**Parvovirus PARV4 visualization and detection**

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The parvovirus PARV4 is the most recently described member of the family *Parvoviridae* that has a human host. To investigate the prevalence of PARV4 in blood, a quantitative TaqMan PCR was developed and plasma, sera or whole blood from a variety of population groups were examined. Eight samples were positive for PARV4, one at high copy number. The high-titre-positive plasma had an approximate viral load of $5 	imes 10^8$ genome equivalents ml$^{-1}$. Two human sera, identified as PARV4 antibody-positive by indirect immunofluorescence, were used in immune electron microscopy to try to visualize native PARV4 within the high-titre human plasma. PARV4 particles were observed using one of these two sera. To our knowledge, this is the first time that native PARV4 has been visualized.

PARV4 is the most recently described member of the family *Parvoviridae* that has a human host (Jones et al., 2005). It is currently a virus without any apparent disease association (Fryer et al., 2005). It was identified by a random amplification of nucleic acids extracted from a patient with acute virus infection who was co-infected with hepatitis B virus (HBV) (Jones et al., 2005). Two further genotypes of PARV4 have now been described (Fryer et al., 2006; Simmonds et al., 2008). Very little is known about PARV4 and its biology. It was initially described as ‘not closely related to any known paroviruses’ (Jones et al., 2005). However, further work has shown that PARV4 is most similar to the recently discovered bovine and porcine HoKo viruses (Lau et al., 2008) and that it groups together with these and also the more distantly related Myanmar erythrovirus (Hijikata et al., 2001), another porcine virus.

The human parvovirus B19 can be present at very high titres in the blood of infected individuals. Plasma and whole-blood samples thought likely to harbour PARV4, namely samples being tested for hepatitis B or C or from human immunodeficiency virus (HIV)-positive patients, were therefore examined. For comparison, samples from UK blood donors were also tested. All samples analysed were anonymized. The frequency of detection of PARV4 in the UK blood-donor population is expected to be low, based on limited data from previously reported surveys (Fryer et al., 2007b; Simmonds et al., 2007; Schneider et al., 2008), although large, formal studies have yet to be performed.

Nucleic acid was extracted from plasma, serum or whole blood, either manually using Qiagen blood kit spin columns or on a Qiagen BioRobot. A quantitative TaqMan PCR (Q-PCR) was designed with the aid of Beacon Designer 3 software (Premier Biosoft International) and optimized for open reading frame (ORF) 2 of PARV4. The Q-PCR was performed on an ABI 7500 platform (Applied Biosystems), using ABgene reagents, and was shown to have linearity of detection over the range $10^1$–$10^8$ copies ml$^{-1}$, with a limit of sensitivity of 50 copies ml$^{-1}$. An oligonucleotide positive control of the target sequence was synthesized (Eurofins MWG Operon), but was subsequently replaced by a biological standard: a high-titre-positive plasma, once one had been identified. Murine cytomegalovirus was used as an internal extraction and amplification control. Samples tested and results are shown in Table 1. Q-PCR conditions were 95 °C for 15 min followed by 45 cycles of 95 °C for 15 s, 60 °C for 60 s. The TaqMan primers used were PWTPARV4.1F (5′-CCTCTCCGAGTCCATTAGCAGA-3′), PWTPARV4.1R (5′-9CTCCATACCTTTCAGCAGTTTC-3′), with a limit of sensitivity of 50 copies ml$^{-1}$.

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In total, PARV4 DNA was detected in eight samples. Quantification of PARV4 in samples was initially carried out against a log$_{10}$ dilution series of the oligonucleotide positive control and subsequently against a high-titre-positive control PARV4 plasma (designated plasma 129). This plasma had a viral load of $5 	imes 10^8$ DNA copies ml$^{-1}$ and was from a hepatitis C virus (HCV) RNA-positive, HCV antibody-negative patient. Viral loads of all eight positive samples are shown in Table 2. The four samples that had a viral load $\geq 760$ copies ml$^{-1}$ were amplified successfully for sequencing, but those with viral loads of $\leq 285$ failed to amplify. Three samples (129, 135 and 342)
were amplified by using a semi-nested PCR to ORF2, initially with primers PARV4Seq1 (5′-CCGGAACC-TTCAAGTCAAGCCA-3′; 2465–2486) and PARV4Seq2 (5′-CCGCTCAAGGTCTGGTTCAACAA-3′; 3010–2988), followed by PARV4Seq1 and PARV4Seq3 (5′-CAAGGTGGACTCCGACATCTGG-3′; 2954–2933). The resulting 490 bp fragments from these three samples were then sequenced with PARV4Seq1 and PARV4Seq3. All three were typed as PARV4 genotype 1. Sample 168 was also confirmed as PARV4 genotype 1 by sequencing with primers PVORF1F and PVORF1R (Fryer et al., 2006).

