Chapter 13

The Detection of Parvoviruses

Sean Doyle

Abstract

Parvovirus B19 is a single-stranded DNA virus which causes severe disease in immunocompromised patients and foetal loss in pregnant women. It is classified as an *Erythrovirus* and this genus also comprises two related viral genotypes (so-called LaLi/A6 (genotype 2) and V9 (genotype 3)) which appear to be immunologically indistinguishable from Parvovirus B19. Serological and nucleic acid test (NAT) systems to detect Parvovirus B19-mediated infection are commercially available; however, some NAT systems are genotype-specific. International standard preparations of Parvovirus B19 IgG and DNA have been produced for assay standardisation purposes, and to ensure consistency of assay manufacture and performance. Immunological assays, such as B-cell ELISpot, T-cell stimulation, and cytokine detection can also be used to confirm exposure to Parvovirus B19. Immunohistochemical techniques, employing commercially available monoclonal antibodies, are used to localise the virus in infected tissue and Parvovirus B19 viral antigen can also be detected in serum and plasma using antigen-specific ELISA. NAT systems have also been described to detect newly identified parvoviruses such as human bocavirus (HBoV), PARV4, and PARV5, although absolute confirmation of clinical diseases associated with these agents is required. This chapter describes the current status of detection systems for all the aforementioned parvoviruses, with particular emphasis on *Erythrovirus* detection by serological, NAT, and immunological approaches.

Key words: Parvovirus, Erythrovirus, ELISA, B-cell ELISpot, Cytokines, NAT testing, Human bocavirus, Recombinant protein

1. Introduction

Human parvovirus B19 (B19) is a non-enveloped, 5.5 kb single-stranded DNA virus (1). It belongs to the family *Parvoviridae*, sub-family *Parvovirinae*, genus *Erythrovirus*. Although initially identified as related genotypes of B19, so-called genotypes 2 (K71/LaLi/A6) and 3 (known as V9) have been reclassified as human parvovirus B19-A6, -LaLi, and -V9, respectively (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_parvo.htm).

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However, for clarity and familiarity, occasionally erythrovirus will be referred to as B19, genotype 2, and genotype 3 (or V9), respectively, throughout this chapter. These strains are immunologically indistinguishable from B19, although they exhibit up to 10–12% sequence divergence at the DNA level (2–4). B19 is a human pathogen, and infection is primarily a concern in both immunocompromised patients (e.g. cancer patients undergoing chemotherapy) and pregnant women, where B19 infection in the first two trimesters of pregnancy can lead to foetal loss (5). Emerging roles for B19 in the pathogenesis of certain kidney diseases and myocardial damage are also emerging (6–9).

B19 transmission may be by respiratory droplets, vertical transmission of mother to foetus or via the receipt of B19-contaminated blood or blood products (e.g. packed red cells, plasma, or platelets) (10). Upon infection with B19, high levels of virus appear in the circulatory system within days to weeks and may exceed 10^{13} viral particles/mL, followed by the appearance of virus-specific IgM and ultimately high-titre IgG (11). B19 is composed of two distinct viral proteins, namely VP1 (83 kDa) and VP2 (58 kDa), which form the 20 nm diameter viral capsid (1). These proteins are co-linearly encoded by the viral genome and are present in the viral capsid in the ratio 5:95 (VP1:VP2) (12). The recombinant expression of B19 VP1 and VP2 capsid proteins in the baculovirus expression system and their application in immunodiagnostic assays, to detect B19 exposure, have been extensively described (12, 13).

B19 DNA detection by PCR is now routinely used to complement patient serology profile and viral DNA can be detected in serum, plasma, tissue extracts, and amniotic fluid (14). The latter has proven to be particularly useful for diagnosing materno-foetal B19 transmission (15). However, B19 viraemia may not be associated with symptoms of infection, so in a blood donor context, an infected person could potentially donate blood within this time window, with obvious implications for blood or blood product recipient exposure to the virus. As a consequence, many blood collection organisations and blood product manufacturers have implemented screening protocols to detect B19 DNA by PCR using minipool screening (i.e. making pools of aliquots of blood serum/plasma and screening same for B19 DNA) (16). Minipool, as opposed to individual specimen screening, is performed to reduce the cost associated with detection of B19 presence. However, despite the high sensitivity of detection associated with B19 detection via PCR, the associated cost, allied to (a) the potential high levels of viraemia leading to cross-contamination during screening and (b) the possibility of false negativity of detection during PCR due to sequence differences between genotypes 1 and 3, means that alternative strategies need to be consider for blood screening (17).

Although a haemagglutination assay has been proposed to detect B19 in plasma (18, 19), this system is apparently too insensitive to detect clinically relevant virus levels, moreover, B19 IgG presence in plasma may lead to virus occlusion and resultant false negativity of detection (2, 17). Consequently, two groups have proposed the use of an ELISA system, utilising anti-B19 IgG to detect the virus in human serum and plasma (17, 20). In addition, Corcoran et al. (17) have demonstrated that viral antigen detection can be enhanced by the use of a specialised diluent which significantly improves virus capture by the IgG[anti-B19] used in their immunoassay system. However, although both assays appear to work satisfactorily, the sensitivity of viral detection is limited to greater than 10^7 particles/mL. Thus, the potential to use emerging technologies (21) to improve the sensitivity of detection of B19 in plasma and serum merits consideration.

