamination of the PCR assay did not occur in any of the negative controls for the experiments described in this thesis.

5.2 APPLICATION OF NESTED PCR

The ability of nested PCR to determine the timing of the acquisition of B19 infection was assessed using a series of diagnostic sera with a known date of onset of symptoms and serial sera from experimentally infected volunteers. Nested PCR was able to detect B19 DNA in 70% of sera with a laboratory confirmed diagnosis of B19 infection. First and second round PCR products were detectable in 16% of diagnostic sera (found to contain B19 DNA by PCR), collected up to 11 days after the onset of

symptoms. Concordant results were obtained with sera collected from the experimentally infected volunteers in whom first and second round PCR products were detectable for up to 8 days after the onset of symptoms. However, there were a number of sera collected within 11 days of the onset of symptoms that either only demonstrated specific PCR product after nested PCR (see figures 7,8) or were not found to contain B19 DNA. The other 84% of sera contained levels of B19 DNA only detectable after both rounds of amplification. The temporal relationship between the presence of second round PCR product and the timing of infection was even more variable. Second round product was detected in sera collected a mean of ten days from the day of onset of symptoms. However a serum collected on the day of onset of symptoms and another collected 150 days after the onset of symptoms contained B19 DNA. The variability of the PCR patterns obtained is shown in figures 7 & 8. In summary, for the testing of individual sera, the PCR fails to accurately predict the timing of infection. However on a population basis there was a statistically significant difference between the timing of infections detected by both rounds of PCR (mean 3 days), second round PCR only (mean 10 days) or those found to be PCR negative (mean 39 days)(figure 8).

The percentage of B19 IgM containing sera found to contain B19 DNA and the long period during which B19 DNA could be detected are in accord with the findings of Clewley. Nevertheless in contrast to Clewley this study did detect a statistically significant association between both the B19 IgM and IgG serostatus of the sera and the PCR result (figures 5,6). B19 DNA was less often detected in samples with low values of B19 IgM. This association was reversed for B19 IgG status, low levels of IgG were associated with a higher probability of B19 DNA being detected. Clearly the duration of illness, B19 IgG and IgM values are not independent variables. Although there is considerable individual variability, B19 IgM and IgG are almost inversely related to each other, and both correlate directly with the interval between

the onset of illness and serum collection.

The association between B19 IgM seropositivity and the presence of B19 DNA was further analysed in the investigation of a B19 outbreak at a local hospital. Sera collected from infected subjects after the decline of the IgM response were assayed by nested PCR. In only one serum could B19 DNA be detected in the absence of B19 IgM during the convalescent phase of infection. The number of sera tested were too small to draw more than tentative conclusions. Nonetheless taking this data, the observations on the level of B19 IgM and presence or absence of B19 DNA and the finding that immune complexes are detectable in serum at the time of development of the IgM response to B19 infection (Anderson *et al* 1985b) a unifying testable hypothesis can be proposed. I postulate the following: 1. B19 IgM is complexed with virus in the convalescent phase of the illness and while B19 IgM may be present in the absence of B19 DNA the reverse is not usually the case. 2. The presence of B19 DNA is a marker of the antigenic stimulation to sustain the B19 IgM response. The first part of the hypothesis could be tested by assaying sera for B19 DNA from a large number of infected individuals at the time when B19 IgM declines to undetectable levels. The second part of the hypothesis could be tested by following up patients with recent B19 infections to determine whether the decay of B19 IgM differs between individuals with B19 DNA detectable by PCR and those without detectable B19 DNA. Insufficient numbers of samples were collected at too few timepoints in this study to test these hypotheses. Additional information could also be obtained by giving a B19 IgM seropositive individual with circulating B19 DNA normal pooled immunoglobulin and determining the effect of this on B19 IgM seropositivity and the detection of B19 DNA.

The relationship between the detection of B19 DNA by nested PCR and the potential infectivity of the material under study remains to be determined. Preliminary

unpublished observations on the ability of sequential sera, collected from infected volunteers, to infect fetal liver cell cultures correlates with B19 dot blot hybridisation positivity (personal communication, Dr A Morey, Oxford). Whether this reflects the situation *in vivo* is not known.

