A study of Parvovirus 4 in Iceland

Hanna Lilja Guðjónsdóttir

Thesis for the degree of Master of Science
University of Iceland
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Program in Biomedical Sciences
School of Health Sciences

HÁSKÓLI ÍSLANDS

Könnun á Parvoveiru 4 á Íslandi

Hanna Lilja Guðjónsdóttir

Ritgerð til meistaragráðu í Líf- og læknavísindum

Umsjónarkennari: Arthur Löve

Leiðbeinandi: Anders Widell

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Instructor: Anders Widell,

Masters committee: Anders Widell, Arthur Löve and Þorgerður Árnadóttir

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Ágrip

Parvoveira 4 (Parv4) er ný veira í Parvoviridae ættinni, fyrst fundin árið 2005 í blóði frá sprautufíkli. Parv4 og Parv4 skyldar veirur úr öðrum prímötum og svínum hafa verið flokkaðar saman í nýja ættkvísl, *Partetravirus*. Parv4 arfgerðir 1 og 2 hafa verið greindar á norðurhveli jarðar, en arfgerð 3 var fundin í Afríku. Á norðurhveli jarðar finnast Parv4 DNA og mótefni gegn Parv4 aðallega í sprautufíklum, en í Afríku virðist dreifing Parv4 almennari. Meinvirkni Parv4 er óþekkt, en Parv4 sýking hefur verið tengd heilahimnubólgu í börnum og fósturbjúg.

Markmið þessarar rannsóknar var að kanna algengi og dreifingu Parv4 á Íslandi, og finna möguleg tengsl við sjúkdóma. Blóðsýni og NPS sýni úr lífsýnasafni veirufræðideildar Landspítala Háskólasjúkrahúss voru skimuð fyrir Parv4 DNA með PCR. Safnað var sýnum úr níu mismunandi hópum.. Þar var um að ræða samanburðarhóp, sem í voru blóðgjafar og háskólanemar, hópur sjúklinga frá göngudeild kynsjúkdóma, lifrarbólguhóp, sem voru einstaklingar sýktir af lifrarbólgu C og/eða B, og sex minni sjúklingahópa. Parv4 jákvæð sýni voru síðan raðgreind og skyldleikatré teiknað. Einnig var gerð langtímarannsókn á Parv4 jákvæðum sænskum sprautufíklum, þar sem endurtekin sýni voru skimuð fyrir Parv4 DNA til að kanna þróun Parv4 blóðsýkingar.

Parv4 DNA fannst einungis í lifrarbólguhópnum, 12 af 128 (9,4%). Það gefur til kynna að Parv4 sé aðeins að finna hjá sprautufíklum og öðrum áhættuhópum fyrir blóðsmitun á Íslandi og að Parv4 dreifist þar með blóði. Faraldsfræði Parv4 á Íslandi er svipuð og í Svíþjóð og annars staðar í Evrópu, þar sem Parv4 er aðallega að finna meðal sprautufíkla. Í þessari rannsókn fundust engar vísbendingar um tengsl Parv4 við sjúkdómseinkenni. Það tókst að raðgreina sjö Parv4 jákvæð sýni af tólf. Allar raðirnar reyndust vera arfgerð 1 og flokkuðust með undirhóp sem inniheldur upprunalega Parv4 veiruna sem var einangruð í San Francisco. Þessi rannsókn sýndi að Parv4 DNA getur verið viðvarandi í blóði í 3 ár eftir sýkingu.

Abstract

Parvovirus 4 (Parv4) is a new virus in the Parvoviridae family, discovered in 2005 in blood from an IVDU. Parv4 and Parv4-like viruses from other primates and swine have been categorized together in a new genus, *Partetravirus*. Parv4 genotypes 1 and 2 have been detected in in the northern hemisphere while genotype 3 was discovered in Africa. In the northern hemisphere Parv4 DNA and Parv4 antibodies are found mostly in IVDUs and others who are parenterally exposed. In Africa Parv4 infection seems to be more widespread. The pathogenicity of Parv4 is unknown, but Parv4 has been linked to meningitis in children and hydrops fetalis.

The aim of this study is to investigate the prevalence and route of distribution of Parv4 in Iceland, and to discover possible disease associations. Blood and NPS samples, from the Biobank of the Virology Department of the Landspitali- National University Hospital of Iceland, were screened for Parv4 DNA with PCR. Samples from nine different groups were collected: a control group, comprising blood donors and students, a STD group, comprising individuals that came to an STD clinic, a hepatitis group, comprising HCV and/or HBV infected individuals, and six smaller patient groups. Parv4 positive samples were subsequently sequenced and a phylogenetic tree drawn. Furthermore, longditutional samples from Parv4 positive Swedish IVDUs were screened for Parv4 DNA to explore the progression of Parv4 viremia.

Parv4 DNA was only detected in the hepatitis group, 12 of 128 (9.4 %) indicating that Parv4 is only found in parenterally exposed individuals like IVDUs in Iceland and is distributed through the parenteral route there. The epidemiology of Parv4 in Iceland is the similar to Sweden and the rest of Europe, where Parv4 is mainly found among IVDUs and is transmitted parenterally. No evidence in this study was found to link Parv4 with disease. Seven Parv4 positive samples out of the twelve were successfully. The seven Parv4 sequences were all genotype 1 and were most similar to the genotype 1 subgroup containing the original Parv4 isolate from San Francisco. This study found that Parv4 DNA can persist in blood for 3 years after infection.

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AAV	Adeno-associated virus
AMDV	Aleutian mink disease virus
AIDS	Acquired immune deficiency syndrome
ARF	Alternate reading frame
B19V	Parvovirus B19
BHoV	Bovine Hokovirus
Bl	Blood
BM/L	Bone marrow and lymphoid
Bp	Base pair
BPV	Bovine parvovirus
Cap	Capsid protein
CMV	Cytomegalovirus
CPV	Canine parvovirus
CT	Cycle threshold
DNA	Deoxyribonucleic acid
DNaseSISPA	DNase sequence-independent singe primer amplification
DRotCongo	Democratic Republic of the Congo
Eh-BtPV-1	Eidolon helvum Parvovirus 1
H-1	Hamster parvovirus
HBoV	Human Bocavirus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
IEM	Immune electron microscopy
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ITR	Inverted terminal repeats
IU	International units
IVDU	Intravenous drug user
MCV	Canine minute virus
mRNA	messenger RNA
MSM	Men who have sex with men
MVM	Minute virus of mice

NA	Not applicable
ND	Not done
NPS	Nasopharyngeal aspirate
NS	Non-structural protein
ORF	Open reading frame
Parv4	Parvovirus 4
PCR	Polymerase chain reaction
PHoV	Porcine Hokovirus
PL	Plasma
PPV 2	Porcine parvovirus 2
PPV	Porcine parvovirus
Rep	Replication protein
RT-PCR	Real-Time polymerase chain reaction
SAA	National Center of Addiction Medicine
Se	Serum
STD	Sexually transmitted diseases
USA	United States of America
UK	United Kingdom
VP	Viral capsid protein
WHO	World Health Organization
TBE	Tris Boreate EDTA

1 INTRODUCTION

A new virus which infects humans was discovered in 2005, Parvovirus 4 (Parv4). The virus was discovered in a serum sample from a homeless intravenous drug user (IVDU) with symptoms corresponding to a human immunodeficiency virus (HIV) infection: fatigue, night sweats, pharyngitis, neck stiffness, vomiting, diarrhea, arthralgias and confusion. The patient turned out to be HIV negative but was infected with Hepatitis B virus (HBV). It is unclear if Parv4 played any part in the patient's symptoms (1).

1.1 Parvoviridae

Parv4 is a new member of the *Parvoviridae* family. Parv4 and Parv4-like viruses have been placed in a new genus, *Partetravirus* (2). Parvoviruses are small (20-25 nm), icosahedral non-enveloped deoxyribonucleic acid (DNA) viruses. Parvoviruses are ubiquitous in nature and infect both vertebrate and invertebrate hosts. They have a small linear, single-stranded genome of ca. 5000 nucleotides with hairpin sequences at each end. Genomes of parvoviruses have 2 large open reading frames (ORF), the one on the 5'end encodes 2 non-structural or Replication (NS or Rep) proteins, and the 3'end on the right encodes two or three viral capsid (VP or Cap) proteins (see Figure 1) (3).

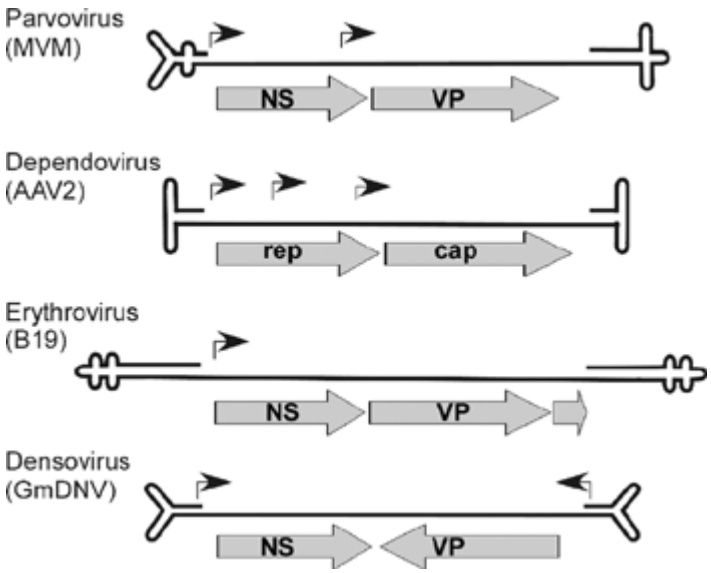


Figure 1 The genome structures of different members of the family Parvoviridae, showing genomes of three of the nine genera: parvoviruses, dependoviruses, and erythroviruses, indicating the promoters and open reading frames of the major genes, non-structural and viral protein. The terminal hairpins are magnified approximately 20 times relative to the intervening single-stranded sequences (3).

Parvoviruses are either autonomous or dependent on co-infection. The small parvovirus genome does not contain a DNA polymerase and parvoviruses cannot stimulate cell division like adenoviruses and papovaviruses. Autonomous parvoviruses require actively dividing host cells, the host cell must go through S phase for viral replication. Other parvoviruses are dependent on co-infection with a helper virus, adenoviruses are the most common but also different kinds of herpesviruses (3, 4). Most parvoviruses encode only two nonstructural proteins so replication depends on the host cells replication machinery, many cellular proteins participate in the replication of the viral genome. Parvoviruses replicate with rolling hairpin replication, which has been studied most in rodent

parvoviruses. Terminal palindromic sequences form hairpin structures at each end that are required for replication. The hairpin duplexes serve as primers from which the cell DNA polymerase starts initiating complementary strands (3).

Parvoviruses are very stable and resistant to many inactivation procedures used for blood-borne viruses. The absence of a lipid membrane and the small DNA genome gives them their stability (5).

Table 1 Taxonomy and characteristics of members of the *Parvovirinae (4).*

	Parvovirus	Amdovirus	Dependovirus	Erythrovirus	Bocavirus	Parv4
5′ and 3′ Hairpin	Dissimilar		Identical	Identical	Not known	Not known
Promoters	2	1	3	1	1	1
Open reading frames (large)	2	2	2	2	3	2
Polyadenylation sites	One	Multiple	One	Multiple	Multiple	Multiple
Packaging strands	Mainly negative	Negative	Both strands	Both strands	Not known	Both strands
Human members	None	None	Several different AAV serotypes	B19V 3 genotypes	HBoV 3+ genotypes	Parv4 3 genotypes

Parvoviridae has two subfamilies: Parvovirinae, which infect vertebrates and Densovirinae, which infect invertebrates (insects and other arthropods). Densovirinae is comprised of four genera and Parvovirinae has six genera (see Table 1). Parvovirus comprises most of the vertebrate parvoviruses; Erythrovirus includes parvovirus B19 (B19V); Amdovirus is comprised only of Aleutian mink disease virus (AMDV), Dependovirus, which has the adeno-associated viruses (AAV) and Bocavirus, includes HBoV. Classification of parvoviruses is currently based on host range and dependence on helper viruses and divides parvoviruses into three groups: autonomous viruses of vertebrates, helper-dependent viruses of vertebrates, and autonomous viruses of insects (6).

In Lukashov et al. 2001 phylogenetic analysis of the Parvovirinae subfamily revealed three major evolutionary groups: primate and chipmunk parvoviruses, including B19V; rodent, pig and carnivore parvoviruses; and AAVs and avian parvoviruses (see Figure 2). Avian parvoviruses that are in the Parvovirus genus were shown to be evolutionary more linked to AAVs in the Dependovirus genus than other autonomous parvoviruses. Therefore, the current classification doesn't always represent the evolutionary relationships between parvoviruses (7). The theory of host-dependent evolution for parvoviruses (8), where parvoviruses co-evolve and co-diverge with their hosts, has been challenged by recent data. The AAV and avian parvoviruses and autonomous primate parvoviruses groups show evidence of host dependent evolution while parvoviruses from carnivores and rodents are more likely independently evolved (7).

Previously only two parvoviruses were known to infect humans, B19V and AAVs but now the list also includes Parv4 and human bocavirus (HBoV) (3, 5, 9, 10).

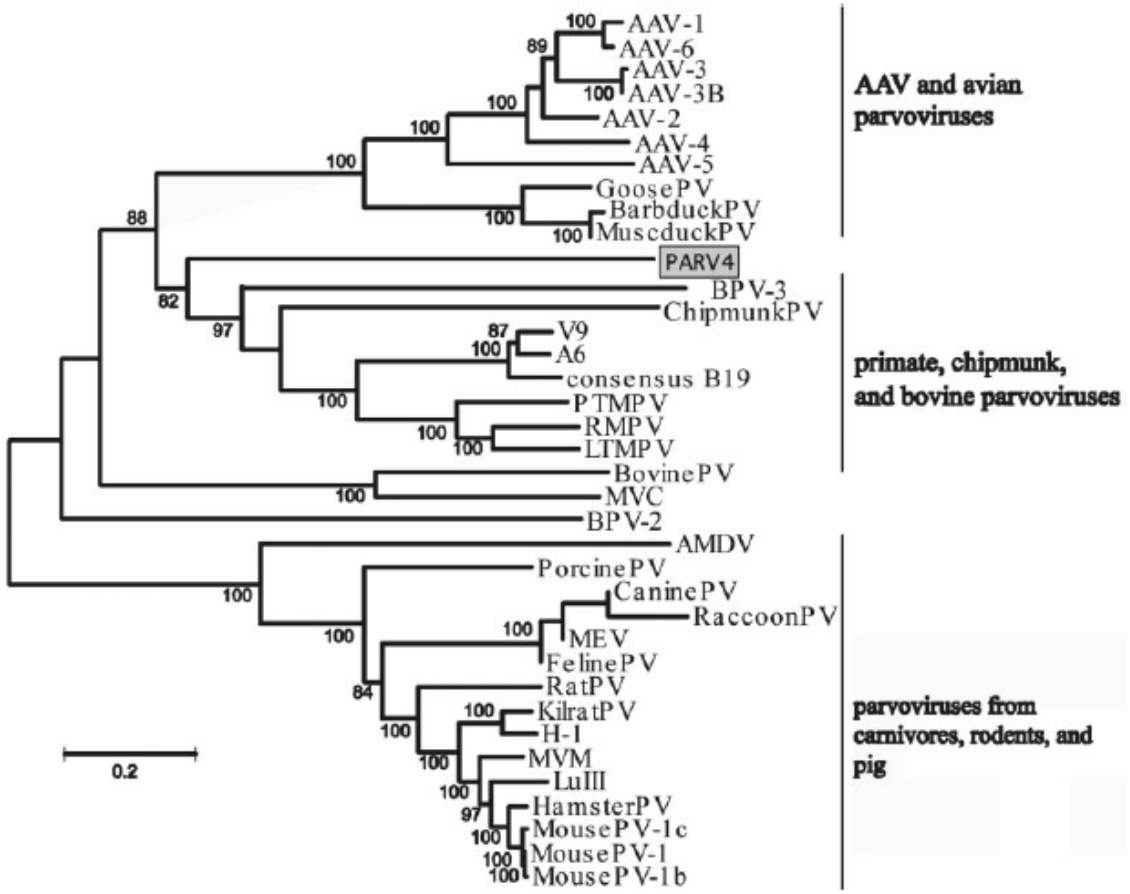


Figure 2 Phylogenetic analysis of the Parv4 genome and other members of the *Parvovirinae* subfamily (1).