In order to standardise B19 serological (IgG) and molecular (DNA) diagnostic test systems and to aid B19 IgG and DNA assay performance, a number of WHO International Standards are available to both assay manufacturers and diagnostic laboratories (22, 23).

A number of parvoviruses, distantly related to the erythrovirus genus have been recently identified. Human bocavirus (HBoV), discovered in 2005, is classified as a member of the family *Parvoviridae* (sub-family *Parvovirinae*, genus *Bocavirus*). Four HBoV proteins are likely to be encoded by the compact viral genome, namely NS1, NP-1, VP1, and VP2 with the latter two proteins co-linearly encoded as is the case for the *Erythrovirus* genus (24). HBoV infection appears to be strongly associated with acute respiratory tract infections (ARTI) with preliminary evidence emerging that the virus may also be a potential cause of gastroenteritis (25). Molecular and serological tests have been developed which allow the sensitive and specific detection of HBoV nucleic acid (26, 27) and HBoV IgG and/or IgM (28–30). Indeed, 17 PCR protocols for HBoV DNA detection, deploying a range of primer pairs and detection strategies, have recently been described (26). HBoV recombinant VP2 protein has been generated by two groups utilising the baculovirus expression system (29, 30), while another group has expressed both HBoV VP1 and VP2 proteins in *E. coli* (28). At present, no cell culture systems for HBoV or virus-specific monoclonal antibody preparations are available to enable direct viral detection in vitro, although high-titre rabbit polyclonal antibodies have been produced which can immunoprecipitate HBoV virions from respiratory specimens (29).

A distinct parvovirus, termed PARV4 (parvovirus 4), was identified in plasma obtained from an intravenous drug user in 2005 (31). This virus has been shown to contaminate approximately 5% of plasma pools, with PARV4 viral loads less than

10^6/mL (31). PARV4 is presently unclassified but is likely to form part of a new genus within the sub-family, *Parvovirinae*. In addition, a related viral genotype, termed either PARV4 genotype 2 or PARV5 has also been identified (32). Although yet to be associated with any specific disease, the potential parenteral transmission route of PARV4 has led to intensive investigations and it appears that tissue persistence of PARV4 is associated with parenteral exposure. Tissue persistence was initially thought to be associated with immunosuppression or HIV/HCV co-infection; however, PARV4 has also been detected, at a low rate, in liver and bone marrow specimens from HIV- and HCV-negative individuals (33). No serological assay systems are presently available for PARV Ig detection.

This chapter will describe detailed serological, cellular, and molecular assay systems for B19 detection and introduce some of the nascent molecular, and serological assays for HBoV and PARV4 detection.

2. Materials

1. WHO Parvovirus B19 IgG International Standard (01/602), contains 77 International units (IU) of B19 IgG per ampoule (NIBSC, Potters Bar, Herts, UK). http://www.nibsc.ac.uk. No B19 IgM standard is currently available.
2. WHO Parvovirus B19 DNA International Standard (99/800), contains 5×10^5 IU of B19 DNA per vial (NIBSC, Potters Bar, Herts, UK). http://www.nibsc.ac.uk.
3. WHO Parvovirus B19 DNA working Reagent (99/736), contains 1,000 IU of B19 DNA per mL (NIBSC, Potters Bar, Herts, UK). http://www.nibsc.ac.uk.
4. Histopaque-1077 (Sigma-Aldrich, Dorset, UK).
5. Nitrocellulose-lined plates (96 well) (Millipore, Bedford, MA, USA).
6. *Staphylococcus aureus* cells (Cowan I strain) SAC (Calbiochem, Darmstadt, Germany).
7. Interleukin-2 (IL-2) (Serotec Ltd, Oxford, UK).
8. RPMI medium and foetal calf serum (http://www.invitrogen.com).
9. [^{3}H]-Thymidine (GE Healthcare, Amersham, UK).
10. Liquid scintillation fluid (GE Healthcare, Amersham, UK.).
11. Streptavidin-conjugated alkaline phosphatase (Sigma-Aldrich, Dorset, UK).

12. Tetramethylbenzidine substrate (BioFX Laboratories Inc., Owings Mills, Maryland).
13. Proteinase K (Sigma-Aldrich, Dorset, UK).
14. Qiagen QiAmp Blood kit (Qiagen, Hilden, Germany).
15. Ethidium bromide (Sigma-Aldrich, Dorset, UK).
16. Polyclonal rabbit IgG [anti-parvovirus B19 VP2] (Code: B-0091) (DakoCytomation, Glostrup, Denmark).
17. *Taq* polymerase and dNTP preparations (Promega, WI, USA).
18. Betaine (Sigma-Aldrich, Dorset, UK).