The nested PCR was applied to 49 sera collected from patients with an unexplained B19 IgM/rubella IgM negative rash illness. This was performed to determine whether the MACRIA assay missed cases of B19 related rash illness. None of these sera were found to contain B19 DNA by PCR.

The hospital outbreak of B19 infection also facilitated a longitudinal study into the persistence of B19 DNA in throat secretions and peripheral blood mononuclear cells. B19 DNA was detectable by nested PCR from all the throat swabs collected within one week of the onset of symptoms and was only detectable in one throat swab at four months. This contrasts with the results obtained by DNA dot blot hybridisation from experimentally infected volunteers (Anderson *et al* 1985b), in whom B19 DNA was detected from day 7 to day 11 post infection. Although three different starting quantities of DNA had to be assayed by PCR to overcome the problem of PCR inhibitors in oropharyngeal secretions; this approach to diagnosis merits investigation of larger numbers of samples to determine if this could form the basis of a non-serum based test of acute B19 infection. This would be useful for investigating rash illnesses in children.

B19 DNA was also detectable in the PBMNC of infected subjects and remained so in three of four individuals to the study endpoint six months after the onset of illness. It was not possible to demonstrate by *in situ* hybridisation which cell type contained the B19 DNA. This could be due to a lack of sensitivity of the *in situ* hybridisation if very low copy numbers of B19 DNA were present in many cells or, due to a

limitation in sampling if there was a high copy number of B19 DNA in a very few cells. A further possibility is that the B19 DNA was present in a form attached to the cell surface, perhaps via a B19 antibody-Fc receptor or complement-Fc receptor interaction and that this complex dissociated during the labelling procedures.

Immunolabelling demonstrated the presence of nucleated erythroid precursors in the PBMNC fraction. The known tropism of B19 virus for erythrocyte precursor cells makes this the most likely cell candidate to contain the B19 DNA detected. Alternatively, the virus may have been phagocytosed by macrophages or neutrophils, with the B19 DNA remaining intact within the cells.

Kurtzman *et al* (1988b) have shown previously that B19 DNA, detected by dot blot hybridisation, could be found in the high-density, granulocyte enriched fraction of blood of acutely infected individuals. This was detected at a time when viraemia was no longer detectable in serum by dot blot hybridisation. In addition, this group was able to demonstrate that the B19 DNA was actively replicating, as indicated by the detection of intermediate replicative forms by Southern blot hybridisation. Occasional granulocytes were seen in the PBMNC fractions collected in the hospital outbreak study so that these cells cannot be excluded as the potential host for the B19 DNA detected by PCR. The question of whether the B19 DNA detected by PCR represents persistent B19 infection or latent infection remains to be answered.

5.2.1 Fetal infections

Nested PCR was applied to the detection of fetal B19 infection. Three questions were addressed, firstly could B19 infections be detected in fetuses with a clinical presentation compatible with B19 infection but that were found to be serum B19

DNA dot blot hybridisation negative? B19 DNA was detected by PCR in fetal serum only in those hydropic fetuses found to be serum dot blot hybridisation positive. B19 DNA was not detected by PCR in any of the 15 other fetuses investigated. This suggests that PCR offers no sensitivity advantage for the detection of fetal B19 infections.

The second question asked was whether the diagnosis of fetal infection could be made by detecting fetally derived B19 DNA in maternal sera? The rationale for this question was based on the observation that Y chromosome sequences of male fetuses can be detected in the maternal circulation by PCR (Lo *et al* 1989). B19 DNA was detectable in maternal serum of only one of five B19 infected pregnancies. It is likely that this DNA was fetally derived because of the absence of maternal B19 IgM antibody. These results suggest that although the infected fetus has an intense viraemia at the time of the hydropic illness, fetal B19 DNA is not usually detectable in the maternal circulation, even at the low level detectable by nested PCR. Possible explanations for this finding are that the placenta does not allow the virus to cross back into the maternal circulation or that maternal lymphocytes and possibly reticuloendothelial cells trap B19 virus crossing the placenta.