Sequence similarity was determined by using the FASTA program at http://www.ebi.ac.uk and searching the Viral Database.

For electron microscopy, 300 μl high-titre plasma 129 was centrifuged at 48 000 g for 45 min. The resultant pellet was resuspended in distilled water and stained with 1.5 % phosphotungstic acid (PTA), pH 6.6. Grids were examined in a Philips 420 transmission electron microscope fitted with an AMT XR60 digital imaging system. Parvovirus particles were not seen. Small, round, featureless virus particles, such as parvoviruses, however, can be extremely difficult to detect, particularly amongst the background debris of plasma or serum. Immune electron microscopy (IEM), a technique that has been employed successfully to detect other small viruses, including parvovirus B19 (Cossart et al., 1975; Curry et al., 2006), was used in a further attempt to visualize the native PARV4 particles. Two serum samples containing antibody to PARV4 had been identified in our laboratory on the basis of their reactivity in an indirect immunofluorescence test (R. P. Parry, unpublished data). These two antibody-positive sera were each mixed with an aliquot of high-titre plasma 129, incubated at room temperature for 1 h and centrifuged at 48 000 g for 45 min. Pellets were resuspended in distilled water and stained with 1.5 % PTA or 2 % methylamine tungstate, pH 6.6, and examined as described above. Parvovirus-like particles that had been aggregated into clumps by one of the sera were seen (Fig. 1a). The particles measured around 20–22 nm in diameter and were morphologically typical of parvoviruses. For comparison, recombinant PARV4 capsids expressed in Sf9 cells by baculovirus (PARV4 capsids provided by Dr Kevin E. Brown, Health Protection Agency) can be seen in Fig. 1(b). The recombinant capsids and the particles found in plasma 129 are similar in size and have the characteristic hexagonal appearance of parvoviruses. Stain has penetrated into several of the recombinant particles, as would be expected, whereas the particles from plasma 129 appear complete.

### Table 1. Samples tested for PARV4 by Q-PCR

<table>
<thead>
<tr>
<th>Population group tested</th>
<th>n</th>
<th>No. (%) PARV4-positive by Q-PCR</th>
</tr>
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<tbody>
<tr>
<td>HCV antibody-negative, RNA-positive blood donors (HCV window phase)</td>
<td>94</td>
<td>3 (3.2)</td>
</tr>
<tr>
<td>Samples for routine HCV RNA testing</td>
<td>88</td>
<td>2 (2.3)</td>
</tr>
<tr>
<td>Samples for routine HBV DNA testing</td>
<td>140</td>
<td>2 (1.4)</td>
</tr>
<tr>
<td>HIV-1 proviral DNA-positive IVDUs</td>
<td>50</td>
<td>0*</td>
</tr>
<tr>
<td>Samples for routine HIV-1 RNA viral load testing</td>
<td>88</td>
<td>1 (1.1)*</td>
</tr>
<tr>
<td>UK blood donors – 20 pooled DNA extracts from 96 donors</td>
<td>–</td>
<td>0</td>
</tr>
</tbody>
</table>

*Overall detection frequency of 1 in 138 (0.7 %) in HIV-1-positive samples tested.

### Table 2. Viral loads of PARV4-positive samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>PARV4 (DNA copies ml⁻¹)</th>
<th>HCV</th>
<th>HCV genotype</th>
<th>HBV</th>
<th>HIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>129</td>
<td>5 × 10⁶</td>
<td>2.70 × 10⁶</td>
<td>2b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>135</td>
<td>760</td>
<td>1.22 × 10⁶</td>
<td>3a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>4.6 × 10³</td>
<td>1.05 × 10⁵</td>
<td>3a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A5</td>
<td>1</td>
<td>5.04 × 10⁶</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C10</td>
<td>5</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>342</td>
<td>3.4 × 10³</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>490</td>
<td>170</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H10</td>
<td>285</td>
<td>+</td>
<td>+</td>
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</table>

**Fig. 1.** Electron micrographs of parvovirus particles. (a) IEM of particles seen in plasma 129; antibody can be seen coating the particles. Stained with methylamine tungstate. (b) Recombinant viral capsids of PARV4, stained with PTA. Bars, 100 nm.
The antibody-aggregated clumps of particles observed in plasma 129 resembled the appearance of B19 virus when visualized by IEM. Plasma 129 and the two serum samples containing antibody to PARV4, however, were negative by PCR for B19 and human bocavirus, and it was concluded that the particles seen were PARV4.