3. Methods

3.1. Detection of B19 IgG and IgM

Detection of B19 IgG and/or IgM is now routinely carried out either by commercially available ELISA, Western blot, or immunofluorescent in vitro diagnostic tests (http://www.biotrin.com/; http://www.ibl-america.com; http://www.mikrogen.de). Indeed, application of these validated and reliable test systems has removed much, if not all, of the variability associated with B19 antibody testing which was evident in the early 1990s. Two FDA-approved immunoassays are available to detect B19 IgM as a marker of recent infection (34), and IgG as a marker of past infection (35), respectively. These microplate immunoassays utilise B19-capsid VP2 and can detect genotype 1, 2, and 3 IgG (4, 36–39).

All commercial B19 IgG detection systems capture B19-specific IgG onto an immobilised, recombinant B19 protein. After B19 IgG binding, removal of unwanted serum proteins and irrelevant IgG is achieved by a wash step. B19-specific IgG is then detected using either an enzyme- (generally horseradish peroxidase) or fluorophore-labelled anti-human IgG antibody (Fig. 1). For microplate ELISA systems 3,3′,5,5′-tetramethylbenzidine (TMB) is now the substrate of choice. Precipitating substrates such as 3,3′-diaminobenzidine (DAB) are used in Western blot assay systems. Detection of B19 IgM by ELISA systems from most commercial manufacturers is also enabled by the aforementioned assay format; however, IgG or rheumatoid factor (RF) removal is essential to avoid assay interference. IgM-capture (μ-capture) technology is utilised in the FDA-approved B19 IgM assay system which appears to confer high assay specificity and avoids the requirement to use additional reagents such as anti-human IgG or RF adsorbents (34).

Thus, accurate laboratory serodiagnosis of recent B19 infection or past exposure relies on testing serum or plasma specimens

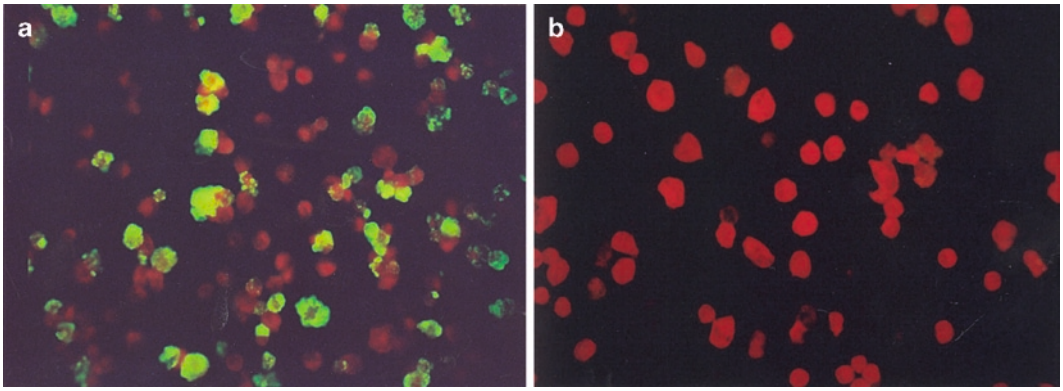

Fig. 1. Immunofluorescent detection of parvovirus B19 IgG using B19 VP1 recombinant baculovirus-infected *Spodoptera frugiperda* cell line (Sf_9). (**a**) B19 IgG reactivity with B19 VP1 protein is detected using FITC-labelled murine anti-human IgG (intense *green* fluorescence against a background of *red* counterstained Sf_9 cells). (**b**) No reactivity observed with B19 IgG seronegative specimen. Courtesy of Biotrin (Dublin, Ireland).

for either specific antibody reactivity against viral capsid proteins, VP2 or VP1, expressed in eukaryotic expression systems (e.g. baculovirus expression system) by ELISA. A specific advantage of the eukaryotic baculovirus expression system is its ability to enable the post-translational protein-folding necessary for the generation of soluble VP2 capsids (12). Unlike B19 VP2, VP1 does not appear to form soluble capsid structures; however, VP1 has been produced as a "conformationally intact" protein which has been shown to retain conformational epitopes present in the native virion (12, 13) (Fig. 1). Co-expression of VP1 and VP2 in eukaryotic expression systems has been proposed to result in the formation of empty capsids, which are antigenically indistinguishable from native B19 virions.

