Evaluation of commercial enzyme linked immunosorbent assay for detection of B19 parvovirus IgM and IgG

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Abstract
An indirect enzyme linked immunosorbent assay (ELISA) (Parvoscan-B19; Sweden) was compared with an in-house MACRIA for the detection of B19 specific IgM. A Parvoscan-B19 IgG test was also evaluated for its ability to detect a recent B19 infection in paired sera. Two hundred and twenty sera submitted to the laboratory for B19 serology and four MACRIA positive control sera were assayed for B19 IgM. Confirmation of the response of sera giving discordant results in the two assays was sought by the use of a "nested" polymerase chain reaction (PCR) for the detection of B19 DNA. The Parvoscan-B19 IgM test was 79% sensitive and 96% specific. Parvoscan-B19 was poor at detecting parvovirus infection in sera collected two to three months after the onset of symptoms. When sera collected more than seven weeks after the onset of symptoms were excluded from the analysis, Parvoscan-B19 IgM was 84% sensitive and 96% specific. Rubella specific IgM positive sera, rheumatoid factor positive sera, and heterophil antibody positive sera were also assayed for B19 IgM. No false positive results were encountered with these problematic sera. By using the cut off criteria for the Parvoscan-IgM test previously advocated by the manufacturers, 90% sensitivity and 87% specificity could be achieved. False positive results, however, occurred with six of the 17 rubella IgM positive sera, four of the 10 rheumatoid factor positive sera, and two of the 11 heterophil antibody positive sera tested.

It is concluded that the Parvoscan-B19 was specific but insensitive when compared with in-house assays.

The human parvovirus B19 virus is 20–25 nm in diameter, icosahedral, non-enveloped and contains a single positive strand DNA genome. The surface of the virus is composed of two major structural proteins, VP1 and VP2, with molecular weights of 84 and 58 kilodaltons respectively.1 VP2 forms 96% of the total capsid.

B19 virus is the cause of erythema infectiosum (Fifth disease),2 aplastic crisis in haemolytic anaemia,1 and fetal loss and fetal hydrops in pregnancy.13 There is no adequate in vitro culture system for the propagation of B19 virus; only very low levels of viral replication are achieved in permissive cell lines such as bone marrow4 and fetal liver.7 The mainstay of B19 diagnosis is the detection of specific IgM and IgG by class specific capture immunoassay.8 The B19 antigen in these tests is obtained from human plasma during the viraemic phase. The consequential scarcity of antigen has limited B19 testing to a few specialised centres internationally.

Recently, an indirect enzyme linked immunosorbent assay (ELISA) was developed for the detection of B19 specific antibodies. This assay uses a synthetic peptide constructed from the N-terminus of the VP2 structural protein (amino acids 284–307).9 The peptide contains two cysteine molecules which are cross-linked to form a circular moiety by treatment with 0-1 M iodine in methanol. This assay has now been developed commercially for the detection of B19 specific IgM and IgG and marketed as Parvoscan-B19 (Ferring Diagnostica; Sweden). We describe a comparative study of Parvoscan-B19 with an IgM antibody capture radioimmunoassay (MACRIA)8 for the detection of B19 specific antibodies. Confirmation of the response of sera giving discordant IgM results with the two assays was sought by the use of a "nested" polymerase chain reaction (PCR) for the detection of B19 DNA.

Methods
Two hundred and twenty sera (183 from adults and 37 from children), submitted for diagnostic B19 IgM testing and four MACRIA positive control sera were tested for B19 specific IgM by the Parvoscan-B19 IgM and MACRIA. The diagnoses given for these samples are shown in table 1. Seventy-four of

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Diagnoses given for samples submitted for B19 testing</th>
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<tbody>
<tr>
<td>Rash + arthralgia</td>
<td>99</td>
</tr>
<tr>
<td>Arthralgia, no rash</td>
<td>92</td>
</tr>
<tr>
<td>Asymptomatic contacts in B19 outbreak</td>
<td>10</td>
</tr>
<tr>
<td>Aplastic crisis</td>
<td>5</td>
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<tr>
<td>Hydrops fetalis</td>
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<tr>
<td>Intratertiary growth retardation/death</td>
<td>3</td>
</tr>
<tr>
<td>Fever of unknown origin</td>
<td>4</td>
</tr>
<tr>
<td>Investigation of anaemia in leukemic patients</td>
<td>3</td>
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<tr>
<td>Cytopaenias</td>
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</table>
| Hepatitis | |}