1.1.1 Parvovirus

There are no known viruses that infect humans in the Parvovirus genus. The viruses of the Parvovirus genus are viruses of mammals and birds. Some of them cause major disease in their animal hosts. Most of the viruses in the genera have negative sense genomes. Their genomes have dissimilar hairpins at the 5' and 3' ends, two messenger ribonucleic acid (mRNA) promoters and a single polyadenylation site at the 3' end. Each virus is usually confined to a single host species (4, 6).

1.1.2 Amdovirus

Aleutian mink disease virus (AMDV) is currently the only virus in *Amdovirus* genus. AMDV is most similar to viruses in the *Parvovirus* and *Bocavirus* genera. AMDV has negative sense genome, with a single promoter and polyadenylation sites in the middle and 3' end of the genome. The virion structure of the AMDV differs from the *Parvovirus* and *Bocavirus* genera and resembles the *Dependovirus* genus. In adult animals AMDV causes persistent infection which results in the degeneration of the immune system and in newborns infection leads to pneumonitis which is often fatal (4, 6, 11).

1.1.3 Erythrovirus

Erythroviruses are named after their propensity for replicating in erythroid cells (12). B19V is the type virus of the *Erythrovirus* genus. Similar viruses have been found in primates. They are up to 60% homologous to B19V, their genomic structure is similar and they behave similarly in their natural hosts

(6). Erythroviruses package both positive and negative DNA strands. Their genome has a single promoter at the 5' end, and polyadenylation signals in the middle and 3' end of the genome. In addition to the two large ORFs in *Erythrovirus* genomes, there are two small ORFs that encode proteins whose function is not known. Nevertheless the B19V Mr 11000 protein is necessary for efficient B19V replication (13).

1.1.3.1 Parvovirus B19

Human Parvovirus B19 (B19V) was discovered in human serum in 1976 (14). B19V only replicates in human erythroid progenitors. B19 can be grown in culture with difficulty. Erythropoietin is required in culture systems for viral replication, most likely by ensuring rapid division of erythroid progenitors (12). B19V infection is a common childhood illness in all parts of the world. The original genotype identified, genotype 1 is dominant almost everywhere. Genotype 3 is dominant in Africa while genotype 2 is rarely detected. Genotype 2 is thought to have been dominant in the past but that genotype 1 replaced it in the 1960s (4, 15). Transmission of B19V occurs through the respiratory route, probably with droplet inhalation and infectivity is highest during the viremic phase. Most infections occur in late winter, early spring and epidemics take place every three to four years (4, 12, 16). B19V infection can also occur through blood products. The risk of infection from single donor blood is probably low and most infections occur through pooled blood products (4). As parvoviruses do not have a lipid envelope and limited DNA content they are resistant to heat, solvent and detergent treatment. More complicated methods are needed to inactivate non-enveloped viruses such as dry heat treatment and nano-filtration methods (4, 12, 17). Nonetheless B19V is less resistant to heat treatment and low pH treatments than other parvoviruses, due to a difference in the capsid structure that renders B19V DNA unstable (18-20). The Food and Drug Administration (FDA) in the US and the European Pharmacopeia require plasma pools to contain less than 10^4 IU/ml B19V (5).

Infection of B19V is often asymptomatic but can cause mild, erythematous disease in healthy adults and children, designated as erythema infectiosum or fifth disease. Arthropathy may accompany B19V infection, more commonly in adults. B19V infection can cause disease that is more serious in certain risk groups: pregnant women, immunocompromised patients, and patients with hematologic disorders. B19V is very erythrotropic and an infection halts erythropoiesis for 7-10 days. In patients with hematologic disorders that cause erythrocytes to have shorter life span, infection can result in aplastic crisis. In immunocompromised patients, B19V infection can become chronic and cause chronic anemia or aplastic crisis (5, 21).

When infection occurs during pregnancy, the risk of B19V crossing the placenta is about 30%. Parvovirus B19V infects the fetal liver where erythrocyte production mainly occurs in early development, causing severe anemia and perhaps resulting in hydrops fetalis. Around 5-9% of maternal infections end in spontaneous abortion, most in the second trimester (21).

1.1.4 Dependovirus

The dependoviruses were the first parvoviruses found in humans. Recent analysis indicates AAVs originate from avian parvoviruses (7). AAVs were first isolated from humans in 1967, before that AAVs were known as contaminants in laboratory stocks of adenovirus (22). Dependoviruses have been

found in both mammalian and avian species, nine different AAV serotypes have been identified in primates and AAVs 1, 2, 3, 8 and 9 are common human infections (23, 24).

1.1.4.1 Adeno-Associated Viruses

AAVs have equal numbers of both negative and positive DNA strands. Their genome has inverted terminal repeats (ITR) at the 5' and 3'end. Adeno-associated viruses are dependent on co-infection with adenoviruses or herpesviruses. AAV integrates into the genome of the host cell in the absence of a helper virus and becomes activated when a helper virus comes along (3). Some replication can occur without a helper virus but efficient replication requires a helper virus. AAVs are common infections in humans and are found in many types of tissue but they have never been associated with disease. Nevertheless the AAVs ability to integrate their genome into the host cell genome and the fact that different AAVs have different cell tropisms, has made them the object of research as vectors for gene therapy (4).

1.1.5 Bocavirus

The *Bocavirus* genus is named after the original members of the genus, bovine parvovirus (BPV) and canine minute virus (MVC). These two viruses infect the respiratory and gastrointestinal tract of young animal. In addition to the two large ORFs that other parvoviruses possess, bocavirus genomes have a third large ORF that encodes a nucleophosphoprotein (NP1) (4).

1.1.5.1 Human Bocavirus

HBoV was discovered in human respiratory samples in 2005 and placed in the Bocavirus genus. In the original study, HBoV was only detected in children, all of which had respiratory symptoms and many had fever. None of the HBoV positive children had gastrointestinal symptoms, conjunctivitis or rashes (25).

HBoV is now thought to be a respiratory pathogen in children, but it is difficult to prove causality without being able to replicate the virus in cells in vitro. Frequent co-detection with other viruses is also a difficulty, HBoV could be reactivated by other infections or detection made easier by them. (26). HBoV respiratory infections are systemic and elicit a B cell response. Low viral loads of HBoV in NPS are correlated with seronegativity while high viral loads correlate with, absence of other viruses, seropositivity and viremia, indicating, that perhaps those were primary infections as viral loads decreased after recovery (26, 27).

HBoV has been detected in many countries all over the world in nasopharyngeal, serum, fecal and urine samples. It is possible that HBoV plays a role in gastrointestinal disease, like MVC and BPV. BPV and MVC have also been linked to fetal infections, so it is possible that HBoV could cross the placenta, as B19 and cause problems in pregnancy in humans (26, 28). HBoV DNA is not detected in bone marrow or lymphoid tissue in autopsy samples. Possibly HBoV infections are not persistent or the spread of HBoV is recent so the study subjects were not exposed during their childhood (16). Nevertheless, high frequencies of seropositivity have been observed in blood donors in the United States (US), 63% (29). Similarly, high frequencies of HBoV homologues are found in nonhuman primates in sub-Saharan Africa (30).

1.2 Discovery of human Parvovirus 4

Parv4 was discovered using the method described by Allander et al. 2001, DNase sequence-independent single primer amplification (DNaseSISPA) for nucleic acids in serum (31). Jones et al. 2005 used the DNaseSISPA method to screen for unknown viruses in plasma samples from 25 patients with symptoms of acute viral infection syndrome. The patients were HIV negative but had at least 11 or more of the 21 potential symptoms of acute retroviral syndrome. 23 of the patients were male, all had potential sexual exposure to HIV in the previous six months and two reported using injection drugs in the previous six months. One of the samples, which came from a homeless IVDU infected with HBV, yielded the Parv4 virus. Phylogenetic analysis revealed that Parv4 is not closely related to any other known parvoviruses and clusters independently of the three main evolutionary groups of vertebrate parvoviruses (see Figure 2) (1).

Table 2 Nucleotide and amino acid identities between the original Parv4 strain (GenBank accession no. AY622943) and Parv4 and Parv5 strains from plasma pools (10).

Virus/strain	Identity (%) (nucleotide/amino acid)	
	ORF1	ORF2
PARV4*		
BR10749-4†	99.7/99.5	99.9/100.0
C51-4‡	99.6/99.5	99.9/99.9
BR11955-4†	98.2/98.6	98.5/99.6
A23-4‡	98.2/99.4	98.5/99.6
PARV5		
BR10627-5†	90.9/96.8	93.5/98.7
C25-5‡	90.9/97.6	93.4/99.2

A variant of Parv4 was discovered in 2006 and termed Parv5. Parv4 and Parv5 share about 92% nucleotide identity (see Table 2), similarly to B19V genotypes 1-3 (32, 33). Phylogenetic analysis found Parv4 and Parv5 to cluster separately from each other (see Figure 6), Parv5 was therefore designated Parv4 genotype 2 (10).

The sequences that cluster with the original Parv4 isolate (GenBank accession no. AY622943) (see Figure 6) belong to genotype 1. Genotype 1 sequences cluster into two subgroups that diverge by about 2% nucleotide difference (see Table 2) (10).

A third genotype of Parv4 was identified in 2008 in two HIV-infected patients that originated in sub-Saharan Africa, sequences NG_OR and CD_BM. Sequences from these two patients form a separate group from genotype 1 and 2 (see Figure 3a), this third genotype is equally distant from both genotypes 1 and 2 (see Figure 3b) (34).

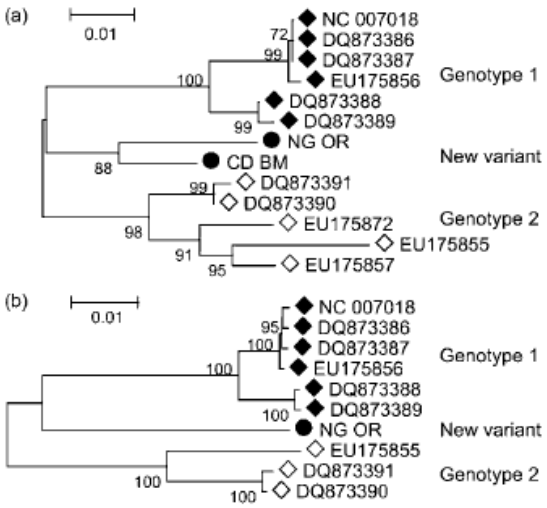


Figure 3 Phylogenetic analysis of concatenated ORF1/ORF2 sequences of NG_OR and CD_BM (a) and near complete genome sequence of NG_OR (b) Trees were constructed by neighbour-joining using Jukes–Cantor corrected pairwise nucleotide differences. Data were bootstrap resampled 1000 times to determine robustness of groupings; values of ¢70% are shown. Bar, corrected P distance. (34).

1.3 Morphology of Parvovirus 4

Small, round virus particles, like parvoviruses, can be very difficult to detect with electron microscopy, especially in the background debris of plasma or serum. Tuke et al. 2010 managed to visualize Parv4 particles with immune electron microscopy (IEM) (see Figure 4a). The Parv4 particles are around 20-22 nm in diameter, icosahedral and morphologically typical of parvoviruses. Recombinant Parv4 capsids were similar in size to Parv4 particles and also had the characteristics of parvoviruses (see Figure 4b) (35).

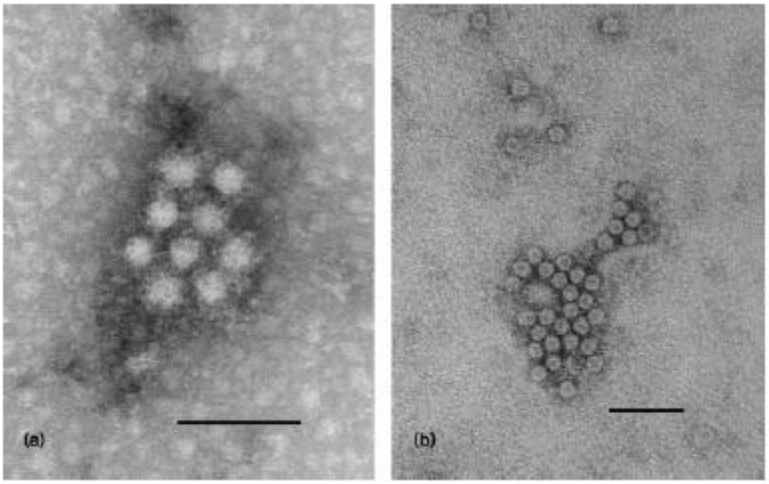


Figure 4 Electron micrographs of parvovirus particles. (a) IEM of particles in plasma. (b) Recombinant viral capsids of Parv4. Bars, 100 nm (35).

1.4 Genomic organization and structure of Parvovirus 4

Parv4 contains two ORFs like B19V, ORF1 contains 1992 nucleotides encoding 664 amino acids and ORF2 contains 2745 nucleotides encoding 915 amino acids. These two ORFs do not overlap, unlike in B19V, and are separated by 103 non-coding nucleotides in genotype 1, 106 in genotype 2 and 107 in

genotype 3 (see Figure 4) (10, 34). ORF1 and ORF2 sequences in genotype 3 are equally different from genotype 1 and 2 sequences as genotypes 1 and 2 are from each other. Translated amino acid difference is similarly different between genotypes (see Table 3) (34). Pairwise comparisons of each ORF of both genotypes 1 and 2 show that ORF2 is more conserved with 91% of the nucleotide difference consisting of synonymous substitutions compared with 89% in ORF1. Synonymous substitutions do not change the amino acid sequence. The amino acid sequences of the genotypes are therefore conserved because most of the nucleotide difference between the two consists of synonymous substitutions, especially the ORF2 encoded amino acid sequences. This indicates that the three genotypes could be a single serotype (10, 34).

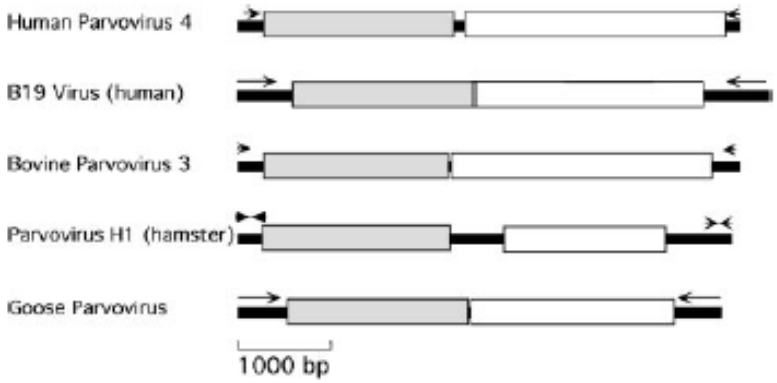


Figure 5 Genetic organization of Parv4 compared to those of B19V, BPV-3, parvovirus H1 and goose parvoviruses. The gray and white boxes represent the genes encoding for NS and VP proteins, respectively. The arrows indicate positions of the terminal repeat sequences. The arrows at the extremities of parvovirus H1 denote that the terminal repeat sequences are dissimilar (1).