The third question addressed was whether PCR could be applied to the detection of B19 DNA in fetal tissues? B19 DNA was detectable by nested PCR and dot blot hybridisation in tissues obtained from two infected fetuses. These results are in accord with those of Salimans *et al* (1989b) who were able to detect B19 DNA by ***in situ*** hybridisation and PCR in placental and fetal tissues from a case of intrauterine fetal death. PCR offers advantages over both dot blot hybridisation and *in situ* hybridisation for the detection of DNA in tissues. Dot blot hybridisation may produce false positive results with "sticky" tissue preparations (personal unpublished observation). *In situ* hybridisation is too labour intensive for routine diagnostic

testing.

5.2.2 Detection of intrauterine infection in neonates

Congenital B19 infection is difficult to diagnose. Only 17% of B19 infected neonates make detectable B19 IgM (PHLS 1990) so that the diagnosis can only reliably be demonstrated by the persistence of B19 IgG after passively acquired maternal antibody has waned. Although B19 virus is not known to cause congenital malformation nor problems in the neonatal period, the difficulty in demonstrating congenital B19 infection has hampered such investigation.

Nested PCR was applied to cord blood samples obtained from neonates following maternal B19 infection in pregnancy to determine whether B19 DNA could be demonstrated in serum and was therefore a marker of congenital infection. Unfortunately it was not known whether fetal infection had occurred during the pregnancies because of the anonymous nature of the study. In addition only limited clinical data was available on the neonates. However, based on a maximum transplacental transmission rate of 33% (PHLS 1990), up to eight of 27 neonates investigated could be expected to have had an intrauterine B19 infection. PCR failed to detect B19 DNA in cord serum from any of the 27 neonates. This could be either because the neonates were not congenitally infected or because persistent B19 DNA in serum is not a feature of congenital infection. Assaying of serum from neonates following a confirmed intrauterine infection would resolve this problem.

5.2.3 Detection of B19 infection in the immunocompromised

Various case reports have described chronic B19 infection in the

immunocompromised. The object of this pilot study was to determine the prevalence, timing and possible source of B19 infection in a cohort of 10 allogeneic bone marrow transplant recipients. Neither B19 DNA nor IgM could be detected in multiple serum samples collected from the bone marrow recipients nor their donors. B19 seroconversions were demonstrable, but these could not be taken as evidence of recent B19 infection because bone marrow recipients develop their donor's immunological memory. In addition the possibility of passive acquisition of B19 IgG from a seropositive blood transfusion cannot be excluded.

Hornsleth, studying 184 allogeneic bone marrow patients, has been able to demonstrate B19 DNA in 15% of subjects by nested PCR (personal communication Prof.A.Hornsleth, Copenhagen). Most of the subjects in the Hornsleth study were suffering from leukemia, similar diagnoses to those subjects in the study group described here. However this pilot study was too small to detect an infrequent B19 infection event.

The detection of two separate episodes of B19 infection in a renal transplant recipient by PCR is the first unequivocal demonstration of secondary B19 infection. A case of possible B19 reactivation has been described previously in a four year old child with acute leukemia (Coulombel *et al* 1989). In that case B19 viraemia was detectable both at the time of development of a pancytopenia and in a serum collected three months previously. No details were given of the patient's B19 status during the three month interval. It may be that the patient was continually B19 viraemic during this time and had a persistent infection rather than a reactivation.

The most likely source of the secondary infection in the renal transplant recipient was his mother, with whom he had close contact at the time of her B19 infection and illness. His viraemia was detected 10 days after her symptoms, a time consistent with

the known incubation period of B19 infection.

5.2.4 B19 infection and malaria

The low B19 seroprevalence observed in the Killifi area would suggest that this cohort of children have yet to acquire B19 infection. This is borne out by the low incidence of B19 infection detected in the children by PCR and IgM testing. This apparent low seroprevalence could also be due, in part, to the demonstrable inhibition of the GACRIA by malaria infected plasmas although the degree of inhibition observed was small.