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these samples were collected within one month of the onset of symptoms, six were collected between one and two months, and 12 were obtained more than three months after the date of onset. The date of onset was unknown for 128 samples. Twelve pairs of acute and convalescent MACRIA positive sera were tested for a rise in B19 specific IgG by Parvoscan-B19. Seventeen sera known to contain rubella specific IgM, 10 rheumatoid factor positive sera, and 11 heterophile antibody positive sera were also tested by MACRIA and Parvoscan-B19.

MACRIA
This assay was modified from a published method. Briefly, polystyrene beads (Northumbria Biologicals, Cramlington, England) were coated with anti-μ by gentle agitation of anti-human IgM (Tago) diluted 1 in 750 in carbonate/bicarbonate buffer, pH 9·6 (0·2 M anhydrous sodium carbonate, 0·2 M sodium bicarbonate), at room temperature for one hour. 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) with test serum diluted 1 in 100 in phosphate buffered saline with 0·05% Tween 20 (Sigma) (PBST) for three hours at 37°C. After this the beads were washed three times in PBST.

Plasma from a B19 viraemic subject was mixed with an equal volume of chloroform and then briefly spun at 13 000 rpm. The supernatant was diluted 1 in 500 in PBST. The beads were then incubated with 180 μl of the mixture at room temperature overnight.

The wash steps were repeated and then the beads were incubated with 180 μl of a 1 in 5000 dilution of mouse monoclonal anti-B19 antibody at 37°C for three hours. The wash steps were again repeated and the beads incubated for 90 minutes at 37°C, with radiiodinated sheep anti-mouse antibody (Dako) diluted in PBST with 5% B19 antibody negative serum at a dilution to produce 50 000 counts per minute in a 180 μl volume. After a final wash the radioactivity bound to the beads was measured by counting for five minutes in an NE 1600 gamma counter (Nuclear Enterprises, UK). Results were expressed as MACRIA arbitrary units of B19 antibody when compared with dilutions of a control positive serum. A result of ≥5 units was considered to indicate recent parvovirus infection.

PARVOSCAN-B19 (Ferring Diagnostica, Sweden)
This assay consists of microwell plates coated with a B19-specific synthetic peptide. After prewashing, 1 in 20 dilutions of patient sera were made in duplicate wells for the IgM test and 1 in 20 and 1 in 200 dilutions for the IgG test. The samples were then incubated for 45 minutes at 37°C. The plate was then washed four times in a wash solution containing 0.9% sodium chloride, 0·005% Tween 20, and 0·01% thimerosal. Goat anti-human IgG or IgM conjugated with alkaline phosphatase was then added to the samples and incubated for 45 minutes at 37°C. The wash step was repeated and p-nitrophenyl phosphate added to each microwell. Following a further incubation for 30 minutes at 37°C in the dark 1 M NaOH was added to the wells and the optical density measured at 405 nm.

DETECTION OF RUBELLA SPECIFIC IgM, RHEUMATOID FACTOR, AND HETEROPHIL ANTIBODY
Detection of rubella specific IgM was performed by MACRIA using a published method. The presence of rheumatoid factor and heterophil antibody were assayed using commercial tests, Rheuma (Wellcome diagnostics) and IM absorption (Mercia), respectively.

"NESTED" B19 POLYMERASE CHAIN REACTION
Sera (50 μl) were added to a 100 μl solution containing 50 mmol potassium chloride, 10 mmol TRIS-HCl, pH 8·3, 2·5 mmol magnesium chloride, 0·1 mg/ml gelatin, 0·45% NP40 (nonidet P-40, Sigma), 0·45% Tween 20 (Sigma) and 0·006 mg proteinase K (Sigma 4914). This was then incubated at 65°C for one hour and then at 95°C for 10 minutes to inactivate the proteinase K. The precipitate formed was then centrifuged at 14 000 rpm for 15 minutes and the supernatant used in the PCR assay.