All three Parv4 genotypes contain a highly conserved region between positions 2800 and 3500 in ORF2, which accounts for ORF2 being less divergent than ORF1. This area contains two sequential alternative reading frames (ARF). The 5' ORF encodes a protein similar to the small alternatively translated (SAT) protein found in members of the Parvovirus genus. The homologous Parv4 SAT protein shared 24-29% amino acid identity with SAT proteins of Porcine parvovirus (PPV), CPV and minute virus of mice (MVM) while within the Parvovirus genus members share 31-38% identity. The conventional ORF2 reading frame, however, shares 11-18% amino acid sequence identity with parvoviruses. The 3' ORF encodes a protein with no known homologues (34). This area also contains phospholipase A2 motifs between amino acids 224-228 and 247-251, required for parvovirus infectivity (10, 36).

The proteins encoded by Parv4 ORF2 are homologous to VP1 of parvoviruses, Parv4 ORF2 proteins share about 41% and 45% amino acid identity with VP1 of B19V-Au genotype 1 and hamster parvovirus (H-1), respectively. ORF1 encoded proteins are homologous to the NS protein of parvoviruses. Parv4 ORF1 proteins share 43% and 50% amino acid identity with goose parvovirus and hamster parvovirus H-1, respectively.

Table 3 Comparison of nucleotide and translated amino acid sequences of Parv4 genotypes. Mean pairwise nucleotide (below the diagonal) and translated amino acid (above the diagonal) sequence differences (%) (34).

ORF1 (positions 282–2271)			
	NG_OR	Genotype 1	Genotype 2
NG_OR		2.9	2.8
Genotype 1	8.0		2.7
Genotype 2	8.3	9.1	
ORF2 (positions 2278–5519)			
	NG_OR	Genotype 1	Genotype 2
NG_OR		1.6	2.0
Genotype 1	6.2		1.4
Genotype 2	6.9	6.6	

Parvoviruses replicate with rolling-circle replication similar to e.g. some bacteriophages and bacterial plasmids, called rolling hairpin (3). Fryer et al. 2007 discovered parvovirus-conserved motifs in the ORF1 amino acid sequence that are associated with rolling-circle replication (10). Sequence analysis on nearly full-length Parv4 genomes indicates that the two ends of Parv4 DNA have (ITRs) (see Figure 5) like many parvoviruses. These ITRs could possibly form a part of the stem structure of hairpins. Unlike many parvoviruses, Parv4 virus capsids package both positive and negative sense strand in similar amounts (10).

1.5 Phylogenetic analysis of Parvovirus 4

Phylogenetic analysis on nearly full-length genomes described in Fryer et al. 2007 revealed that both Parv4 genotypes 1 and 2 are equally distant from human/primate autonomous parvoviruses and the AAVs and avian parvoviruses (see Figure 6). Parv4 is most closely related to porcine parvovirus 2 (PPV-2) (10). PPV-2 was identified in swine sera from Myanmar (37). Analysis of the two viruses revealed that PPV-2 and Parv4 are not recombinants of other viruses. At the protein level Parv4 shares the greatest amino acid identity with hamster parvovirus H-1, which causes morbidity and mortality in neonatal hamsters (10, 38).

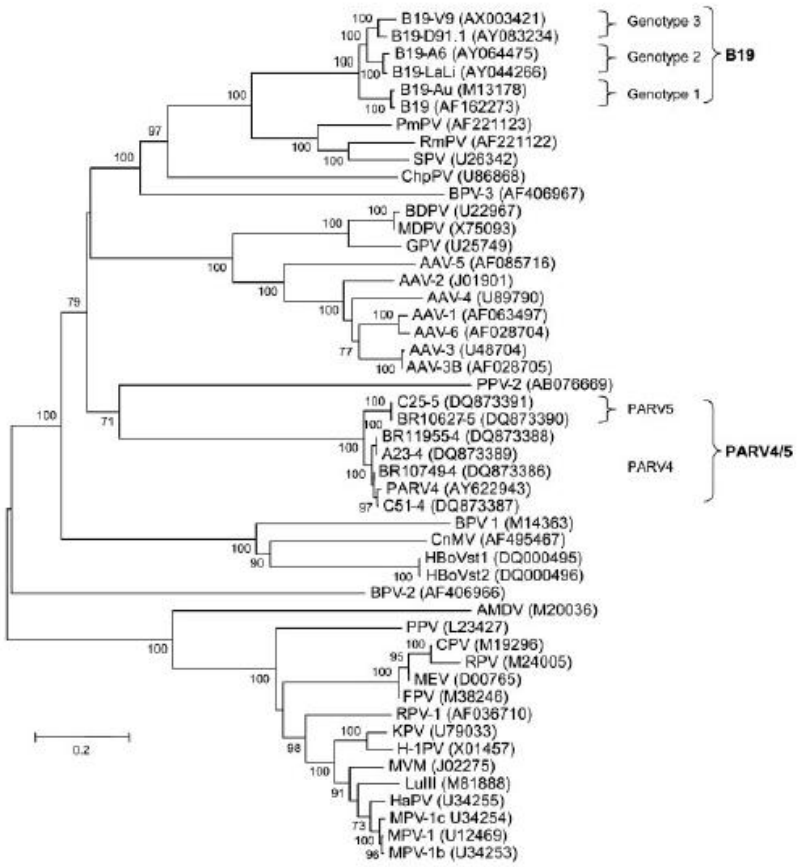


Figure 6 Phylogenetic analysis of nearly full-length Parv4 and Parv5 sequences and other members of the subfamily *Parvovirinae* (10).

1.5.1 Parvovirus 4-like viruses

Since the discovery of Parv4 in 2005, Parv4-like viruses have been isolated in swine, cattle and nonhuman primates. Porcine hokovirus (PHoV) and bovine hokovirus (BHoV) were identified in Hong Kong, the term Hokovirus is derived from the city Hong Kong. PHoV and BHoV share ~62% and ~63% nucleotide identity with Parv4, respectively. PHoV and BHoV form two distinct clusters and Parv4, PHoV and BHoV also form a separate cluster among parvoviruses, distantly related to PPV-2 (see Figure 7) *(30, 39)*.

Genome structure of PHoV and BHoV is similar to Parv4 and other parvoviruses. They have two large, non-overlapping ORFs and in ORF2, the same highly conserved region with phospholipase A2 motifs like Parv4 has. PHoV and BHoV have ITRs at both ends of their genomes and ORF2 more conserved than ORF1 (39). Porcine Parv4 homologues have also been identified in plasma and clotting FVIII concentrates in the UK. Porcine Parv4-like strain share 98-99% nucleotide identity amongst themselves and 97-98% with PHoV (see Table 4). Porcine Parv4-like isolates from the UK were found to belong to a different cluster from PHoV (40). Amino acid sequences of the SAT protein are 84 amino acids long in porcine and bovine Parv4-like viruses while 67 amino acids long in human Parv4. Parv4 strains in cattle, swine and humans are all substantially different (see Table 4), indicating that they diverged a long time ago (40).

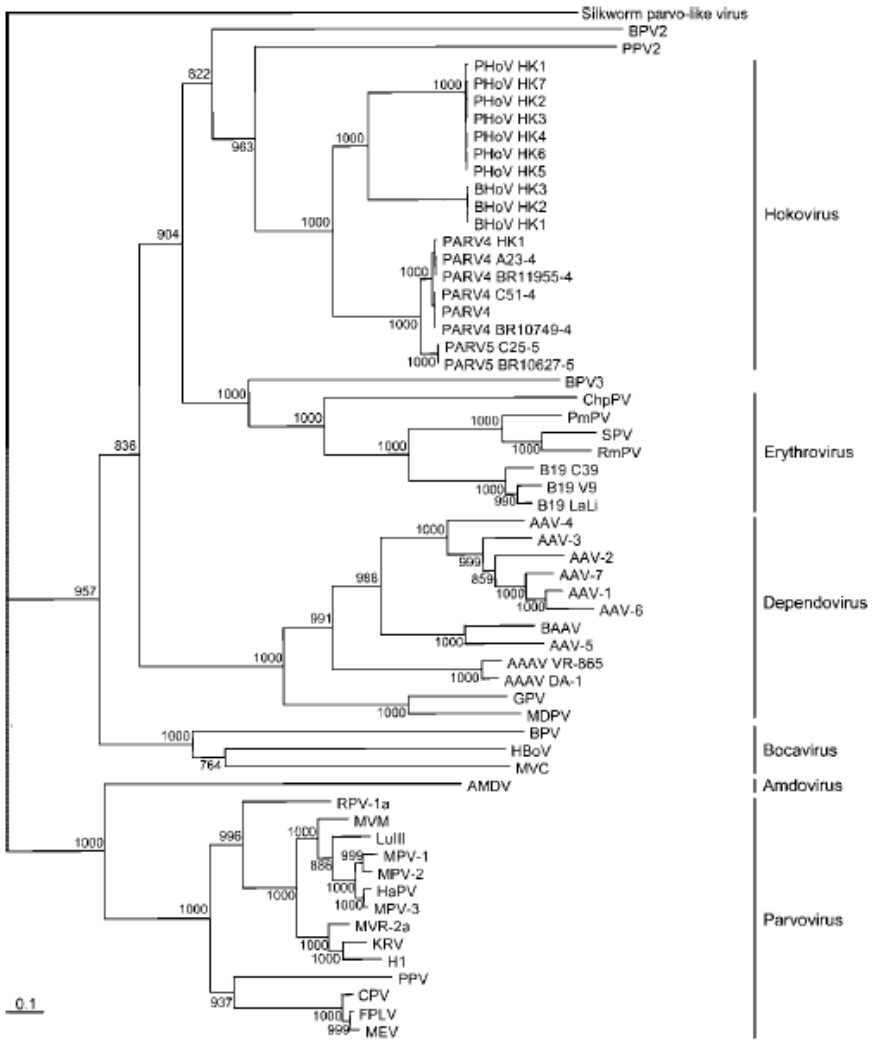


Figure 7 Phylogenetic analysis of nearly full-length genome sequences of PHoV, BHoV and Parv4 identified in the present study (39).

Sharp et al 2010 detected Parv4, B19V and HBoV antibodies in non-human primates. Parv4 seroprevalence was highest in chimpanzees 63%, 18% in gorillas and not detected in various Old World monkeys tested. The Parv4 seroprevalence found in chimpanzees and gorillas indicates cross-reactivity with the human Parv4 antigen (30). A homologue of Parv4 was identified in a chimpanzee and a baboon and homologues of HBoV were identified in chimpanzees and gorillas. In ORF1 and ORF2, the chimpanzee variant differs from human Parv4 genotypes by 18.2% and 15.2%, respectively. Nucleotide difference from the porcine and bovine homologues in ORF1 was 41.5% and 43.3%, respectively and in ORF2 36.9% and 37.8%, respectively. The chimpanzee variant is considerably more similar to the human Parv4 genotypes than PHoV and BHoV. Although, not as similar as Parv4 genotypes are to each other (see Table 3). Phylogenetic analysis shows the chimpanzee variant as a close outgroup to Parv4 while a short sequence isolated from a baboon occupies an intermediary position more divergent from Parv4 and more similar to PHoV and BHoV. The phylogeny of Parv4-like viruses seems to mirror the phylogeny of their hosts (30). A novel Parv4-like parvovirus has been detected in fruit bats, Eidolon helvum Parvovirus 1 (Eh-BtPV-1). Phylogenetic analysis suggested that Eh-BtPV-1 forms a separate branch, within the genus of Parv4-like viruses,

closest to the root of the genus indicating a zoonotic transmission in the past, with a Parv4-like virus spreading from bats to other mammals. (41).

Parvoviruses were thought to be stable, with little genetic variety within host species. Now B19V is known to encompass three genotypes and, with canine parvovirus (CPV), to have a much more rapid rate of nucleotide substitution, more similar to the rate of ribonucleic acid (RNA) viruses (32, 42, 43). Hence, parvoviruses can evolve more rapidly and generate new viruses and genotypes in a short time. The relationships between Parv4 and its homologues in non-human primates are consistent with the host-dependent evolution theory whereas the discovery of different parvoviruses infecting cattle and swine complicates the issue (30, 39).

Table 4 Percentage diversity of genome sequences of Parv4-like viruses (40).

Genotype	PARV4-p	PHoV	BHoV	PARV4-g1	PARV4-g2
PARV4-p	98–99				
PHoV	97–98	98–99			
BHoV	62	62	99		
PARV4-g1	58	58	60	98–100	
PARV4-g2	58–59	58	59–60	91–92	96–99
PARV4-g3	58	58	60	92	91–92

In West Africa where opportunities for cross species transmission are plentiful Parv4-like viruses remain species specific, which indicates that the risk of zoonotic transmission from nonhuman primates to humans is low (44). However, there is evidence that members of the Parvoviridae family have crossed the species barrier in the past (43) which argues for possible zoonosis in the future.

1.6 Epidemiology of Parvovirus 4

1.6.1 Tissue distribution of Parvovirus 4

Parv4 has been detected in various tissues (see Table 5). Parv4 has been found in both healthy and pathological skin samples, 8% and 12% respectively. This is similar to B19V genotype 2, which is also detected in skin tissue seemingly without disease (45, 46). Parv4 DNA has been detected in liver tissue samples from HIV negative patients, 15% of liver tissue specimens, mostly from HCV related liver transplantations (47) and 41% of liver autopsy samples (48). Parv4 viremia was not detected in patients alongside Parv4 DNA in liver tissue.

In a study by Manning et al. 2007, three different types of autopsy tissue were screened for viral DNA. Parv4 DNA was found in lymphoid and bone marrow tissue from HIV infected individuals but not in brain tissue. In the same study, HBoV was not detected in any tissue types while B19V was found in all three from both HIV infected and uninfected individuals. Both Parv4 and B19V viral loads were higher in bone marrow than in other tissue types, which is consistent with the tendency of parvoviruses to target dividing cells. Plasma samples from a similar group of patients all tested negative for Parv4 and B19V (16). Another comparison of Parv4 and B19V tissue tropism was performed in a study by Corcioli et al. 2010. The frequency of B19V was higher than Parv4 in all tissue types except for the liver where the frequency was the same for both viruses. This could indicate that the liver is an important target of Parv4 infection (48). To date Parv4 has not been successfully cultured in vitro (4).

Table 5 Types of tissue in which Parv4 DNA has been detected.

Types of tissue	Parv4 DNA positive (%)	Reference
Brain (front/occipital lobe)	0	Manning et al. (16)
Bone marrow	5,5-65	Corcioli et al. (48), Manning et al. (16), Simmonds et al. (49)
Liver	15-41	Schneider et al. (47), Corcioli et al. (48)
Lung	23	Corcioli et al. (48)
Lymphoid (lymph node or spleen)	39-61	Simmonds et al (49), Manning et al. (16)
Myocardium	49	Corcioli et al. (48)
Skin	4-12	Corcioli et al. (48), Botto et al. (45)
Synovium	5	Corcioli et al. (48)

1.6.2 Prevalence of Parvovirus 4

Prevalence of Parv4 has mostly been estimated by PCR studies screening for Parv4 DNA (see Table 6). As it seems likely that Parv4 persists in tissue, possibly lifelong, without viremia, it is not surprising that the highest frequencies of Parv4 DNA have been detected in tissue. The highest frequencies have been detected in tissue from HIV-infected IVDUs (16, 49). Frequencies of Parv4 viremia are much lower than frequencies of Parv4 in tissue. In most studies no viremia has been detected in blood donors and other groups at low risk for parenteral exposure, with some exceptions 2% in blood donors in USA (9), 4% in blood donors in Thailand (50), 4% in blood donors in Italy (45) and notably 22% in China (51) (see Table 6). Higher frequencies of viremia have been detected in highly exposed individuals 6% (9), 8% in IVDUs (50) and up to 41,5% in HCV and HBV infected patients (51).