The low seroprevalence of B19 infection in this age group is at variance with other studies of B19 infection from other regions of Africa. It is also at variance with the observed seroprevalences of other childhood diseases such as measles in African populations. Schwarz *et al* (1989b) studied the seroprevalence of B19 infection in Malawi and Mascarene Islands and found that between 30% and 50% of children from one to 10 years of age had detectable B19 IgG. No difference in seroprevalence was observed between rural and urban populations. Concordant results were also obtained in Niger from the small pilot study of Jones *et al* (1990) in which 13 of 24 (54%) of young children were seropositive.

It proved unfounded that plasmas collected from malaria infected individuals would inhibit the detection of B19 IgM by MACRIA and so only limited PCR testing was performed. The PCR was used to test plasmas collected from those individuals with severe anaemia to determine whether the MACRIA test was missing cases of B19 infection. B19 DNA was only detected in one such subject, who was B19 IgM negative/IgG positive. The PCR finding was repeatable. This finding is unusual, B19 DNA is not usually found in B19 IgM negative/IgG positive sera. This could

indicate rapid loss of specific IgM following B19 infection or chronic B19 infection. The very low haemoglobin (2.3g/dl) would be more consistent with a chronic infection, although the anaemia may simply be related to the malaria infection.

The PCR was also able to confirm the specificity of the IgM response detected in one of the two B19 IgM positive subjects.

The hypothesis that the severity of anaemia observed with malaria infection is due to concurrent B19 infection was not confirmed by this study. It remains to be seen whether B19 infection is an additional cofactor for severe anaemia when B19 infection coincides with malaria infection.

5.2.5 B19 infection in patients with arthritis

This study examined both the prevalence and incidence of B19 infection in individuals attending a rheumatology clinic. It also attempted to determine whether B19 infection modified the course of pre-existing arthritic disease. Previous studies have focused on patients with early joint symptoms while this study examined all attenders at the clinic.

If B19 virus was the aetiogical agent of rheumatoid arthritis or an important cofactor in its genesis then the IgG seroprevalence of B19 would be expected to be higher in rheumatoid patients than in control populations. Jones *et al* (1986) have reported an increased B19 IgG seroprevalence in patients with early rheumatoid arthritis. Contary to this finding Lefere *et al* (1985) were unable to show a statistically significant difference in B19 IgG seroprevalence between rheumatoid arthritis patients and healthy adult controls. The B19 IgG seroprevalence in the study reported here was

similar for each of the patient groups examined and therefore is at variance with the Jones *et al* (1986) study.

Recent B19 infections, identified by the presence of B19 IgM, were detected in all the diagnostic groups except the non-inflammatory joint group. Again, if B19 infection were to be implicated in the aetiology of rheumatoid arthritis then it might be expected that the seroprevalence of B19 IgM would be high in the early rheumatoid arthritis group. The fact that the diagnostic classification of all the rheumatoid and inflammatory joint disease patients with evidence of B19 infection remained unchanged at six months follow up suggests that B19 infection was an intercurrent illness superimposed on an existing arthritic condition in these subjects.

A further observation made while testing these sera and not previously documented was that the MACRIA test was found to produce false positive reactivities with four of the sera containing rheumatoid factor. This was removed by rheumatoid factor absorption. Confirmation of recent B19 infection was provided by PCR in six of 11 samples tested, a proportion similar to that obtained by assaying of diagnostic sera found to be IgM positive. Nine of the 11 B19 IgM positive patients were female. This striking sex ratio was also observed by White *et al* (1985) who identified 19 cases of recent B19 infection in an early synovitis clinic, all were women.

PCR was performed on 96 unselected sera collected from patients attending the clinic to determine whether recent B19 infections could be detected by PCR that the MACRIA test failed to detect. It was postulated that B19-specific immune complex formation could be the cause of arthritis in some individuals and that this could interfere with the detection of B19 IgM by the MACRIA test. None of the B19 IgM negative samples was found to contain B19 DNA by PCR.