Exposure to B19 in immunocompetent individuals results in the appearance of high-titre B19 IgM. The appearance of B19 IgG then coincides with a diminishing IgM antibody response, and B19 IgG reactivity against conformational epitopes of VP1 and VP2 persists post-infection. However, for both capsid proteins, reactivity against linear epitopes disappears abruptly against VP2, but more slowly against VP1 (13, 40–42) – an observation which has significant consequences for diagnosis. Antibody reactivity against linear VP2 epitopes, predominantly directed against a heptapeptide (amino acids 344–350; KYVTGIN) identified by analysis of acute-phase sera (40), usually disappears within 6 months of B19 infection (41). This heptapeptide sequence is also present in V9 (genotype 3). Thus, detection of B19-specific IgG, directed against linear epitopes of VP2, may assist in timing B19 exposure to within a 6-month period. Although the antibody response wanes against linear epitopes on B19 capsid proteins it persists against conformational epitopes of both capsid proteins. Indeed, this observation has been extended to a so-called second

generation epitope type-specific (ETS) EIA which determines the ratio of B19 IgG reactivity against capsid VP2 and aforementioned heptapeptide (an ETS index). According to the authors, a cut-off value of 10 will distinguish between acute infection (ETS index ≤10) or past infection (ETS index ≥10) (43).

The V9 VP1 and VP2 open reading frames have been cloned and expressed in the baculovirus expression system (37). These authors observed self-assembly of V9 VP1/VP2 and VP2 capsids into parvovirus-like particles (diameter approximately 23 nm). A panel of 270 clinical specimens were screened for the presence of V9 IgM and IgG antibodies in ELISA and showed 100% serologic cross-reactivity between B19 and V9 when comparing V9 VP2 capsids to the B19 VP2 ELISA. Thus, V9 and B19 antibody responses may be diagnosed equally well by ELISA using either V9 or B19 recombinant capsids as antigen source (37). Moreover, Heegaard et al. noted that most genetic variation between genotypes 1 and 3 (Table 1) resulted in silent mutations resulting in 96–97% identity at the protein sequence level.

Controversial data was presented in 2004 which suggested that enzyme immunoassays utilising B19 VP2 capsids derived from genotype 1 did not detect a subset of V9-derived IgG (36). This preliminary finding was based on the use of an unvalidated in-house IgG ELISA which used a V9 VP1 protein as a diagnostic antigen. Candotti et al. (36) also noted that the small numbers of amino acid substitutions in VP1u and NS1 regions of genotype 3 strain, compared to genotype 1, did not modify

Table 1
Parvovirus nomenclature

Name	Genotype	Additional name
Parvovirus B19-Au	1	Parvovirus B19
Parvovirus B19-A6	2	K71, LaLi, A6
Parvovirus B19-V9	3	V9
Human Bocavirus	–	HBoV
PARV4	–	–
PARV5	–	PARV4, genotype 2

Parvoviruses belong to the family *Parvoviridae* (sub-family *Parvovirinae*, genus *Erythrovirus*). Genotypes of B19 (genotype 2 (K71/LaLi/A6) and 3 (V9) are now classified as human parvovirus B19-A6, -LaLi, and -V9, respectively (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_parvo.htm). Parvovirus B19 has been officially classified as B19 virus but this nomenclature has failed to gain widespread use. A number of companies manufacture in vitro diagnostic tests, based on B19 capsid proteins, which detect human antibodies produced upon infection with each of the parvovirus B19 genotypes (1–3). HBoV falls within the genus *Bocavirus* and classification of PARV4 and 5 is outstanding

protein hydrophilicity or antigenicity. This again underlines the fundamental identity and indistinguishable immunological nature of both B19 and V9-derived proteins. The findings of Candotti et al. relating to proposed differences between B19 and V9 IgG detection were disputed (38) and work was completed to resolve this significant issue (39). Resultant data, from analysis of blinded specimens using microplates coated with various combinations of parvovirus antigens (again blinded), confirmed that B19 VP2 capsids can indeed detect all V9-derived IgG, a finding which confirms the diagnostic utility of this validated immunoassay system for all B19 IgG detection (35). Moreover, the findings of Parsyan et al. (39) made it clear that the use of the VP2 capsid facilitates optimal antibody detection and that the additional presence of the VP1 protein did not improve diagnostic accuracy. In separate studies, all three erythrovirus genotypes were shown to both haemagglutinate human red cells and infect myeloid cells (KU812Ep6 or UT7/EpoS1 cells) with equal efficiency leading to the conclusion that the three virus genotypes belong to the same species – parvovirus B19 (4). Moreover, utilisation of erythrovirus genotype 1 capsids enabled detection of IgG from genotype 2-infected individuals ($n = 25$) and erythrovirus genotype 2 capsids detected IgG from genotype 1-infected individuals ($n = 24$) (4).

Thus, it can be concluded that present data support the utility of B19 immunoassay systems to efficiently detect both genotype 2 and genotype 3 IgM and IgG, respectively.

3.2. B-Cell Memory

In the absence of robust cell culture methods to detect B19 in test specimens, alternative techniques can be used to both confirm B19 IgG detection and immune status. One such technique is determination of the B19-specific memory B-cell population in the patient and a memory B-cell ELISpot technique has been developed for this application (44–46) (Fig. 2).