Extracted serum (5 μl) was added to a 50 μl PCR reaction volume containing 10 mmol TRIS-HCl, pH 8·3, 50 mmol potassium chloride, 1·5 mmol magnesium chloride, 0·01% (weight/volume) gelatin, 1 unit recombinant Taq DNA polymerase (Perkin Elmer Cetus), 200 μmol of each dNTP and 300 ng of each primer. Following first round PCR amplification 1 μl of first round PCR product was transferred into a second 50 μl PCR reaction mix. The second round reaction mix contained the same constituents as the first round mix, but substituting the first round primers for 300 ng of each second round primer.

The oligonucleotides primers used in the first round of amplification were 5'-CTT TAG GTA TAG CCA ACT GG-3' nucleotide positions(n) 2905-2931 and anti-sense 5'-ACA CGT AGT TTA CTA GTG GG-3' nucleotides 4016-3997, yielding a 1112 base pair product. Second round PCR was performed using the previously published sequences11 5'-CAA AAG CAT GTG GAG TGA GG-3' nucleotides 3187-3206 and anti-sense 5'-CCT TAT AAT GGT GCT CGT GG-3' nucleotides 3290-3271 to produce a product of 104 base pairs. Thirty five cycles of both first and second round amplification were performed using the following conditions, 95°C for one minute, 55°C for one and a half minutes and 72°C for one minute.

Samples (15 μl) of the first and second round PCR products were then analysed by electrophoresis on a 2% agarose gel. Bands were visualised by ethidium bromide staining.

Results

igM tests
Sixty four sera were determined to be
copositive and 134 negative by both tests. Nine sera were initially reactive in the Parvoscan-B19 IgM but negative in the MACRIA. The sensitivity and specificity of the Parvoscan-B19 IgM assay on initial testing of routine diagnostic sera were 79% and 94%, respectively. These discordant sera were retested by MACRIA and the Parvoscan-B19 IgM test (as recommended by the manufacturers); all remained MACRIA negative while only six remained as reactive in the Parvoscan-B19 IgM test. The sensitivity and specificity of the Parvoscan-B19 IgM test following repeat testing of reactive sera was 79% and 96%, respectively (table 2). All discordant sera were tested for the presence of rheumatoid factor, heterophile antibody, and rubella specific IgM, but none was found to contain these antibodies. The discordant sera were also tested by "nested" PCR and none was found to contain B19 DNA.

Seventeen sera were found to be Parvoscan-B19 IgM negative and MACRIA positive. Eleven of the sera contained only low titres of B19 specific IgM (>5, ≤11 arbitrary units) as detected by MACRIA. Eight of these were retested in the Parvoscan-B19 IgM test and MACRIA, one retested as Parvoscan-B19 IgM positive, and all repeated as MACRIA positive. None of these contained rheumatoid factor, heterophile antibody, or rubella specific IgM.

"Nested" PCR was also performed on these eight sera; four contained B19 DNA giving further confirmation of recent B19 infection.

There was, in general, concordance between the calculated MACRIA units obtained with the in-house test and the optical density obtained in the Parvoscan-B19 IgM test (fig 1).

None of the rheumatoid factor positive sera, heterophile antibody containing sera, or rubella specific IgM positive sera was falsely positive in either the Parvoscan-B19 IgM or MACRIA tests. Some of these sera were reactive, however, in the Parvoscan-B19 IgM test but just below the cut off for the test (fig 2). These sera were unreactive in the MACRIA test.

### IgG Tests

The Parvoscan-B19 IgG test was formulated to detect a diagnostic rise (50% increase in OD) in B19 IgG antibody, indicative of acute B19 infection. Twelve pairs of sera were tested from 12 patients. The time interval between the date of onset and the first serum collection was known for seven patients (median three days, range one to 21 days). The acute and convalescent sera were collected at least a week apart; all the acute sera contained B19 specific IgM. Of the 12 pairs of sera tested, a diagnostic increase in optical density was seen in six pairs when tested at a 1 in 20 dilution. On retesting five of the pairs negative at a 1 in 20 dilution, at a 1 in 200 dilution, two exhibited a diagnostic increase in optical density.