5.2.6 Detection of B19 DNA in clotting factor concentrates

PCR was able to detect B19 DNA contaminating clotting factor concentrates. B19 DNA was found in seven concentrates by PCR, only two of these contained sufficient DNA to be detectable by dot blot and Southern blot hybridisation. B19 DNA was detected in both treated and untreated concentrates. It was detected in steam treated, TNBP treated and monoclonally purified concentrates. It was not detected in pasteurised concentrates or in the small number of chloroform and dry heat treated concentrates tested.

Two factors affect the detection of B19 DNA in the samples, firstly whether the concentrate contains plasma collected from a viraemic subject and secondly whether the DNA survives the anti-viral treatment procedures. The first factor was not addressed by this study and so only limited conclusions can be drawn about the effectiveness of different treatments to inactivate B19 virus. In addition, the infectivity of the DNA detected by PCR is unknown. The effectiveness of various treatments could be assessed by adding B19 virus to plasma pools and determining by PCR whether viral DNA was detectable at the end of the procedure.

The ability to screen for B19 DNA by PCR in concentrates is, however, potentially useful. The inability to detect B19 DNA by such a sensitive technique is likely to indicate that the concentrate does not contain sufficient B19 virus to be infectious. The value of universal screening for B19 DNA in clotting factor concentrates is marginal. It is of course undesirable to give blood products containing any potentially infectious material to patients. However B19 infection is only likely to pose a threat to the immunocompromised and pregnant women. For these groups screening of clotting factor concentrates by PCR may be warranted.

5.3 CONCLUDING REMARKS

The development and application of nested PCR for the detection of human parvovirus B19 DNA has been described. The development of nested PCR included the optimisation of the assay and the development of quick and simple methods for the extraction of sample DNA. Attention was paid to ensuring that DNA contamination of the samples or the PCR did not occur. The assay was highly sensitive, detecting 1-10 copies of B19 DNA. This exceeds the sensitivity of existing (non-PCR based) B19 DNA detection systems by 1000-10,000 fold.

It was hoped that the design of the first and second round PCR assays would allow nested PCR, when applied to sera collected from infected individuals, to accurately determine the length of time that individuals had been infected and augment existing diagnostic tests. To this end the PCR was applied to a series of diagnostic sera with a known date of onset of symptoms and serial sera from experimentally infected volunteers. The assay failed to achieve this objective because of variation between individuals in their ability to clear B19 DNA from their serum. However on a population basis the timing of infection could be inferred from the pattern of PCR results obtained.

Nested PCR was also applied to PBMNC and throat swabs collected from infected individuals to characterise the presence of B19 DNA at these sites during the course of infection. The PCR assay was able to detect B19 DNA persisting in the PBMNC fraction of blood at the study endpoint, six months after infection. The biological significance of this finding remains to be determined. Combined immunolabelling and *in situ* hybridisation studies failed to reveal the cell type infected with B19, either because of a lack of sensitivity of the *in situ* hybridisation assay or, due to a limitation in sampling if there was a very high copy number of B19 DNA in a very few cells.

B19 DNA was also detected by PCR in throat swabs obtained from infected individuals at the time of their symptoms and so PCR could form the basis of a non invasive diagnostic test of recent B19 infection.

PCR was applied to the diagnosis of B19 infection in a variety of situations in which existing tests have shortcomings. In fetal and congenital infections in the neonate PCR only offered advantage in the detection of infection in fetal tissues. In the small population of immunocompromised patients tested, the conclusions that can be drawn are limited, but those tested did not appear to develop primary or recurrent B19 infection.

PCR was also used to confirm the specificity of existing B19 diagnostic tests for "problematic" sera obtained from patients with malaria and arthritis. It provided a low sensitivity (70%), high specificity confirmatory test. It was also hoped that PCR might provide clues to the pathogenesis of the arthritis of B19 infection. It failed to achieve this goal as the prevalence of B19 DNA was no different between sera collected from B19 infected patients with arthritis attending rheumatology outpatient clinics and sera from unselected B19 infected individuals with a variety of B19 related illnesses.

The assay was useful in providing a sensitive screening test for the detection of B19 DNA in clotting factor concentrates. Although PCR is unable to determine the infectivity of the B19 DNA containing concentrates, it is unlikely that concentrates, not found to contain B19 DNA by such a sensitive test, are infectious.