3.2.1. Isolation of PBMC and Stimulation of Cells

Isolate peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation as follows:

1. Pipette Histopaque-1077 (20 mL) into each of three sterile 50 mL conical centrifuge tubes.
2. After collecting 60 mL blood in heparinised tubes, slowly layer 20 mL blood on top of each Histopaque layer. Maintain centrifuge tubes at a 45° angle and allow specimens to run down side of tube until it fills to the 40 mL mark.
3. Centrifuge specimens at $1{,}000 \times g$ for exactly 30 min at room temperature (25°C), then carefully remove the tubes from the centrifuge so that the gradient is not disturbed.
4. Using a sterile pipette, slowly aspirate the upper layer (plasma) to within 0.5–1 cm from the opaque interface containing the PBMC.

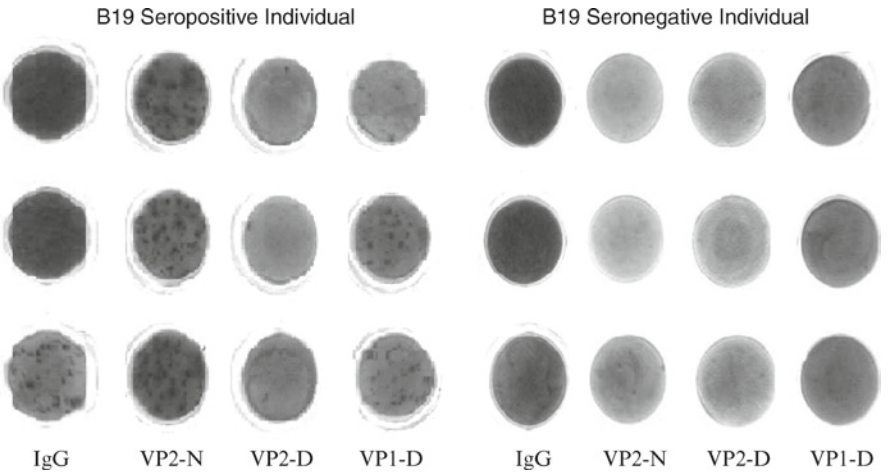

Fig. 2. B-cell ELISpot using PBMC from a B19 seropositive and seronegative blood donor. Plasma from the seropositive donor was strongly reactive against conformational epitopes of capsid VP2 (VP2-N) and linear epitopes of VP1 (VP1-D). No memory B-cells were detectable against linear epitopes of VP2 (VP2-D). Distinct ELISpot wells were coated with anti-human IgG as a positive control to verify B-cell stimulation. All incubations with PBMC at 6×10^6 cells/mL (in triplicate).

5. With a sterile transfer pipette, carefully transfer the opaque interface (Buffy layer) to a fresh 50 mL conical tube, avoiding carryover of the lower clear layer (Histopaque). The interface (containing PBMC) from all three tubes can be pooled into one 50 mL conical tube for washing.
6. Wash PBMC with RPMI medium at room temperature and mix gently by inversion. Following centrifugation at $250 \times g$ at room temperature for exactly 10 min, remove the supernatant and discard (leaving small amount of liquid at bottom). Using a pipette to aid in dispersion, gently resuspend the lymphocyte pellets in complete RPMI (12 mL). (Complete RPMI is prepared made up with 500 mL RPMI 1640 Medium, 8%(v/v) heat-inactivated FCS, 100 U/mL penicillin, 100 U/mL streptomycin, 2 mM l-glutamine, and 50 μM 2-mercaptoethanol.)
7. Conduct a cell count using a haemocytometer with approximately 10 μL of cell suspension and using a 10× microscope lens. A good yield is about 2.4×10^6 cells/mL (representing 1 million cells/mL whole blood).
8. Culture cells using a minimum of 5×10^6 cells/mL in 2 mL per well complete RPMI in 24-well tissue culture plates.
9. Culture PBMC (5×10^6 cells/mL) for 5 days in complete RPMI in the presence of heat-killed *Staphylococcus aureus* cells (Cowan I strain) (SAC) diluted 1:5,000 and 10 ng/mL interleukin-2 (IL-2). SAC and IL-2 jointly function to induce generalised antibody production in resting memory B cells (46).

10. Coat nitrocellulose-lined plates (96 well) with either native B19 VP2 (10 μg/mL), denatured VP2 (10 μg/mL), denatured VP1 (10 μg/mL), or rabbit anti-human IgG (10 μg/mL) in 50 mM sodium carbonate buffer pH 9.6, overnight at 4°C.
11. Wash ELISpot plates with sterile phosphate-buffered saline (PBS) and block with sterile 20%(v/v) FCS in RPMI medium.
12. Add PBMC (post-stimulation) at concentrations of 1×10^6, 1×10^5, and 1×10^4 cells/well to the plates and incubate in complete RPMI for 18 h at 37°C (100 μL per well).
13. Wash ELISpot plates with PBS (4 × 200 μL per well) to remove unbound cells.
14. Detect memory B cells (secreted IgG) by incubation with biotinylated rabbit anti-human IgG (1 μg/mL in (1% (w/v) BSA in PBS)), for 1 h at room temperature, followed by PBS washing (4 × 200 μL per well).
15. Add streptavidin-conjugated alkaline phosphatase at a dilution of 1/1,000 (1% (w/v) BSA in PBS) and incubate for 1 h at room temperature, followed by PBS washing (4 × 200 μL per well).
16. Addition of 5-bromo-4-chloro-3-indolyl phosphate/(nitro blue tetrazolium (BCIP/NBT) substrate (100 μL per well) facilitates memory B cell detection as spots. This development step proceeds for 15 min after which spots can be counted under light microscopy (Fig. 2).
17. The number of spots are averaged from triplicate wells and resultant data reported as the mean number of spot-forming cells (SFC)/million starting cells (44).