Three studies have been published, Sharp et al. 2009, Lahtinen et al. 2011 and Simmonds et al. 2012 that screened for Parv4 antibodies (52-54). Serological studies have for the most part confirmed results from DNA studies (see Table 7). Frequencies of Parv4 antibodies are 0% in low risk, university students in Finland (53) and 0% in a low risk group in UK (52). The absence of Parv4 antibodies in these groups is further evidence that the frequency of Parv4 is low in the general population. Parv4 antibodies are more frequent among high-risk groups, 67% for HCV and HIV infected IVDUs in UK (52), 78% among HIV infected IVDUs in Finland (53) and 95% among HCV infected IVDUs in Swiss (54) (see Table 7).

In Ghana 8.6% of the children tested, were found positive for Parv4 DNA. The children were between 14 to 24 months old. Children from seven out of nine villages were tested positive, suggesting that Parv4 infection is widespread there (55). A serological study found high frequencies of seropositivity, in blood donors and other low risk groups, in four sub-Saharan African countries, 4-37% (see Table 9). Prevalence of Parv4 in Africa is therefore very different from the picture presented in Europe and the United States. Prevalence appears to be high in the general population in Africa, especially central Africa while next to nonexistent in the Northern Hemisphere.

Table 6 Frequency of Parv4 DNA in blood samples among various groups

Risk Group	Type of Tissue	HIV	AIDS	HCV	Parv4 DNA (%)	Country	Reference
Blood donor	Pl	-	NA	-	2	USA	Fryer et al. (9)
Blood donor	Se	-	NA	-	4	Thailand	Lurcharchaiwong et al. (50)
Blood donor	Se	-	NA	-	1	Italy	Vallerini et al. (56)
Blood donor	Bl	-	NA	-	4	Italy	Botto et al. (45)
Low risk	Pl	-	NA	-	0	UK	Manning et al. (16)
Low risk	BM/L	-	NA	-	0	UK	Simmonds et al. (49)
Exposed subjects	Pl	-	NA	-	6	USA	Fryer et al. (9)
Exposed subjects	Pl	+	-	ND	0	UK	Manning et al. (16)
IVDUs	Se	ND	NA	+	8	Thailand	Lurcharchaiwong et al. (50)
IVDUs	BM/L	-	-	+	8	UK	Simmonds et al. (49)
IVDUs	BM/L	+	-	ND	55	UK	Simmonds et al. (49)
IVDUs	BM/L	+	+	ND	85	UK	Simmonds et al. (49)
MSM	BM/L	+	+	-	0	UK	Simmonds et al. (49)
Hemo-philiacs	BM/L	+	+	+	1of2[a]	UK	Simmonds et al. (49)
Infants	Bl	ND	NA	-	8,6	Ghana	Panning et al. (55)
HCV patients	Se	-	NA	+	33	China	Yu et al. (51)
HBV patients	Se	-	NA	-	41,5	China	Yu et al. (51)
Healthy control	Se	-	NA	-	17-22	China	Yu et al. (51)

[a] only 2 samples tested, BM/L (bone marrow and lymphoid tissue), Bl (blood), MSM (men who have sex with men), ND (not done), NA (not applicable), Pl (plasma), Se (serum).

1.6.3 Route of transmission

Parv4 DNA has been detected in lung tissue but not in bronchoalveolar lavage (BAL) and other respiratory samples, which suggests that the respiratory route is not the route of transmission for Parv4 (45, 48, 57).

1.6.3.1 Parenteral route of transmission

The high frequency of Parv4 among IVDUs indicates that Parv4 is transmitted parenterally. Higher frequencies correlate with increased parenteral exposure. In Simmonds et al. 2007 the highest frequency 85%, of Parv4 DNA is found among the HCV and HIV infected IVDUs that have already developed AIDS, 55% among pre-AIDS IVDUs, and 8% among HIV negative, HCV infected IVDUs (see Table 6). IVDUs with AIDS have possibly been exposed most. Parv4 DNA has not been reported in serum from HIV infected homosexuals with AIDS without a history of parenteral exposure (49). IVDUs can easily become infected with HCV while HIV is less common because HIV seems to be transmitted less efficiently than HCV through the bloodborne route. HIV infected IVDUs are therefore

more likely to be infected with Parv4 because they have been exposed more (49, 58). Transmission by blood exposure has been shown to be less effective than HCV transmission, 0.3% vs. 3% in a healthcare setting (59).

Table 7 Distribution of Parv4 Antibodies in Europe

Risk group	HIV	HCV	Parv4 Ab (%)	Country	Reference
IVDUs	+	+	67	UK	Sharp et al. (52)
IVDUs	-	+	35	UK	Sharp et al. (52)
MSM	+	-	24	UK	Sharp et al. (52)
Heterosexuals	+	-	0	UK	Sharp et al. (52)
Hemophiliacs	+	+	55	UK	Sharp et al. (52)
Hemophiliacs	-	+	27	UK	Sharp et al. (52)
Sibling control	-	-	3	UK	Sharp et al. (52)
Low Risk	ND	ND	0	UK	Sharp et al. (52)
Low Risk	ND	ND	0	Finland	Lahtinen et al. (53)
IVDUs	+	-	78	Finland	Lahtinen et al. (53)
IVDUs	-	+	34,5	Finland	Lahtinen et al. (53)
General population	ND	ND	0	UK	Sharp et al. (60)
Blood Donors	-	-	0	France	Sharp et al. (60)
IVDUs	+	+	95	Swiss	Simmons et al. (54)
MSM	+	-	11	Swiss	Simmons et al. (54)
IVDUs[a]	-	+	31	UK	Simmons et al. (54)

[a] 66% IVDUs, ND (not done), MSM (men who have sex with men).

Parv4 antibody and Parv4 DNA frequencies are highest in HIV infected IVDUs (see Table 7). Lahtinen et al. 2011 found that Parv4 seropositive IVDUs are more likely to have injected more drugs, injected over longer periods and shared their injection equipment more often than seronegative IVDUs. They are also less educated and more likely to have been unemployed or incarcerated (53). Sharp et al. 2009 screened hemophiliacs, who had been exposed to untreated and unscreened blood in the 1970s, and their siblings for Parv4. Frequency of Parv4 antibodies among HIV and HCV infected hemophiliacs is 55% and 27% among HIV negative, HCV infected hemophiliacs. Only one sibling control subject had Parv4 antibodies, giving the hemophiliac sibling controls a seroprevalence of 3% (52). The very low seroprevalence among siblings of hemophiliacs is strong support for parenteral transmission for Parv4. Parv4 is not transmitted efficiently between individuals, even in close domestic contact. The one sibling, seropositive for Parv4, may possibly have been infected parenterally in a domestic setting by accident, although this would be a rare occurrence (52).

Table 8 Distribution of Parv4 Antibodies in Asia

Risk group	HIV	Parv4 Ab (%)	Country	Reference
IVDUs	+	74	Taiwan	Yang et al. (61)
MSM	+	71	Taiwan	Yang et al. (61)
Heterosexuals	+	59	Taiwan	Yang et al. (61)

MSM (men who have sex with men).

1.6.3.2 Alternative Parv4 transmission routes

In Africa where only genotype 3 has been detected, the distribution of Parv4 is very different. Originally, Parv4 genotype 3 was detected in two HIV infected individuals without a history of injection use. One was a heterosexual woman from Nigeria, HIV infected but HCV and HBV negative, and the other was a heterosexual man from the Democratic Republic of Congo, HIV infected but HCV negative (34). In sub-Saharan Africa Parv4 infection seems to be ubiquitous. Antibody measurements among HIV and HCV uninfected populations found high frequencies of seropositivity, especially in central Africa (see Table 9). There must therefore be another route of transmission for Parv4. Parenteral transmission is highly improbable as the source of Parv4 infection in Africa. Parv4 frequency is significantly higher among older children than younger, thus making perinatal infection improbable. The children had a lower risk of Parv4 infection if they did not live close to a river and had access to a kitchen, which indicates better hygiene in the household. For that reason food-borne or smear (contact with contaminated objects) transmission is a possible culprit (55). The discovery of Parv4 DNA in nasal swabs and fecal samples in children in Ghana suggests that Parv4 could be transmitted by the fecal-oral route or the respiratory route (62).

Table 9 Distribution of Parv4 Antibodies in Africa

Risk group	HIV	HCV	Parv4 Ab (%)	Country	Reference
Blood Donors	-	-	37,1	Burkina Faso	Sharp (60)
General population	ND	ND	24,8	Cameroon	Sharp (60)
Military population	(2)	-	35,3	DRotCongo	Sharp (60)
Blood Donors	+	-	36,4	South Africa	Sharp (60)
Blood Donors	-	-	4,4	South Africa	Sharp (60)

ND (not done).

The difference in seropositivity between HIV infected and HIV uninfected blood donors in South Africa, 36% and 4% respectively, is difficult to explain (see Table 9). The majority of these blood donors would have acquired their HIV infection through sexual contact. In the UK 27% of HIV infected homosexuals without a history of injection drug use were found to be Parv4 seropositive compared to 0% among HIV infected heterosexuals also without a history of parenteral exposure (see Table 7).

As seen with hemophiliacs and their siblings Parv4 is not transmitted even with close contact between members of the same household. A possible transmission route for Parv4 is arthropod-borne or parasite-associated transmission but on the other hand no known parvoviruses are borne by vectors (49, 60). There is high frequency of Parv4 seropositivity in chimpanzees and gorillas. Consequently, Parv4 homologues are even more widespread in nonhuman primates than Parv4 is in humans. If there is an environmental source of Parv4 infection, it could possibly be the reason for high seropositivity in non-human primates. Sub-Saharan Africa seems a possible place of origin for Parv4 in IVDUs in the northern hemisphere (30).

There are fewer studies on Parv4 prevalence from Asia than Europe and Africa; however, some geographical differences can be seen. Frequency of Parv4 DNA in blood donors and healthy control is higher in Asia than seen in studies from Europe (see Table 6). In Taiwan HIV infected heterosexuals

(61) have a high rate of anti-Parv4 while none was found among HIV infected heterosexuals in the UK (52). Some other different transmission route than the parenteral route may be dominant in Asia as well as Africa. It has been shown that Parv4 is able to transmit through the placenta from mother to fetus (63). It is unknown if placental transmission is responsible for the high frequencies of Parv4 found in African populations.

1.7 Molecular epidemiology

Currently genotypes 1 and 2 have been detected in the US, Europe and Thailand while genotype 3 has been detected exclusively in Africa. A pattern of temporal succession of genotype 2 replacing genotype 1 has been observed (16, 47, 48, 64), most distinctly in a study from Edinburgh where genotype 2 sequences were isolated from individuals born before 1956 and genotype 1 from individuals born after 1956. A similar pattern is seen for B19V, temporal succession of with genotype 1 replacing genotype 2 in the 1960's (16). Parvoviruses have a fast rate of sequence change (42) and the genotype 1 sequences from Edinburgh had a very restricted variability (16). The authors estimated that the Parv4 variants diverged from the original Parv4 strain in the last 20 years, indicating that Parv4 infection may have appeared and spread rapidly among IVDUs in Edinburgh in the 1980s-1990s (16). The temporal succession of Parv4 genotypes is also supported with data on pooled blood products. Older pools collected before the 1980's are more likely to contain genotype 2 whereas more recent pools from the 2000's were more likely to contain genotype 1 (65, 66).

1.8 Parvovirus 4 role in disease

The original Parv4 strain was isolated from a symptomatic, homeless, HBV infected IVDU that was lost to follow-up, Hence, it was not possible to study this individual further and determine the cause of his symptoms (1).

In a study by Fryer et al. 2007, 6% of symptomatic, highly exposed patients were positive for Parv4 DNA compared to 2% of blood donors. The symptomatic patients had symptoms related to acute HIV infection after engaging in high-risk sexual behavior or injecting drug use but tested HIV negative. It is possible that Parv4 infection had a role in their symptoms, but more likely the frequency of Parv4 is higher in this group because Parv4 is a blood-borne virus. Therefore, this group is more likely to become Parv4 infected (9).

In a study by Lahtinen et al. 2011, no difference was found between Parv4 IgG positive and negative IVDUs with regard to symptoms (fever, tiredness, sweating, cough, diarrhea, shortness of breath, swallowing complaints, muscle weakness, dizziness, skin abscesses or herpetic lesions, loss of eyesight, or headache) in the six months prior. In the same study two HCV infected IVDUs had a primary Parv4 infection. They had detectable IgM, increasing IgG levels, low IgG avidity and Parv4 DNA in serum, which the authors presented as diagnostic criteria for Parv4 primary infection. The authors applied the kinetics of B19V infection to these two Parv4 cases determining the time of infection to approximately 2005. Neither of those patients sought any medical care during that time, however, as these patients were IVDUs, they probably would not have done so unless they were extremely ill (53).

In Panning et al. 2010 the infants that tested positive for Parv4 genotype 3 DNA had no signs of acute infection (rash, fever, myalgia) (55). In another study, a patient with suspected viral disease but negative for most common viruses, tested positive for Parv4 DNA. However, this patient suffered from Wegener granulomatosis and died one month later of multiple organ failure, Parv4 role in this patients symptoms is uncertain (56).

Two recent studies have linked Parv4 with disease in children. Benjamin et al. 2011 screened cerebrospinal fluid (CSF) samples from children with symptoms of central nervous system (CNS) infection of unknown etiology and found two of the samples positive for Parv4 DNA (67). A study from Taiwan found Parv4 DNA in plasma from three mothers and their newborn children with hydrops, The study raises the possibility that Parv4 can be linked to non-immune hydrops fetalis, like CMV and B19V (63). Parv4 is not yet clearly associated with disease and so far Parv4 infection seems to be subclinical.

1.9 Parvovirus 4 in blood Products

Parv4 has been detected in blood products. Manufacturing plasma pools from European and North American donors have tested positive for Parv4, 4% of recent pools from 2006 and 21% of archived pools from 1990 to 1993. The frequency of Parv4 was similar for both recent and archived pools if one manufacturer was discounted (9). The high frequency for one manufacturer could indicate that Parv4 infections are seasonal or occur in epidemics as B19V. Higher frequencies of Parv4 are found in blood products expiring before the 1990s, 33% and 23% compared to 9% and 2% of blood products expiring after the 1990s (65, 66). Higher viral loads are also found in older pools compared to recent ones (9, 65, 66). The difference in frequency and viral load could be explained by blood safety measures approved in response to the HIV epidemic in the mid-1980s. Parv4 is more frequent in IVDUs and the removal of risk groups from donating blood could have lowered the frequency of Parv4 in donated blood. Parv4 has also been detected in 26% of recent plasma pools (2007 to 2010) from China. The different results from Chinese plasma pools compared with North American and European could be due to geographical or seasonal differences (68).

It is unknown what effect various virus inactivation methods have on Parv4. A study screening coagulation factor concentrates for Parv4 found no Parv4 DNA in nanofiltrated or pasteurized products whereas the solely solvent detergent treated product had the highest frequency of Parv4 (66). This result could be an indication that solvent detergent treatment is ineffective against Parv4. Solvent detergent treatment is effective against enveloped viruses but is less effective against non-enveloped viruses (17). B19V is inactivated in pooled plasma by neutralizing B19V antibodies (69). However, the lack of Parv4 antibodies in blood donors indicates that Parv4 DNA would not be inactivated by antibodies in pooled plasma. Nanofiltration is possibly less effective for removing Parv4 from blood as small viruses are easier to remove if complexed with antibodies (70). Although Parv4 DNA is present in blood products, the Parv4 present is not necessarily infectious. The viral loads detected in plasma pools and blood products were as high as 106 copies per ml (9, 65, 66). The viral loads found for Parv4 are lower than those found for B19V are. B19V loads can be up to 1000-fold higher (9, 65). The consequences of Parv4 being transmitted by blood, and blood products, are unknown.