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APPENDIX

TRIAL 1007: HUMAN VOLUNTEER INOCULATION OF PARVOVIRUS B19

Patou,G., Pattison, J.R. & Tyrrell, D.A.J.

1989

Introduction

Parvovirus infection is the cause of erythema infectiosum, the aplastic crisis seen in haemolytic anaemia, fetal loss in pregnancy and hydrops fetalis.

Currently the diagnostic tests available for the detection of parvovirus are IgG and IgM immunoassays and DNA dot-blot hybridisation. The department of Medical Microbiology, University College and Middlesex School of Medicine is one of only four centres in the UK currently offering these diagnostic tests.

Human parvoviruses are non-cultivatable and attempts to produce synthetic antigen in the UK have been unsuccessful to date. Since infected individuals are asymptomatic or have only mild non-specific constitutional symptoms during the viraemic phase of parvovirus infection, it is serendipidous when viraemic individuals are detected following community acquired infection. Almost invariably infection in haematologically "normal" individuals is detected at the "late" antibody phase of the illness.

A current stock of parvovirus antigen required for the immunoassay tests, was obtained by screening 4,000 units of blood donated to the National Blood Transfusion Service (NBTS). A subsequent screen of 20,000 samples failed to yield a single viraemic unit.

At present stocks of parvovirus antigen are severely depleted and this has necessitated reducing the diagnostic service offered to referring laboratories (formerly approximately 60 nationally and internationally) to a regional service only. Within 12 months there will no longer be sufficient antigen to provide a diagnostic service for patients.

Aim

The aim of this study is to generate large quantities of parvovirus antigen by inoculation of a healthy male seronegative volunteer for use in diagnostic and research immunoassays.

Volunteer selection

The volunteer, designated SM is a healthy 30 year old parvovirus seronegative male. He is medically qualified and a clinical virologist. His knowledge and experience allow him maximum understanding of the background of the study and his consent could not be more informed. He has a past medical history of jaundice at 6 years, tonsillectomy at 12 years and suffers from hayfever. He is married and has a male child 14 months old. Physical examination of the volunteer revealed no abnormality.

Study

Ethical committee approval was obtained at the relevant institutions. The study was performed at the MRC Common Cold Unit and designated trial 1007. Fully informed consent was obtained and the volunteer reserved the right to withdraw from the study at any time.

Pre-inoculation investigations 1/8/89

Haemoglobin 13.9g/dl	White blood count $6.4x10^9/l$
Platelets $286x10^9/l$	Mean corpuscular volume

(MCV) 78.9fl

Biochemical screen: No abnormalities detected

Day 0 Inoculation 25/9/89

Half a millitre of viraemic plasma KCHA1 diluted 1/5 in Hank's buffered saline was inoculated into each of the volunteer's nostrils. KCHA1, known to contain parvovirus, had been subjected to and passed the normal screening procedures for blood donation for transfusion purposes.

Day 1 26/9/89

Isolation at MRC Common Cold Unit

Clinical: No symptoms

Day 2 Day 4 27/9/89 - 29/9/89

Clinical: No symptoms

Day 5 30/9/89

Clinical: Headache and fatigue

Laboratory: CIE; B19 antigen negative

Day 6 1/10/89

Clinical: Generalised myalgia and fatigue

Laboratory: CIE; B19 antigen weakly positive

Day 7 2/10/89

Clinical: Generalised myalgia and fatigue

Laboratory: CIE ; B19 antigen strongly positive

Pre-venesection investigations:-

Haemoglobin 13.9 g/dl White blood count $5.3x10^9/l$

Platelets $230x10^9/l$

Seven hundred millilitres of whole blood venesected into heparinised blood collection pack, 350mls plasma was separated and stored at -70°C.