3.3. B19 T-Cell Proliferation Assays and Cytokine Detection

1. Isolate peripheral blood mononuclear cells (PBMC) from individuals by density gradient centrifugation as described in Subheading 3.2.1.
2. Culture PBMC (2×10^6 cells/mL) in triplicate with purified recombinant VP1 (10 μg/mL) or VP2 (10 μg/mL) for 72 h (previously 0.2 μm filtered). Optional: parallel cultures can be set up for (a) proliferation assays and (b) cytokine analysis from cell culture supernatants.
3. Cells cultured with medium alone or with a combination of phytohemagglutinin (PHA; 2 μg/mL) serve as negative and positive controls, respectively.
4. Add 0.5–1 μCi of [^{3}H]-Thymidine for the final 4 h of culture, to facilitate labelling of cells, before harvesting onto glass fibre filters using automatic cell harvesting.
5. Liquid scintillation counting (LCS) is carried out using 5 mL LCS fluid per glass fibre filter. Background values for negative

control samples are typically between 200 and 600 cpm, and always less than 1,500 cpm. Results are expressed as stimulation indices (S.I.) representing the proliferative response for test samples divided by the response obtained from the negative controls.

6. IL-2, IL-10, IL-13, IFN-γ, and other cytokines can, in parallel cell culture supernatants, be assayed by commercially available ELISA with pg/mL sensitivity of detection or IFN-γ ELISpot (35, 47–50).

3.4. Nucleic Acid Testing

The main technique used for B19 detection is PCR and many groups have described both qualitative and quantitative PCR strategies for detection of individual, or all three, B19 genotypes (51–54). Apart from total genotype detection, the balance between PCR assay sensitivity and specificity to facilitate detection of acute infection only is important (2).

3.4.1. Specimen Preparation and B19 DNA Amplification (See Note 1)

1. Obtain stock proteinase K and dilute to 20 mg/mL, or prepare stock proteinase K (20 mg/mL) by resuspending in either 25 mM Tris–HCl or sterile high purity water.
2. Obtain clinical specimens (ideally serum or plasma) and add 2 µL proteinase K (20 mg/mL) per 100 µL, mix by gentle vortexing and incubate at 56°C for 1 h. Specimens can be pre-diluted (10^{-4}–10^{-10}) in phosphate-buffered saline (PBS) prior to proteinase K digestion to facilitate semi-quantitative B19 DNA titre evaluation.
3. After incubation, boil all treated specimens at 100°C for 5 min to inactivate proteinase K and centrifuge (13,000 × *g* for 30 min at 4°C) to remove precipitated protein. It should be noted that biological specimens (plasma, amniotic fluid or soft tissue) can also be extracted using the Qiagen QiAmp Blood kit, or similar DNA extraction kits, as described by the relevant manufacturer.
4. Add template DNA (5 µL), present in each specimen supernatant, to the following mixture: 10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1%(v/v) Triton X-100, 2.0 mM $MgCl_2$, 200 µM of each dNTP, 1 M betaine, 1.0 µM of each oligonucleotide primer (Table 2 (54)), specific for regions within the NS1 coding region of the B19 genome, and 1.25 U of *Taq* polymerase in a total volume of 50 µL.
5. PCR amplification uses the following programme: 95°C for 6 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min terminating in 72°C for 5 min.
6. 10 µL of each PCR product is then subjected to 1% (w/v) agarose gel electrophoresis containing 0.5 µg/mL of ethidium bromide for 30 min at 100 V. Amplicon (202 bp) visualisation

Table 2
PCR primers for high sensitivity, single-round amplification of parvovirus B19 (53, 54)

Primers	Gene	Position	Sequence 5′–3′
F1	NS1	1399–1422	AATACACTGTGGTTTTATGGGCCG
R1	NS1	1600–1576	CTAAAATGGCTTTTGCAGCTTCTAC

These primers do not efficiently amplify either genotype 2 or 3 due to base-pair mismatch

can be performed using an Eagle-Eye II gel documentation system (Stratagene, CA, USA) or similar instrument.

7. This assay can detect at least 200 IU B19 DNA (4×10^4 IU/mL) (23) and sensitivity can be further improved by use of PCR-ELISA detection (54).