Sharp et al. 2011 documented hemophiliacs that seroconverted for Parv4 after receiving virally inactivated clotting factor concentrates demonstrating that Parv4 is transmissible by blood products and resistant to viral inactivating methods (71).

Porcine Parv4 like viruses have been detected in porcine plasma and factor VIII (FVIII) concentrates used by hemophiliacs with autoimmune antibodies against human FVIII (40). Transmission of parvoviruses between species has been seen with feline parvovirus to dogs to form a new canine parvovirus (72). Possibly different Parv4 strains could evolve and recombine to form new, unknown strains. Parenteral transmission, particularly between species, could therefore be a concern (40).

1.10 Parvovirus 4 and the immune system

1.10.1 Parvovirus 4 in immunosuppressed patients

Parv4 is detected more often among HIV infected individuals (see Table 6); one possible reason is that because of immunosuppression Parv4 is detected more easily. In Corcioli et al. 2010 viremia was found more frequently among immunosuppressed hematological patients than immunocompetent although the difference was not statistically significant (48). However, in Simmonds et al. 2007 Parv4 DNA was not detected in HIV infected homosexuals with AIDS while Parv4 DNA was detected in 85% of HIV infected IVDUs with AIDS (49) indicating that the Parv4 is not detected more frequently in HIV infected individuals because of immunosuppression. It is therefore more likely that HIV and Parv4 infection have a shared route of transmission. Manning et al. 2007 found that Parv4 and B19V viral loads are not higher in immunosuppressed individuals than immunocompetent. The suppression of the immune system does not seem to affect the replication of Parv4. This behavior is different from latent, persistent DNA viruses like CMV, which reactivate and cause disease in immunosuppressed individuals (16).

1.10.2 Persistence of Parvovirus 4

A study on the T cell response to Parv4 found a high level of $CD8^+$ T cell response to Parv4 (73), similar to low-load persistent viruses like CMV (74) and Epstein Barr virus (EBV) (75). EBV and CMV both establish lifelong latent infections. The T cell responses to Parv4 were sustained for up to 3 years. T cell responses to B19V virus are also sustained for up to 3 years. B19V responses actually increase after resolution of acute symptoms which is different from most other viruses (76). B19V is not thought to be a classical persistent virus like e.g. Hepatitis B but does persist in various tissues (15) and at very low levels in blood (77). Persistent B19V viral replication in tissue could be a source of continuous supply of antigen to stimulate sustained T cell responses (76). Parv4 has been detected in different tissue types without viremia. Contained viral replication in tissue could also be the source of antigen to stimulate persistent T cell responses against Parv4.

1.11 Blood-borne viruses in IVDUs

Intravenous drug use was practically unknown in Iceland before 1980. In the early 1980's Icelandic IVDUs returned from abroad and sought medical aid in Iceland. Shortly after amphetamines began to

be smuggled into the country and since then intravenous drug use has only increased. Over 90% of IVDUs in Iceland are thought to have sought aid at the SAA (National Center of Addiction Medicine). SAA has admitted 1786 IVDUs between 1991 and 2009. SAA estimates that Iceland has 700 active IVDUs today, most in the Reykjavik area, which is similar to cities in other countries. IVDUs in Iceland are mainly men; women comprise 30% of IVDUs. The average age of current and former IVDUs in Iceland in 2009 is 31.9 years. In the early '00s, the average age of new IVDUs admitted to SAA lowered, although this trend reversed a few years later. In 2009 the average age of new IVDUs was 30,2 years (78).

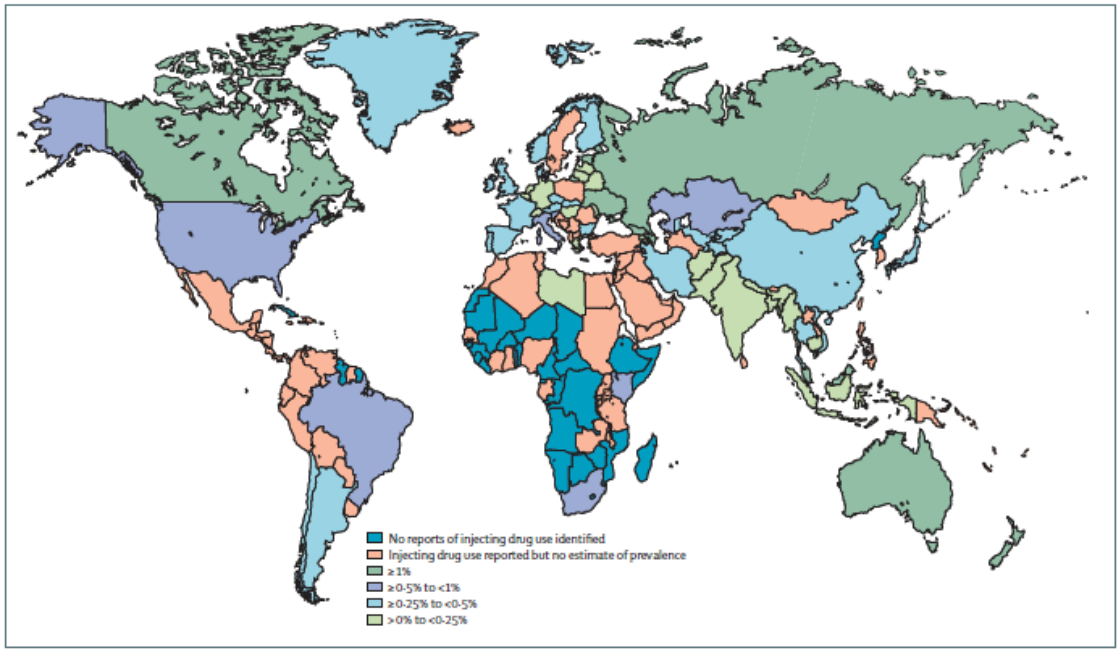


Figure 8 Prevalence of injecting drug use (79).

There are an estimated 16 million people worldwide that inject drugs and they are present in most countries (see Figure 8) (79). IVDUs are at risk of infection with blood borne pathogens through sharing of injection equipment. HIV, HCV and HBV are transmitted by IVDUs and prevalence increases with longer duration of injection (80, 81).

1.11.1 Hepatitis C and B viruses in IVDUs

Around a 170 million people worldwide are chronically infected with HCV. HCV is mainly transmitted through exposure to blood. Infections rarely occur through transfusions any more, intravenous drug use is the main risk factor today. HCV infections rarely occur through sexual contact. High-risk sexual behavior is a risk factor, possibly because of an association with herpes simplex type 2 infection (82). Egypt (22%), Pakistan (4.8%) and China (3.2%) have particularly high rates of chronic HCV infection. The explanation for higher rates in these countries is due to contaminated injection equipment (83). HCV infection in Iceland occurs almost exclusively in IVDUs, and over 60% of those have been infected with HCV (78, 84). Prevalence of HCV in IVDU populations worldwide ranges from 10% to 97% (see Figure 9). Worldwide 67% of IVDUs have been HCV infected (85).

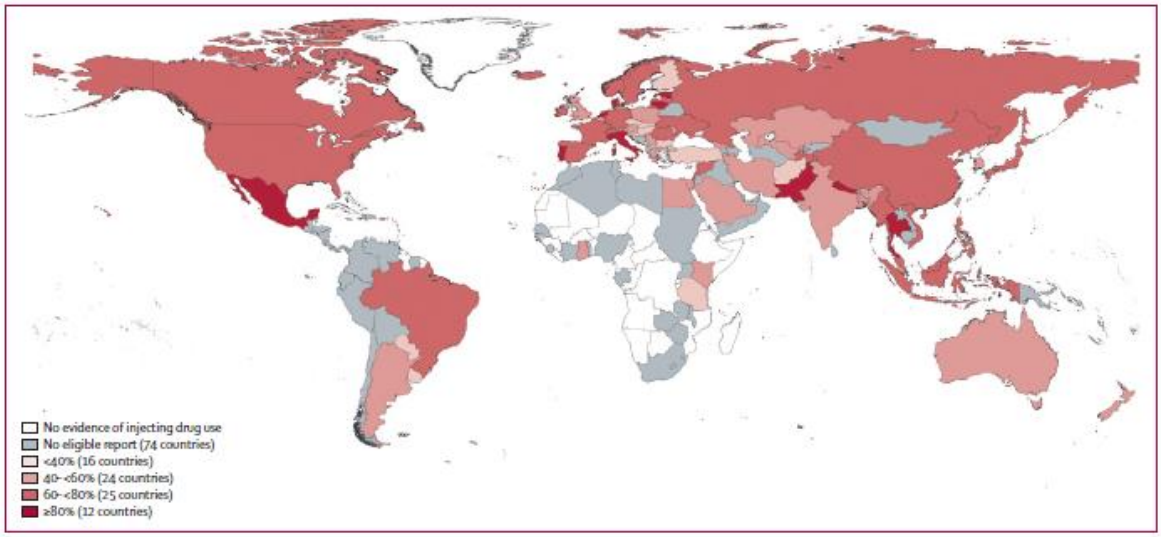


Figure 9 Prevalence of Hepatitis C antibodies in IVDUs (85).

HBV is one of the most common infections in the world. One third of the world's population or 2 billion people have been infected with HBV and 350 million are chronically infected. HBV is transmitted by contact with blood and other body fluids. In highly endemic areas like China and parts of Asia 8% to 10% of the population is chronically infected. HBV infections are mostly acquired at birth and in early childhood there. In North America and Western Europe, less than 1% is chronically infected and most HBV infections occur during adolescence and adulthood through intravenous drug use and sexual contact. (86, 87). Prevalence of HBV is lower among IVDUs than the prevalence of HCV. Globally 8.4% of IVDUs have been HBV infected. In many countries, 2% to 10% IVDUs are HBV infected (85). No study has evaluated HBV prevalence in IVDUs in Iceland (see Figure 10). HBV infection occurs mostly in immigrants and IVDUs in Iceland. About half of diagnosed HBV cases are immigrants (88) but the rest are thought to be mainly due to intravenous drug use. There have been two HBV epidemics of in Iceland among IVDUs in 1989-1991 and 2007 (78, 89, 90).

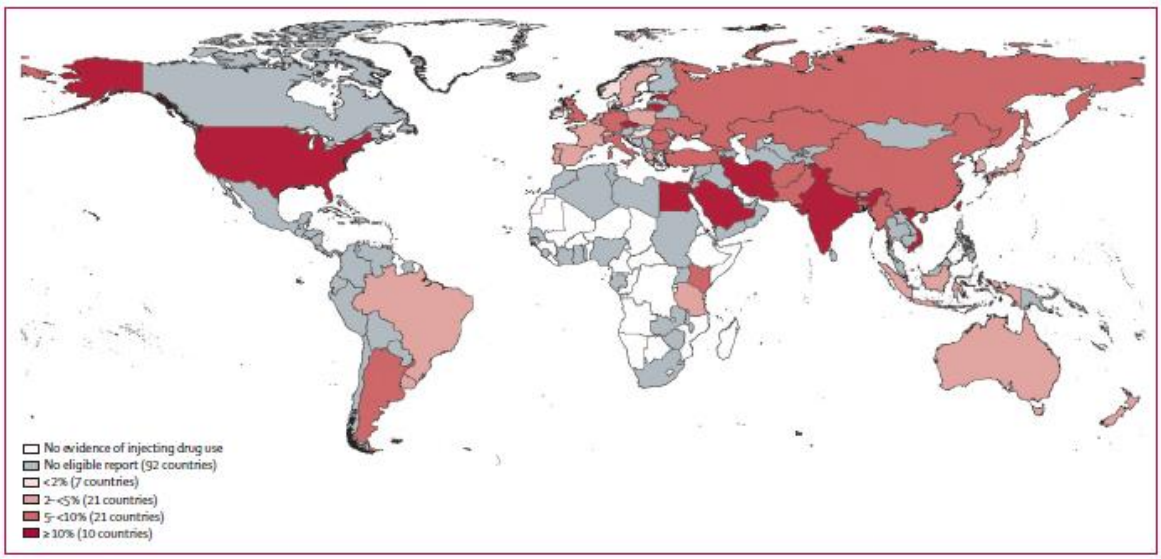


Figure 10 Prevalence of Hepatitis B surface antigen in IVDUs (85).

1.11.2 HIV in IVDUs

There are 34 million HIV infected people in the world. HIV is transmitted by close contact with various body fluids, such as blood or semen. Transmission can occur through sexual contact, contaminated injection equipment or from mother to child (91). There are an estimated 3 million IVDUs with HIV worldwide. Prevalence of HIV among IVDUs varies among countries (see Figure 11). A few countries in South America, eastern Europe and southeast Asia have very high prevalence of HIV, over 40% while in other IVDU populations HIV has not yet been introduced (79). The prevalence of HIV among IVDUs is lower than the prevalence of HCV. HCV spreads easily among IVDUs and can become endemic in a short time while HIV spreads slower (58). In Iceland, HIV is not widespread among IVDUs and is associated with transmission by sexual contact. However, the number of HIV cases among IVDUs in Iceland has been increasing in the last years (78, 90).

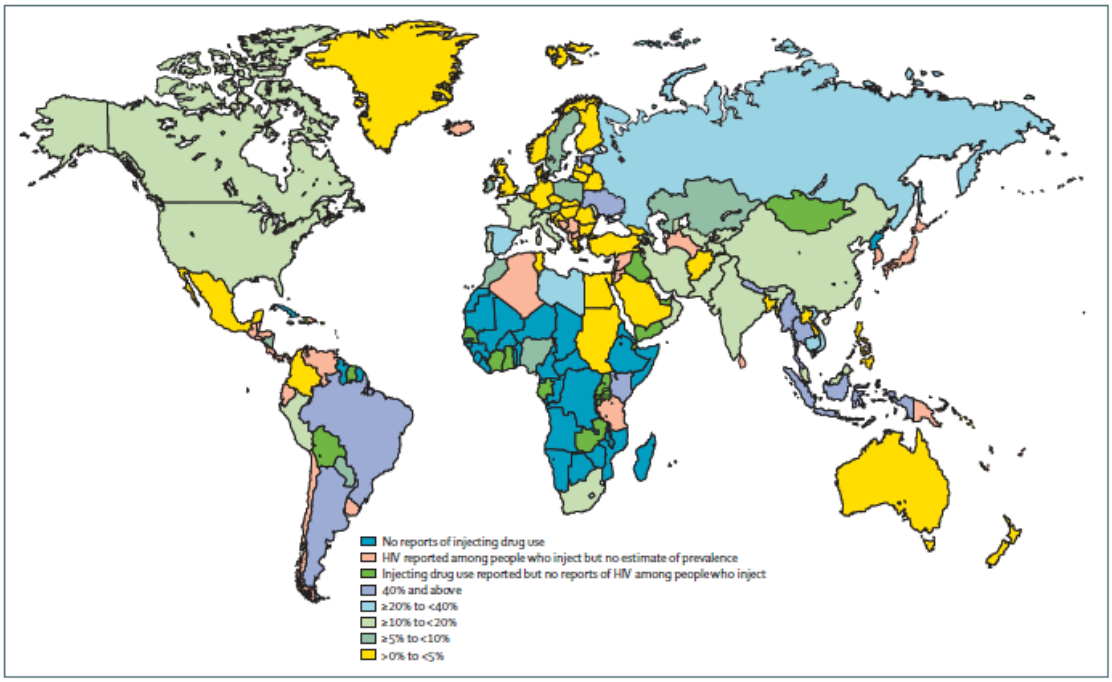


Figure 11 Prevalence of HIV infection among IVDUs (79).