### Discussion

Parvoscan-B19 was an easy test to perform and could be completed in two hours. The instructions for the test were generally clear and concise. The suggested format for the sera in the wells was complicated, however, and presupposed that each serum sample would be screened for both IgM and IgG at two dilutions. This is rarely necessary or helpful unless paired sera are obtained from the patient.

The Parvoscan-B19 conjugate was supplied in a single bottle, which, when reconstituted was only stable for two weeks. This limits the shelf life of the kit considerably. The microtitre
wells were provided in 16-well strips. One of the strips proved totally unreactive on testing and presumably was not adequately coated with antigen.

Although the MACRIA was developed as the first serological assay for the detection of B19 specific IgM and is the "gold standard" by which other assays will be judged, it may produce false positive and negative results. We have addressed this question by performing "nested" PCR to detect B19 DNA in the discrepant samples. In our experience B19 DNA is detectable by this technique in 72% of B19 MACRIA positive sera collected up to 150 days after the onset of symptoms and in no MACRIA negative samples tested (unpublished data). In this study B19 DNA was detected in four of eight of the Parvoscan-B19 IgM negative/MACRIA positive sera tested, confirming the specificity of the MACRIA result. B19 DNA, however, was not detectable by PCR in any of the six Parvoscan-B19 IgM positive/MACRIA negative sera tested.

Dates of onset of symptoms were available for 10 of the 17 Parvoscan-B19 IgM negative/MACRIA positive sera, five of which were two to three months before serum collection. The dates of onset of symptoms were known for 43 of the positive sera; 42 of the sera were collected within seven weeks of the date of onset while only one was collected at 2 months. It may be that the Parvoscan-B19 IgM test is relatively poor at detecting parvovirus infections two to three months after the onset of symptoms. If all sera known to have been collected more than seven weeks after the onset of symptoms are excluded from the analysis then the test is 84% sensitive and 96% specific.

The Parvoscan-B19 IgM test has a low sensitivity using the kit cut off for a positive B19 specific IgM result calculated as five times the mean of the negative control wells. As the manufacturers had previously used three times the mean of the negative control wells as a cut off in the kit, we also analysed our results using this criterion. Using this cut off the test was 90% sensitive and 87% specific. If, in addition, the test is only used for sera collected within the seven weeks of the date of onset of symptoms then the test was 96% sensitive and 87% specific. When the "problematic" sera tested were analysed using this cut off criterion, however, two of 11 heterophil antibody positive sera, four of 10 rheumatoid factor positive sera, and six of 17 rubella specific IgM positive sera were falsely positive in the Parvoscan-B19 IgM test.

The Parvoscan-B19 IgG test was less useful in diagnosing acute B19 infection. ELISA s can only detect a narrow range of antibody concentrations so that two dilutions of serum were required to screen for B19 IgG. In the limited testing performed the test was only 67% sensitive; although the test may have a role in confirming equivocal IgM serology. The Parvoscan-B19 IgG test is not intended for determining immunity or past exposure to B19 virus and should not be used for this purpose. Seventy one sera tested by both an in-house B19 GACRIA and the Parvoscan-B19 IgG test showed no correlation between the optical density obtained from the Parvoscan-B19 IgG test and the arbitrary B19 GACRIA units calculated from the GACRIA (data not shown).

Parvoscan-B19 performed disappointingly in detecting B19 specific IgM when compared with a B19 MACRIA. The test could distinguish recent rubella infection from a recent B19 virus infection, an important problem in the clinical setting, but some of the rubella specific IgM positive sera exhibited reactivities only just below the test cut off. False positive B19 IgM results have been reported previously when rubella IgM positive sera were tested in the B19 MACRIA. This was not found to be the case with the rubella specific IgM positive sera used in this evaluation.

Parvoscan-B19 is the first commercially produced test for the detection of B19 infection in the United Kingdom. Unfortunately the test has a low sensitivity in its present format and results should be interpreted with care. The use of a lower cut off enhances the sensitivity of the test but produces false positive reactivities due to the presence of rubella specific IgM, rheumatoid factor and heterophyl antibody.