3.4.2. Quantitative Detection of Genotypes 1–3

At present, two commercial quantitative B19 PCR assays are available, namely the Artus Parvo B19 PC Kit (http://www.qiagen.com) and the Lightcycler® Parvovirus B19 Quantification Kit (http://molecular.roche.com). According to the manufacturers, both systems operate at high sensitivity and specificity of detection and the Artus Parvo B19 PC Kit is capable of detecting all three genotypes of parvovirus B19. However, at least one non-commercial, quantitative B19 PCR assay has been extensively validated for detection of B19 DNA (51) and further evaluated and optimised to detect all three genotypes (52). Details of these non-commercial assays, which amplify a 113-bp fragment in the VP1 region, are presented herewith:

1. Primers: 5′-GACAGTTATCTGACCACCCCCA-3′ (forward) and 5′-GCTAACTTGCCCAGGCTTGT-3′ (reverse).
2. Target specific probe (5′-FAM (6-carboxyfluorescein)-labelled and 3′-TAMRA (6-carboxytetramethylrhodamine) (quencher)): 5′-CCAGTAGCAGTCATGCAGAACCTAGAGGAGA-3′.
3. PCR conditions: 7 mM $MgCl_2$; 200 µM dATP, dCTP and dGTP; 400 µM dUTP; 300 nM each primer; 100 nM probe; 0.01 U Amperase and 0.025 U AmpliTaq Gold DNA polymerase.
4. Template, 10 µL extracted DNA, resulting in a total volume of 50 µL.
5. Amplification: 50°C for 2 min, followed by 95°C for 10 min and subsequently 45 cycles at 95°C for 15 s and annealing and extension at 58°C for 1 min (51).

   An internal control probe can also be used (51)

   or

Amplification: 95°C for 15 min and subsequently 45 cycles at 95°C for 15 s and annealing and extension at 60°C for 1 min (52). These authors further showed that reduction of annealing/extension temperature to 56°C contributed to an improvement in the detection of genotype 3 isolates.

6. Amplification and detection can be carried out using the ABI Prism 7700 Sequence Detection System (51) or Lightcycler® instrument (52). In addition, Baylis et al. (52) modified the original B19 PCR assay to function with Sybr Green I (60°C (using a commercial Lightcycler Faststart DNA Master^{PLUS} SYBR green I kit) and a Lightcycler instrument and found concordance between genotype detection using the above primers.

3.5. Detection of B19 Viral Antigen

A limited number of reports have appeared in the literature which describe cell culture conditions that facilitate B19 replication and the ability of B19 IgG to diminish virus infectivity (55–57). Although excellent research methodologies, to date, these methods have not gained widespread applicability for B19 antigen detection in routine diagnostic laboratories. Consequently, two alternative technologies have been proposed to detect B19 antigen in human serum and plasma, namely (a) B19 antigen detection immunoassays (17, 20) and (b) a red blood cell agglutination assay termed receptor-mediated haemagglutination assay (RHA) (18, 19). Since the latter system (i.e. RHA) occasionally fails to detect B19 antigen in the presence of B19 IgG, thereby producing false negativity, it will not be further discussed.

However, B19 antigen assays have been shown to be easy to perform and, in addition, one assay is capable of detecting all three genotypes of human parvovirus B19. Moreover, combined B19 antigen and IgM detection in clinical specimens by a B19 antigen assay and a B19 IgM assay facilitated the identification of 91% of acute B19 infections in a test population (17). This B19 antigen detection strategy is presented in Subheading 3.5.1.

3.5.1. B19 Antigen ELISA

1. Dilute test plasma and control specimens (1 in 5) in a low pH proprietary commercial diluent (Biotrin International Ltd., Dublin, Ireland) and add to IgG [anti-B19 VP2] sensitised microwells (100 µL/well) for 1 h.
2. Following a wash step (4× PBST), rabbit IgG [anti-B19 VP2]-horseradish peroxidase conjugate is incubated in the wells for 30 min.
3. Following a further wash step (4× PBST), add TMB substrate (100 µL/well) to the microwells for 30 min.
4. Terminate the reaction using 1 N sulphuric acid (100 µL/well) and measure absorbance at 450/630 nm.

5. The presence of B19 antigen in a specimen is determined by the absorbance ratio of specimen sample to cut-off calibrator specimen (Index value). Specimens which yield index values ≥1.0 are classed positive while those ≤1.0 are deemed negative.

3.6. Immunohistochemical Detection of B19

Immunohistochemistry is perhaps the least frequently used technique to detect parvovirus B19 as it requires extensive optimisation. It is also dependent on many factors such as tissue type or source, previous tissue treatment, available instrumentation and most significantly, the quality of anti-parvovirus B19 antibody utilised for virus detection. Consequently, the procedure presented below reflects the strategies deployed by numerous authors to detect parvovirus B19 in paraffin-embedded tissue sections using immunohistochemical techniques (58–64).