2 AIMS OF STUDY

The aim of the study was to evaluate the prevalence, transmission route, possible disease links and clinical importance of the new human parvovirus 4 in Iceland. Parvovirus 4 is a recently discovered virus and no previous studies have been conducted on this virus in Iceland. The study was conducted at the University Hospital MAS in Malmö where a comparable study was underway. Iceland and the city of Malmö are of similar population size and are serviced by one hospital and one Clinical Virology Laboratory. It is interesting to compare the prevalence of Parv4 between the two places. The demographics of Malmö and Iceland on the other hand are quite different. Malmö has a large immigrant population, whereas Iceland is more homogenous. In Sweden, there was also an opportunity to carry out a longditutional study on blood from Swedish IVDUs, to investigate the progression of Parv4 infection.

3 MATERIALS AND METHODS

3.1 Sample collection

Serum and NPS (Nasopharyngeal Aspirate) samples were collected from the Biobank of the Virology Department of the Landspitali - National University Hospital of Iceland. The samples were stored at -20°C and ranged from between the years 2006 to 2008. Permits were received from the Bank and the Science Ethics Committee and Data Protection Authority. The study was implemented by unlinked anonymous testing so results could not be linked to identities of the subjects. The only information supplied about individual subjects was age, sex and the patient group. No contact was initiated with patients and no further information about the subjects was obtained. About 250 µL of serum or NPS was collected from each subject.

Table 10 Patient groups

Patient group	Type of sample	HCV/ HBV Ab	Number of patients
Control	Serum	-	211
Hepatitis	Serum	+	128
STD ward	Serum	-	119
Dialysis	Serum	-	34
Respiratory symptoms	Serum	-	35
Immunosuppressed patients	Serum	-	24
Neurological symptoms	Serum	-	24
Febrile patients	Serum	-	23
NPS	NPS	-	26

Various patient groups were selected to find the prevalence in these groups, indications of transmission route and disease associations. Nine groups of samples were collected; three large subject groups and six smaller patient groups (see Table 10). First, a control group, consisting mainly of blood donors and University students was selected to estimate prevalence of Parv4 in the general population in Iceland. The control group comprised 211 healthy subjects: 44 blood donors, 158 University students, (students that came to the Virology Department for the practical part of their virology course), 4 insurance applicants, 2 students applying for university entrance and 3 taking part in another study. Secondly, HCV and HBV infected patients were chosen to investigate the transmission route of Parv4 and association with HCV and HBV. The hepatitis group was comprised of 128 HCV and HBV infected subjects, 79 subjects were HCV infected, 38 HBV infected and 11 were both HCV and HBV infected. The third large group consisted of HCV, HBV and HIV uninfected individuals that came to the sexually transmitted diseases (STD) clinic, usually because of recent high-risk behavior. The STD clinic group consisted of 119 HCV and HBV uninfected subjects. The Dialysis group comprised of patients on dialysis. The Respiratory symptoms group consisted of patients with respiratory symptoms of yet unknown etiology. The immunosuppressed group consisted of

immunosuppressed patients because of treatment for hematological malignancies. The Neurological symptoms group consisted of patients with neurological symptoms of yet unknown etiology. The Febrile patient group was comprised of patients with fever of yet unknown etiology. The NPS group was comprised of children with respiratory disease of yet unknown etiology (see Table 10). The samples were then transported to the University Hospital MAS in Malmö where they were stored at -20°C until analysis.

In Sweden, there was also an opportunity to do a longditutional study on Swedish blood samples from HCV infected IVDUs, enrolled in a needle exchange program at the University Hospital MAS. The Swedish Parv4 study had already detected 12 Parv4 positive IVDUS (unpublished data, Anders Widell, Viktor Dahl). A 100 µl from available samples from these IVDUs were collected from the Malmö Microbiology Biobank, where they were stored at -20°C.

3.2 Nucleic acid extraction

The serum samples and NPS samples were extracted with the MagNA Pure LC machine and using the Total Nucleic Acid Isolation Kit from (Roche Applied Science, Mannheim Germany). Total nucleic acid was extracted from 100 µL of sample, according to the manufacturer's instructions (Roche Applied Science), and eluted into 50 µL of extracted sample. Negative and positive controls were used in each run.

3.3 Real-time Consensus Polymerase Chain Reaction

Originally, both PCR methods, Consensus real-time and nested PCR were used to screen samples. The real-time PCR was observed to be more sensitive. Therefore, real-time consensus PCR was used to screen all the samples while the nested PCR was used to confirm positive and uncertain results, and used for sequencing. Positive results in the real-time PCR were defined positive if fluorescence was detected by the 40th cycle (cycle threshold (CT) < 40).

Table 11 Nucleotide sequences of primers used in this study

Name of Primer	Nucleotide sequence of Primer
PV4NS1F	5′-AAGACTACATACCTACCTGTG-3′
PV4NS1R	5′-TGCCTTTCATATTCAGTTCC-3′
PV4NS1Fn2	5′-GTTGATGGYCCTGTGGTTAG-3′
PV4NS1Rn2	5′-CCTTTCATATTCAGTTCCTGTTCAC-3′
ConsensusFwd	5′-CTAAGGAAACTGTTGGTGATATTGCT-3′
ConsensusRev	5′-GGCTCTCCTGCGGAATAAGC-3′

Consensus real-time PCR was based on the PCR procedure as described in Fryer et al. 2007 (9). The real-time PCR was performed in a Roche Lightcycler 2.0 machine, (Roche Applied Science). The forward primer ConsensusFwd was located between nucleotides 3285 and 3310 of ORF2 (GenBank Accession Number AY622943). The reverse primer ConsensusRev was located between nucleotides 3368 and 3387 of ORF2 (see Table 11). This region was highly conserved between genotypes 1 and

2. The primers amplify a 103 base pair (bp) product. A fluorogenic hydrolysis probe (ConsensusProbe 5′-(FAM) TGTTCAACTTTCTCAGGTCCTACCGCCC (TAMRA)-3′) was mapped to nucleotides 3313 to 3340 of ORF2. The amplification reactions were performed with QuantiTect Probe PCR kit (Qiagen, Hilden, Germany). The procedure for performing the real-time PCR mixture is shown in Table 12. Then 5 µL of extracted DNA were added to the mixture and the PCR reaction run on the Roche Lightcycler 2.0 machine as follows, 95°C for 15 minutes and then 45 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Table 12 Procedure for real-time consensus PCR for each sample.

Reagents	Volume (µL)
Qiagen H_2O	3
Forward Consensus primer (10 pmol)	1
Reverse Consensus primer (10 pmol)	1
Consensus probe 1 pmol	1
Qiagen probe 2X Master mix	10
Total volume	15 + (5 of sample)

3.4 Nested Polymerase Chain Reaction

Positive and uncertain samples were screened for Parv4 by a nested PCR gel method as described by Fryer et al. 2007 (9). The primers amplified a conserved region in ORF1, first round primers were PV4NS1F and PV4NS1R and second round primers were PV4NS1Fn2 and PV4NS1Rn2 (see Table 11). The PCR mixture for the first round of PCR was prepared as shown in Table 13. After the PCR mixture was prepared, 2 drops of oil were added on top of the mixture and lastly 10 µL of extracted DNA added under the oil, which makes the final volume 50 µL. The first round of PCR reaction was performed on a Perkin Elmer GeneAmp 9600 Thermal Cycler (Perkin Elmer Massachusetts, USA) at 96°C for 9 minutes, then 45 cycles at 96°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, and lastly 7 minutes at 72°C (see Table 14).

Table 13 1st PCR Procedure for each sample

Reagents	Volume (µL)
H_2O	24,5
Gold PCR buffer X2 (Applied Biosystems, California USA)	5
Gold $MgCl_2$ (Applied Biosystems)	4
dNTP (10 mM) (Applied Biosystems)	1
PV4NS1Fn2 (1 mM)	2,5
PV4NS1Rn2 (1mM)	2,5
AmpliTaq Gold DNA polymerase (Applied Biosystems)	0,5
Total volume	45 + (5 of sample)

The mixture for the second round of PCR reaction was prepared as seen in Table 15. As in the first half of the procedure, two drops of oil were added and then 3 µL of product from the first half PCR procedure for a final volume of 50 µL. The same cycler and cycling program as before were then used for the second PCR round. The PCR products were then analyzed by agarose gel electrophoresis.

Table 14 Cycling conditions for nested PCR.

Step	Temperature (°C)	Time
Polymerase activation	96	9 min
45 cycles:		
Denaturing	96	30 sec
Annealing	55	30 sec
Elongation	72	1 min
Final elongation	72	7 min

3.4.1 Agarose Gel Electrophoresis

Agarose gel (2%) was prepared to analyze the PCR products from the nested PCR. First 1X TBE (Tris Borate EDTA) buffer was made from 10X TBE buffer (Applied Biosystems). A 100 mL of 10X TBE were added to 900 mL deionized water. Then 2 grams of agarose (Agarose for routine use, Sigma-Aldrich, Missouri USA) were added to 100 mL of 1X TBE buffer and then heated in a microwave at 650 watt for 2 minutes shaken, heated again at 650 watt for 1 minute, shaken and finally heated at 450 watt for 1 minute. 1.5 µL of ethidium bromide 10 mg/mL (Sigma-Aldrich) were added to the agarose solution. Afterwards the whole solution was poured into a casting tray and a comb was added. 1.5 µL of loading buffer bromophenol blue (Sigma-Aldrich) was added to 7 µL of amplified product, size marker 1 kb DNA ladder (Invitrogen, California USA) and positive control, respectively. When the gel plate had solidified, the comb was taken out. The gel plate was placed in a horizontal gel box and 1X TBE buffer was pored over. The size marker, positive control, and samples were then added to the wells and current applied. The electrophoresis took about 45-60 minutes and then the gel was placed in UV light and photographed.

Table 15 2nd PCR Procedure for each sample.

Reagents	Volume (µL)
H_2O	31,5
Gold PCR buffer X2 (Applied Biosystems)	5
Gold MgCl2 (Applied Biosystems)	4
dNTP (10 mM) (Applied Biosystems)	1
PV4 NS 1Fn2 (10 mM)	2,5
PV4 NS 1Rn2 (10 mM)	2,5
AmpliTaq Gold DNA polymerase (Applied Biosystems)	0,5
Total volume	47 + (3 of sample)

3.5 Sequencing of Parvovirus 4 PCR products

The PCR products of the 12 positive samples were analyzed again with gel electrophoresis as before but this time with 1% agarose gels. The Parv4 sequences were extracted with Qiaquick Gel Extraction Kit (Qiagen) using a microcentrifuge according to the manufacturer's protocol. Parv4 positive samples were sequenced with ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol. Primers were diluted to 0.8 mM to have 3.2 pmol in the reaction and the PCR mixed as seen in Table 16. However, for samples that had weaker bands in the nested PCR 6 µL of template DNA and 2 µL of H_2O were used instead of 4 µL of template DNA. The sequencing reaction was run on Perkin Elmer GeneAmp 9600 Thermal Cycler as follows: 96°C for 10 minutes, 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes, and lastly it was kept at 4°C until it was ready for purification (see Table 17). The sequencing products were then purified with isopropanol precipitation. The sequencing products were stored in a freezer until sent to the sequencing facility at the Department of Clinical Chemistry at the University Hospital in Malmö.

Table 16 Sequencing procedure for each sample

Reagents	Volume (µL)
Sigma H_2O	3
PV4NS1Fn2 (0,8 mM)	1
PV4NS1Rn2 (0,8 mM)	1
Terminator Ready Reaction Mix	8
Total volume	16 (+ 4 of PCR product (3-10 ng))

Table 17 Cycling condition for sequencing reaction.

Step	Temperature (°C)	Time
Polymerase activation	96	10 min
25 cycles:		
Denaturing	96	10 sec
Annealing	50	5 sec
Elongation	60	4 min
Stored until ready	4	-

3.6 Sequence analysis

The sequences were edited with Factura (Applied Biosystems) and (Sequence Navigator, Applied Biosystems). Further analysis was done with Bioedit 7.0 (Ibis Biosciences, Carlsbad California) and MEGA5. Sequences were aligned with Clustal W, pairwise distances calculated and a Neighbour Joining tree calculated.

3.7 Statistical analysis

Statistical analysis was done with Office Excel 2007 software (Microsoft, Washington USA). T tests were used to compare mean age between control and hepatitis groups. Chi square test was used to evaluate frequencies of Parv4 DNA between groups, association between presence of Parv4 DNA and hepatitis co-infection, and sex ratio between groups.

4 RESULTS

4.1 Detection of Parv4 in low risk subjects in Iceland

No Parv4 DNA was detected in serum samples in the control group (see Table 18). This group predominantly consists of blood donors and University students. The students were not screened for HCV, HBV or HIV, but were considered low risk subjects. These subjects were chosen to represent the general population in Iceland. The sex ratio was skewed towards females 60%, as there were more female University students than male. The average age in this group of 26.8 years was also low as the majority of the University students were in their early twenties.

Table 18 Parv4 screening of Icelandic samples.

Patient Group	Number of subjects	Average age (range) (years)	Sex ratio (M/F)	Parv4 positive samples (%)
Control group	211	26,8 (19-64)	85/126	0 (0)
Hepatitis	128	38,3 (18-92)	80/48	12 (9,4)
STD Unit	118	28,4 (16-60)	68/50	0 (0)
Patients on dialysis	33	64,6 (10-88)	24/9	0 (0)
Respiratory symptoms	34	32,8 (0,9-79)	14/20	0 (0)
Cancer patients	24	48,1 (4-81)	13/11	0 (0)
Neurological symptoms	21	51,8 (19-75)	17/4	0 (0)
Febrile patients	23	40,9 (16-89)	10/13	0 (0)
NPS from children	25	2,8 (0,4-16)	15/10	0 (0)
All	615			12 (2,0)

4.2 Detection of Parv4 in HCV and/or HBV infected subjects

Parv4 DNA was detected in 12 or 9.4% of serum samples of HCV and/or HBV infected subjects (See Table 19). The frequency of Parv4 in HCV/HBV infected subjects was significantly higher than in the control group (χ^2-test $p<<0.01$). Age and gender were not comparable between the control and the hepatitis group (see Table 18). The mean age of the control group was significantly lower than the mean age of hepatitis group (unpaired t-test $p <<0.01$). The sex ratios between the two groups were significantly different, (χ^2-test $p<<0.01$). The sex ratio in the hepatitis group was skewed towards males.

All twelve positive subjects were found positive with real-time PCR and seven with the nested PCR method. Seven Parv4 positive subjects were HCV infected, two were HBV infected and three were both HBV and HCV infected. The mean age of the Parv4 positive subjects, 27.6 years, was significantly lower than the mean age, 39.4 years, of the Parv4 negative subjects (unpaired t-test, p=0,0075,). The mean age of the Parv4 positive subjects, 27.6 years, was significantly lower than the

mean age, 38.3 years, of the HCV/HBV infected subjects (95% confidence limits, (31,8-44,7). Gender was not a significant risk factor for the presence of Parv4 DNA (χ2-test p = 0.75). HCV and HBV infected individuals in Iceland are predominantly IVDUs, so it is reasonable to assume that these subjects are IVDUs.

Table 19 Parv4 positive subjects.

Sample no.	Sex ratio	Age (years)	Hepatitis C/B	RT-PCR (CT)	Nested PCR
45	M	18	HCV	37,48	Negative
65	M	28	HBV	34,80	Negative
69	F	34	HCV	37,03	Positive
301	M	30	HBV	29,33	Negative*
318	M	21	HCV/HBV	31,99	Positive
611	F	39	HCV	29,10	Positive
617	M	28	HCV/HBV	37,62	Low Positive
623	M	26	HCV	33,78	Positive
630	M	30	HCV	36,27	Positive
632	F	35	HCV	33,74	Positive
635	F	21	HCV/HBV	37,21	Negative
641	M	21	HCV	37,63	Negative

4.3 Detection of Parv4 in samples from the Sexually Transmitted Diseases Clinic

None of the 118 samples from the STD unit contained Parv4 DNA. The average age of the subjects in this group was 28.4 years and 58% of the subjects were male. Samples are sent anonymously from the STD unit for HIV testing. It is reasonable to infer that these subjects have engaged in high-risk behavior.