Failure to detect virus particles with the second serum may have been related to the titre of the reagents. The sera were only tested at one dilution by immunofluorescence, but results from a prototype ELISA suggested that this second serum had a lower antibody titre to PARV4. For IEM purposes, the titre of PARV4 in plasma 129 was also low and probably near the limits of sensitivity for IEM detection. This may account for the fact that virus particles from this sample were not seen with PTA staining, rather than any difference between the stains.

PARV4 was detected at low frequency in samples from the blood of patients infected with HIV-1, HCV and HBV. In a study of the three human parvoviruses, B19, bocavirus and PARV4, in HIV-1-infected and non-infected individuals, Manning et al. (2007) established that a high proportion (70.8%) of HIV-1-infected individuals harbour PARV4 in lymphoid and bone-marrow tissues, but none had viraemia. It is interesting to note that seven of the eight individuals in whom PARV4 was detected in the plasma were co-infected with hepatitis viruses (Table 1). The original discovery of PARV4 was in an intravenous drug user (IVDU) from the USA. The 94 HCV window-phase plasma samples analysed in our study for PARV4 were USA-sourced plasmas and the donors may have been remunerated financially. PARV4 was not detected in any of the UK blood donors tested.

These data contrast with those of a recent study in Thailand, which revealed PARV4 in sera both from IVDUs (8%) and in blood donors (4%) (Lurcharchaiwong et al., 2008). Both of these figures are higher than those reported previously from the UK and elsewhere. It is again of interest that the majority of the PARV4-positive IVDUs in the Thai study, seven of eight (87.5%), were HCV-co-infected; this may of course simply be coincidental, as the proportion of HCV positives within this group of IVDUs was very high (88.6%). The determination of the prevalence of past infection with PARV4 in these different populations awaits the results of serological studies. Whether co-infection is a reflection of the natural history of the virus infection, a commonality of transmission routes or a consequence of underlying disease also awaits further elucidation.

The high viral load found in sample 129 (5 × 10⁸ DNA copies ml⁻¹) suggests that this patient was experiencing active virus replication and may represent primary infection. The only other known high-level samples were from the original patient, which contained 6 log₁₀ copies ml⁻¹ (E. Delwart, personal communication), and from archived plasma pools with 6.58 log₁₀ copies ml⁻¹ (Fryer et al., 2007b). It is not known whether the lower viral loads found in this (Table 2) and other studies represent virus replication, waning virus levels as antibody develops or a chronic virus carrier state. Fluctuating low levels of B19 DNA were observed in the plasma of 7.9% of patients with congenital haemoglobinopathy. It has been postulated that this may be due to minor reactivation from sites of virus persistence (Lefrère et al., 2005), which may also explain the 1% of pregnant women (Lefrère et al., 2005) and blood donors (Candotti et al., 2004) who are B19 DNA-positive. A similar phenomenon may be occurring with PARV4. Further development of antibody assays and follow-up studies on PARV4-positive patients are required to investigate these hypotheses.

The high level of sequence conservation observed within the samples that tested positive for PARV4 is consistent with the findings of other groups. This argues for a recent evolutionary origin or a high conservation pressure. Manning et al. (2007) observed an apparent temporal shift in PARV4 genotypes, with genotype 1 representing the current ‘modern’ infection and genotype 2 the older strain. Study subjects positive for genotype 1 were all born after 1958 and those infected with genotype 2 were born between 1949 and 1956. A similar situation has recently been described for B19 variants, with genotype 1 superseding genotype 2 in the skin (Norja et al., 2006). Demographic information on the patients and donors in our study was not available, as all samples were obtained in a random, anonymized manner.

The three genotypes of PARV4 now identified (Simmonds et al., 2008) have not yet been related to any disease. However, 8 years elapsed between the discovery of B19 and its association with fifth disease (erythema infectiosum) (Anderson et al., 1983). Our findings and those of others suggest that a parenteral transmission route is likely. It remains to be seen where PARV4 replicates and whether there are any disease associations.

References


Fryer, J. F., Delwart, E., Hecht, F. M., Bernardin, F., Jones, M. S., Shah, N. & Baylis, S. A. (2007b). Frequent detection of the parvoviruses, PARV4