1. Paraffin-embedded tissue sections can be dewaxed as follows: Immerse in xylene (60°C, 10 min, twice) followed by 5 min sequential immersions, two per treatment, in absolute ethanol, 95%(v/v) ethanol and distilled water (30 s) at room temperature.
2. Block endogenous peroxidase activity by immersion of slides in 3% (v/v) hydrogen peroxide in distilled water, with gentle stirring for 30 min at room temperature.
3. Remove residual hydrogen peroxide by gentle rinsing in distilled water.
4. To facilitate antigen retrieval, heat slides at 96°C for 30 min in 10 mM sodium citrate pH 6.0 and then cool to room temperature for 20 min.
5. Immerse in TBST (0.1 M Tris–HCl, 0.2 M NaCl, and 0.05% (v/v) Tween 20, pH 7.4), twice, and cover with blocking solution (1% (w/v) skim milk powder, 1% (w/v) BSA in TBS) and incubate in a humidified chamber for 1 h at 37°C.
6. Immerse slides in optimised polyclonal rabbit IgG [anti-B19] antibody solution (DAKO; 1/50–1/300) (58–60) and incubate in a humidified chamber for 16 h at 4°C.
7. Wash slides twice in TBS, for 5 min on each occasion and probe using the appropriate secondary antibody–enzyme conjugate and precipitating substrate (e.g. 3,3′-diaminobenzidine (DAB)) detection system.
8. Ensure appropriate using of negative control sera to ensure validity of observed results.

Other anti-parvovirus B19 antibody preparations have been used for immunohistochemistry and include: (a) mouse monoclonal IgG [anti-VP1/VP2] from Dade Behring, Marburg, Germany) (61), (b) mouse monoclonal IgG [anti-VP2] from Chemicon International, USA (62) and (c) parvovirus B19

primary antibody (code R92F6; NCL-Parvo, Novocastra, Newcastle upon Tyne, UK) (63).

3.7. HBoV and PARV4 Detection

3.7.1. HBoV PCR

Seventeen PCR protocols for HBoV DNA detection, deploying a range of primer pairs and detection strategies, have recently been reviewed and described (26). More recently, the relative sensitivities of conventional vs. quantitative PCR have been compared (27). These authors describe a primer set comprising: Fwd primer 5′-TGGCTACACGTCCTTTTGAACC-3′ and Rev primer 5′-GACTTCGTTATCTAGGGTTGCG-3′ using the following PCR reaction mixture and amplification conditions:

DNA extract (4 µL), 5 µL 10× PCR buffer, 400 µM of each dNTP, 1 µL Taq polymerase, primers (10 pM each) in nuclease-free water to 50 µL.

PCR: 95°C for 15 min, 35 cycles of amplification (94°C for 30 s; 55°C for 30 s and 72°C for 30 s) plus a final extension step at 72°C for 10 min.

Choi et al. (27) demonstrated that this PCR assay yielded a PCR product of 384 bp and was approximately two logs less sensitive than a real-time PCR assay described in the same publication, nonetheless, the aforementioned conventional HBoV PCR assay represents a useful starting point for initial forays in HBoV detection.

3.7.2. PARV4 PCR

A quantitative PCR assay utilising consensus primers to amplify a region of ORF2 of PARV4 and PARV5, which is highly conserved between the two genotypes, has been developed (31, 32).

Fwd: 5′-CTAAGGAAACTGTTGGTGATATTGCT-3′ located between nucleotides 3285 and 3310 of ORF2 (GenBank Accession Number AY622943).

Rev: 5′-GGCTCTCCTGCGGAATAAGC-3′ located between nucleotides 3368 and 3387 of ORF2.

These primers amplify a 103-bp product.

Probe5′-(FAM)TGTTCAACTTTCTCAGGTCCTACCGCCC (TAMRA)-3′) which hybridises to nucleotides 3313–3340 of ORF2.

Amplification reaction mixture: 1× QuantiTect Probe (Qiagen, Germany) PCR master mix, 10 pmol of each primer, 0.05 µM of probe, and 5 µL of template DNA. Final volume: 20 µL. Amplification conditions: 95°C for 15 min and then 45 cycles of 95°C for 15 s and 60°C for 1 min.

Extensive primer details and PCR conditions for PARV4 and 5 detection have also been presented elsewhere (64, 65).

3.8. Concluding Remarks

Standardised and validated ELISA and immunofluorescent detection systems are now commercially available to detect parvovirus B19 IgM and IgG resulting from exposure to all erythrovirus

genotypes. Validated molecular assays are also available to accurately detect viral DNA. In addition, WHO International Standards are now in place and enable inter-laboratory standardisation of B19 IgG and DNA detection. Furthermore, a battery of cellular, immunohistochemical and infectivity assays can facilitate ongoing research into B19 pathogenicity. Although numerous validated research assays have been developed to detect HBoV, PARV4, or 5 DNA or antibody responses (HBoV only), no commercial tests are yet available; however, it is clear that many of the lessons learned from B19 assay development will find resonance in future studies on, and detection systems for, these emerging pathogens.

4. Note

1. DNA extraction should always be performed in separate laboratory to that of PCR reagent preparation and nucleic acid amplification.

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