4.4 Detection of Parv4 in samples from various smaller patient groups

None of the samples in the smaller patient groups tested positive for Parv4 DNA (see Table 18). The number of subjects in each group was quite small, around 25. The mean age was 2.8 years in the NPS group. The oldest child in this group was 16, but most of the subjects were less than two years old. The mean ages of the other smaller groups were higher than the control group and the hepatitis group except for the respiratory symptoms group and the febrile patients' group. The sex ratios of the small groups were fairly even except in the dialysis patient group and the neurological symptoms group, which were predominantly male.

4.5 Sequencing analysis of Icelandic Parv4 sequences

Table 20 The sequences from the seven samples that were successfully sequenced

Sample	Sequences
69	CCCAGCATCAAGAAGCTTGCAGACAAGAATAACCATGTTTCAGTTTCAGAGAATGGTTCCGGATGGCTTAGCTCCACTTCCTGAAGAGGAAGTGAGAAGCTTTTTTAAGCTAGGTGAACAGGAACTGAATATGAAAGGA
301	CCCAGCWTCAAGAAGCTTTGCWGACAAGAATAACCATGTTTCAGTTTCAGAGAATGGTTCCGGATGGCTTAGCTCCACTTCCTGAAGAGGAAGTGAGAAGCTTTTTTAAGCTAGGTGAACAGGAACTGAWTATGAAAGGA
318	CCCAGCATCAAGAAGCTTTGCAGACAAGAATAACCATGTTTCAGTTTCAGAGAATGGTTCCGGATGGCTTAGCTCCACTTCCTGAAGAGGAAGTGAGAAGCTTTTTTAAGCTAGGTGAACAGGAACTGAATATGAAAGGA
611	AGAAGCWTGCVGACVAGAATAACCATGTTTCAGTTTCAGAGAATGGTTCCGGATGGCTTAGCTCCACTTCCTGAAGAGGAAGTGAGAAGCTTTTTTAAGCTAGGTGAACAGGAACTGAATATGAAAGGA
623	CACCCAGCATCAAGAAGCTTTGCAGACAAGAATAACCATGTTTCAGTTTCAGAGAATGGTTCCGGATGGCTTAGCTCCACTTCCTGAAGAGGAAGTGAGAAGCTTTTTTAAGCTAGGTGAACAGGAACTGAATATGAAAGGA
630	CACCCAGCATCAAGAAGCTTTGCAGACAAGAATAACCATGTTTCAGTTTCAGAGAATGGTTCCGGATGGCTTAGCTCCACTTCCTGAAGAGGAAGTGAGAAGCTTTTTTAAGCTAGGTGAACAGGAACTGAATATGAAAGGA
632	CCCAGCATCWAGAAGCTTTGCAGACAAGAATAACCATGTTTCAGTTTCAGAGAATGGTTCCGGATGGCTTAGCTCCACTTCCTGAAGAGGAAGTGAGAAGCTTTTTTAAGCTAGGTGAACAGGAACTGAATATGAAAGGA

The sequencing was successful for seven of the twelve samples (see Table 20). The sequences were from a conserved region in ORF1. Six of the sequences were short, about 140 bp, while sequence 611 was even shorter, 129 bp. The pairwise distances between the sequences were 0%. The overall mean distance between the sequences was 0.4%.

Phylogenetic analysis showed that the Icelandic Parv4 sequences clustered with genotype 1 sequences. The sequences grouped among the genotype 1 subgroup including the original Parv4 isolate AY622943 (see Figure 12).

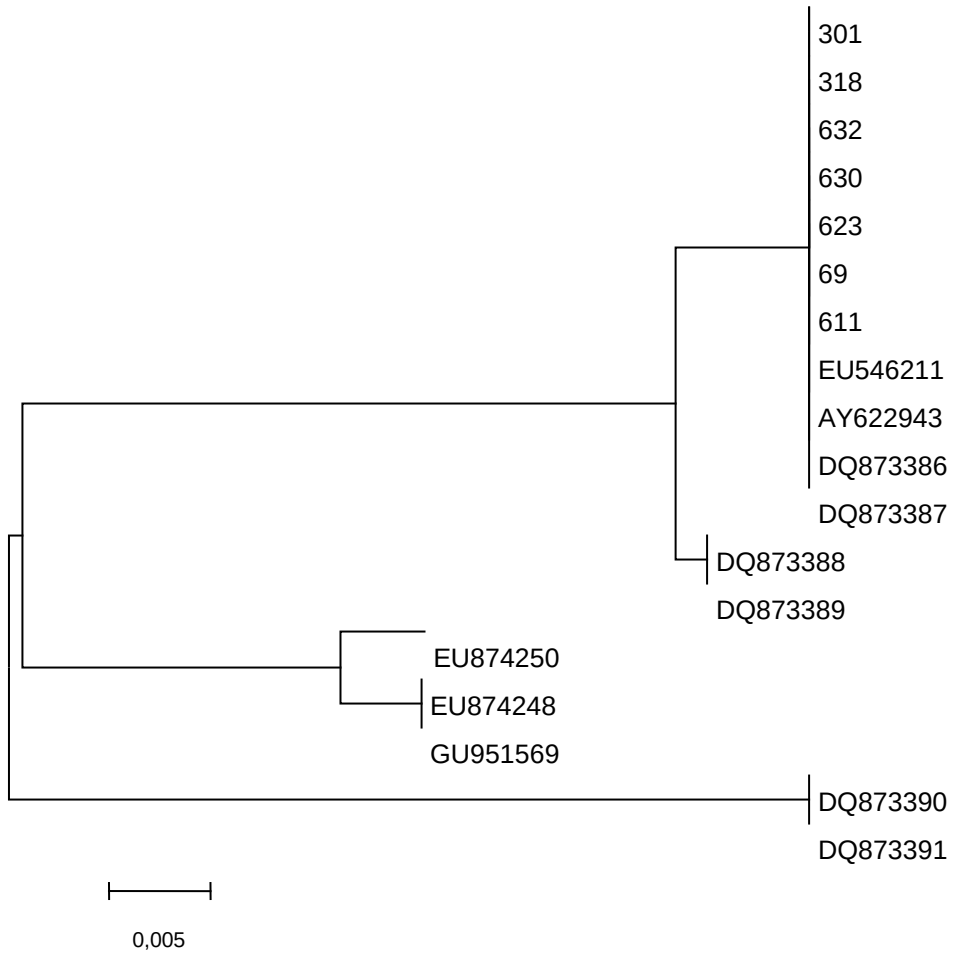
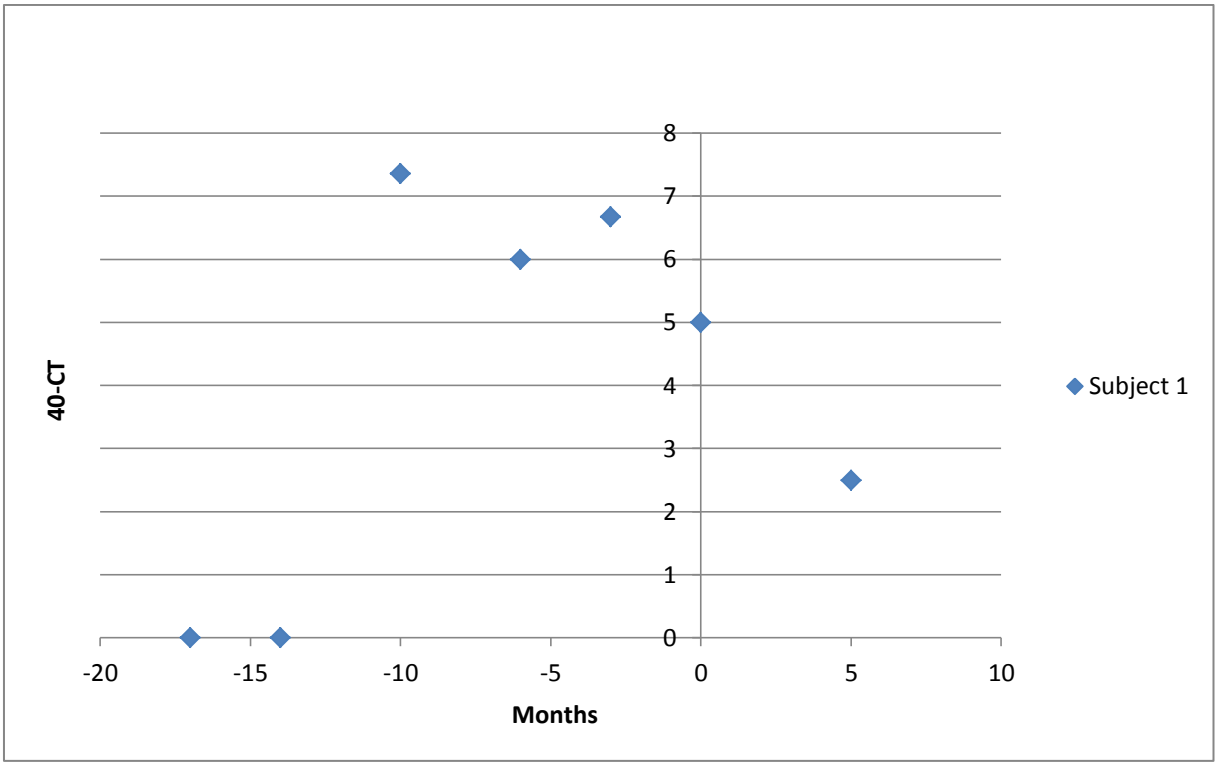


Figure 12 Phylogenetic tree constructed by the Neighbour-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The strains from this study are identified by their sample numbers and the reference strains by their GenBank accession numbers.

4.6 Parv4 in Swedish, longitudinal samples

A Swedish study, conducted at University Hospital MAS in Malmö, found twelve Parv4 DNA positive IVDUs. This study used the same methods as above, screening 249 blood donors and 100 HCV positive IVDUs (unpublished data, Anders Widell, Viktor Dahl). Then, as a part of this study, other available blood samples from eleven Parv4 positive subjects were collected and screened for Parv4. The original samples tested were those with the highest HCV RNA titer to screen samples where the subject was seroconverting. The real-time consensus PCR was used to screen available samples and the CT-values minus 40 versus time were plotted on graphs.


8
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Months
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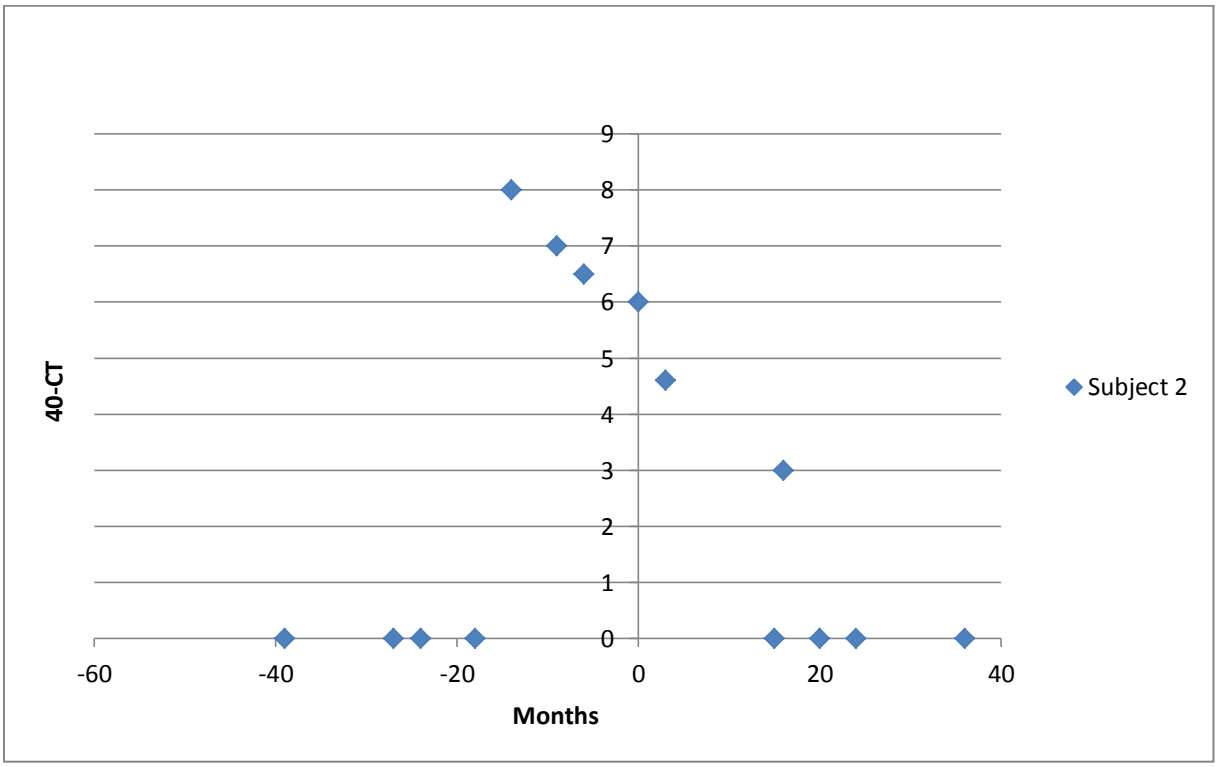
9
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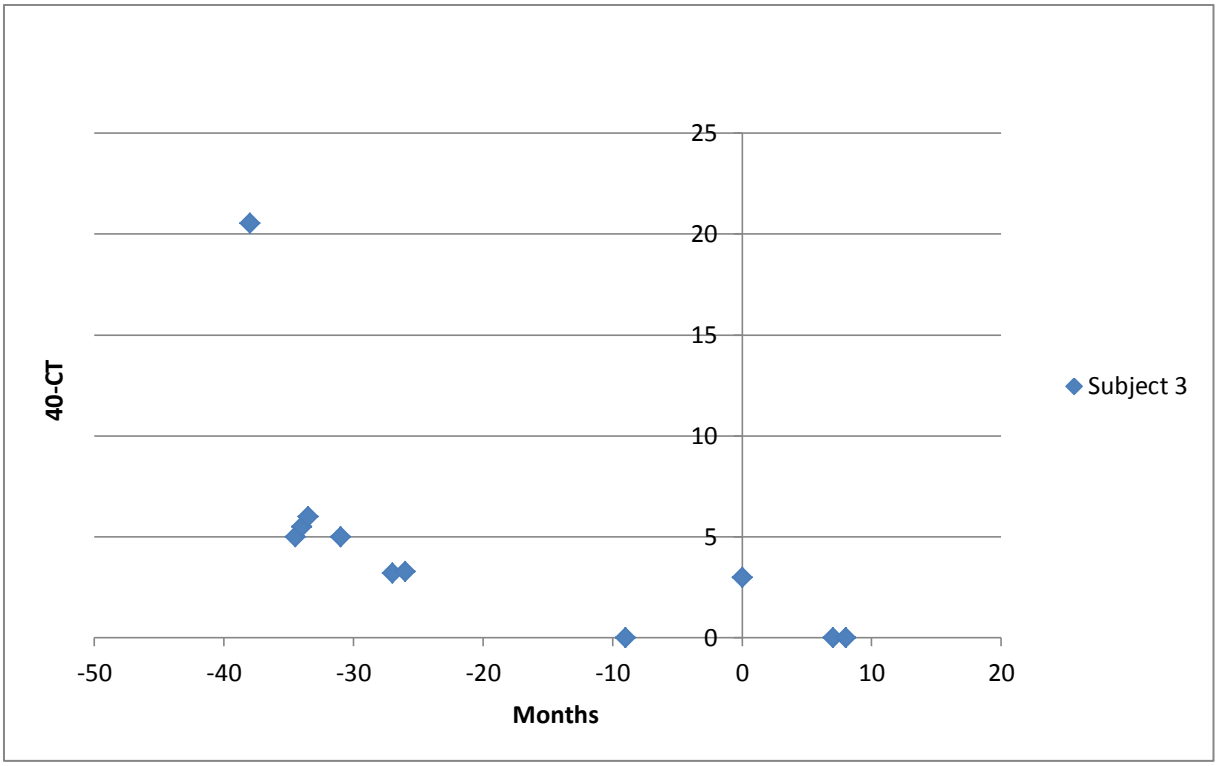
Subject 2