Day 8 3/10/89

Clinical: No symptoms

Laboratory: CIE; B19 antigen strongly positive

Day 9 4/10/89

Clinical: Sneezing. No other symptoms

Laboratory: CIE; B19 antigen weakly positive

Post-venesection investigations:-

Haemoglobin 12g/dl White blood count $3.5x10^9/l$

Platelets $226x10^9/l$ MCV 77fl

Target cells seen in blood film

Day 10 5/10/89

Clinical: Acute self-limiting diarrhoea, vomiting and abdominal cramps.

Sneezing and post-nasal drip

Laboratory: CIE; B19 antigen negative

Day 11 6/10/89

Clinical: Sneezing and post nasal drip. No systemic symptoms

Laboratory: Haemoglobin 12g/dl White cell count $3.6x10^9/l$

Platelets $230x10^9/l$ MCV 74fl

Blood film shows borderline hypochromia

CIE; B19 antigen negative

Day 12 7/10/89

Clinical: Arthralgia both knees

Day 13 - 14, 8/10/89 - 9/10/89

Clinical: No symptoms

Day 15 10/10/89

Clinical: No symptoms

Laboratory: Haemoglobin 11.2g/dl White blood count $8.2x10^9/l$

Platelets $425x10^9/l$ MCV 78.6fl

Day 16 11/10/89

Clinical: Arthralgia both knees

Day 17 12/10/89

Clinical: Malaise

Laboratory: Haemoglobin 11.4g/dl White blood count $11.4x10^9/l$

Platelets $492x10^9/l$ MCV 78fl

Reticulocyte count 1.4% Differential white cell count: not available

Volunteer commenced on ferrous sulphate 200mg t.d.s.

Day 18 13/10/89

Clinical: "Puffy" face, malaise,myalgia. Arthralgia knees and ankles bilaterally. Soreness plantar surfaces of feet

Day 19 14/10/89

Clinical: As for day 18

Day 20 15/10/89

Clinical: Increasing pain, plantar aspects of feet and generalised myalgia

Day 21 16/10/89

Clinical: Increased arthralgia, now involving elbows, ankles and knees. Decreased myalgia and swelling of cheeks

Laboratory: Haemoglobin 11.9g/dl White cell count $8.1 \times 10^9/l$

Platelets $422 \times 10^9/l$ MCV 80.3fl

Reticulocyte count 1%

Day 22 17/10/89

Clinical: Continuing arthralgia but decreasing constitutional symptoms.

Day 23 18/10/89

Clinical: Decreasing arthralgia and constitutional symptoms.

Day 24 19/10/89

Clinical: Minimal arthralgia. No constitutional symptoms.

Day 25 20/10/89

Clinical: No symptoms. Clinical illness fully resolved.

Day 30 25/10/89

Laboratory:	Haemoglobin 12.6g/dl	White cell count $7.5x10^9/l$
	Platelets $436x10^9/l$	MCV 76.6fl
	Retic count 1.6%	

One millilitre of whole blood was collected daily from day 5 to day 15 and twice weekly from day 16 to day 40 for use in the B19 PCR assay.

Validation of B19 antigen collected as plasma from volunteer in MACRIA assay for B19 IgM detection

The plasma collected from SM was titrated from 1/200 to 1/1600 and used as B19 antigen in a B19 IgM antibody capture radioimmunoassay. It was compared to reference B19 antigen KCHA1 used at optimal dilution (1/400). Plasma SM was antigenic in this assay and gave comparable performance to plasma KCHA1 over the full dilution range (data not shown).

Summary

Volunteer SM developed a B19 infection following inoculation with B19 virus. He

developed a biphasic illness; a 'flu-like illness developed from day 5 to day 7 and a transient arthralgia with constitutional symptoms from day 16 to day 24. Transient upper respiratory and gastrointestinal symptoms also occurred but B19 virus may not have been the cause. The volunteer's haemoglobin concentration dropped to 11.2g/dl by day 15 with microcytosis and target cells present, suggestive of iron deficiency revealed by the B19 infection and 700ml venesection. This had subsequently recovered to 12.6g/dl by day 30 following oral iron supplimentation and continues to be monitored. Three hundred and fifty millilitres of plasma were collected during the peak of the viraemia and contained B19 antigen in concentrations utilizable in a B19 IgM capture assay.