25
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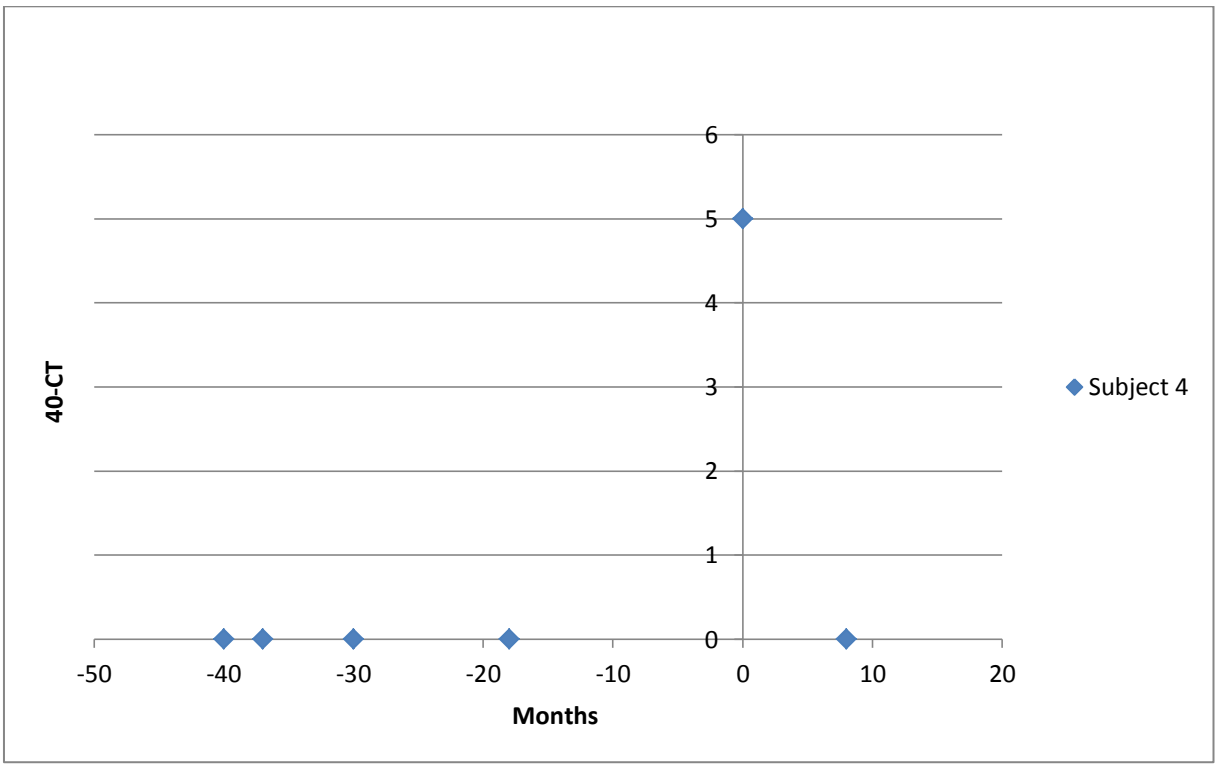
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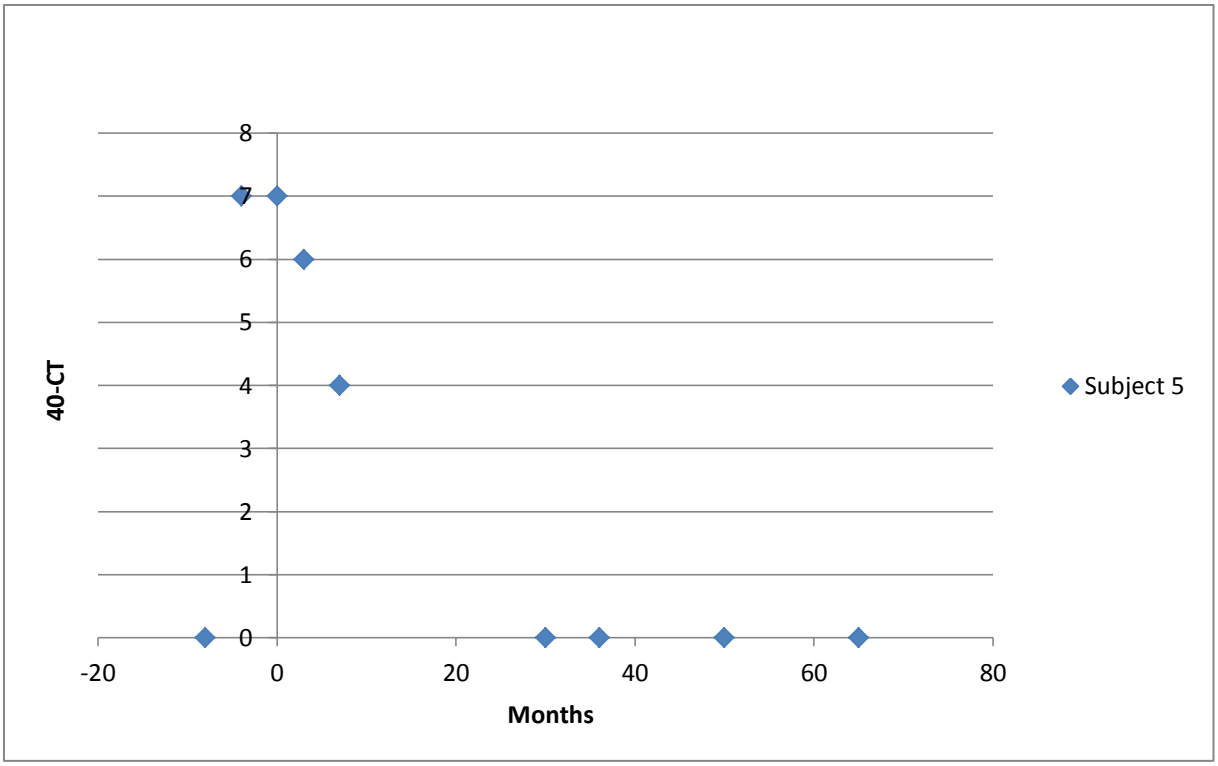
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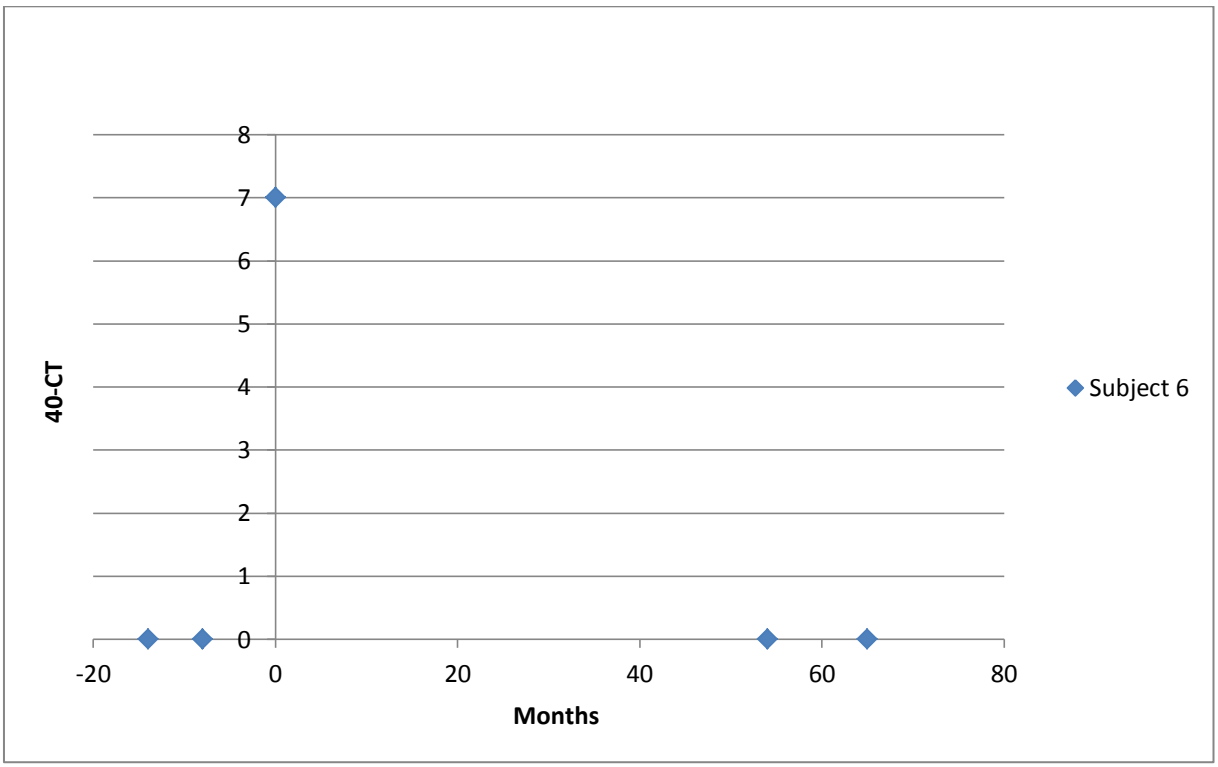
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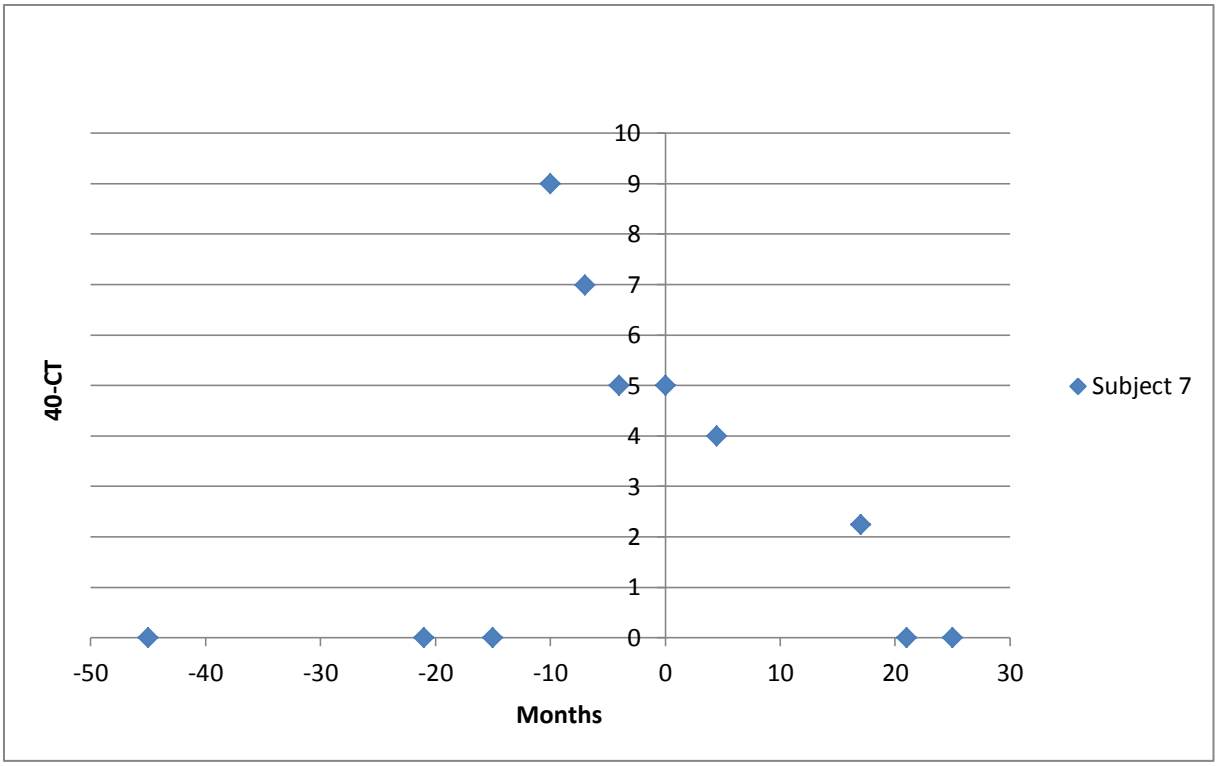
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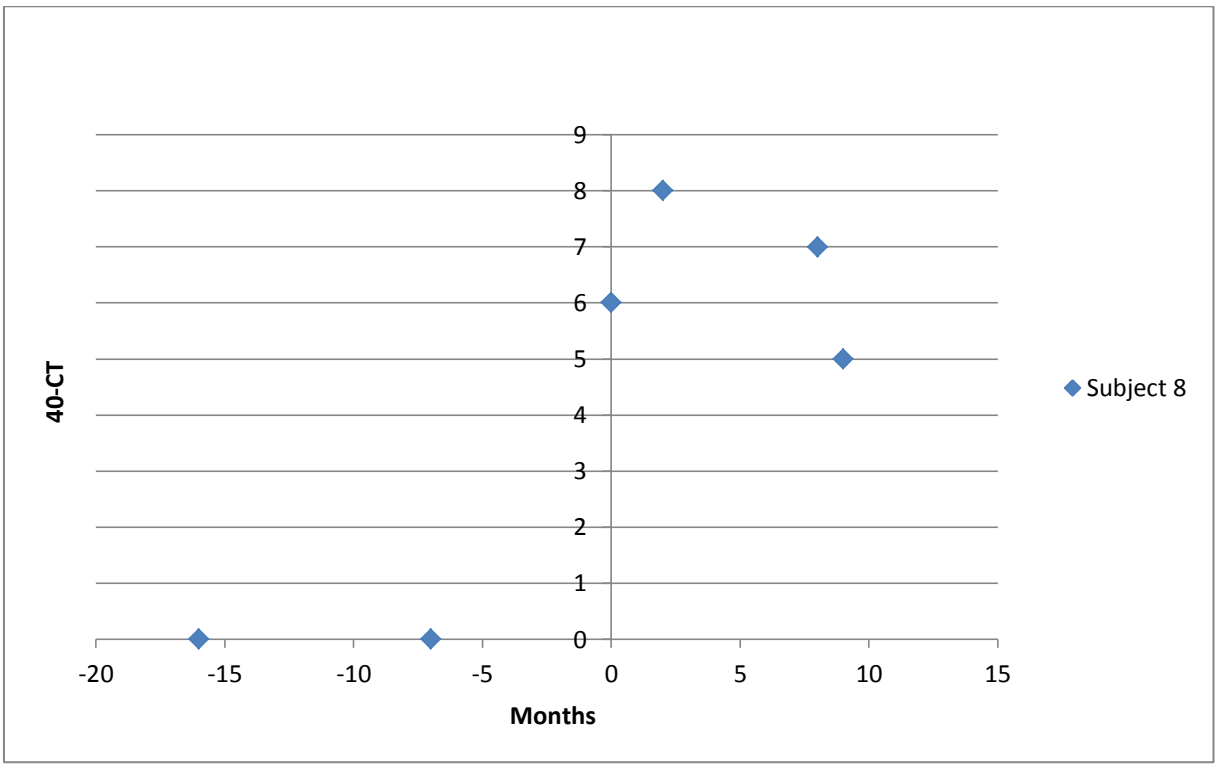
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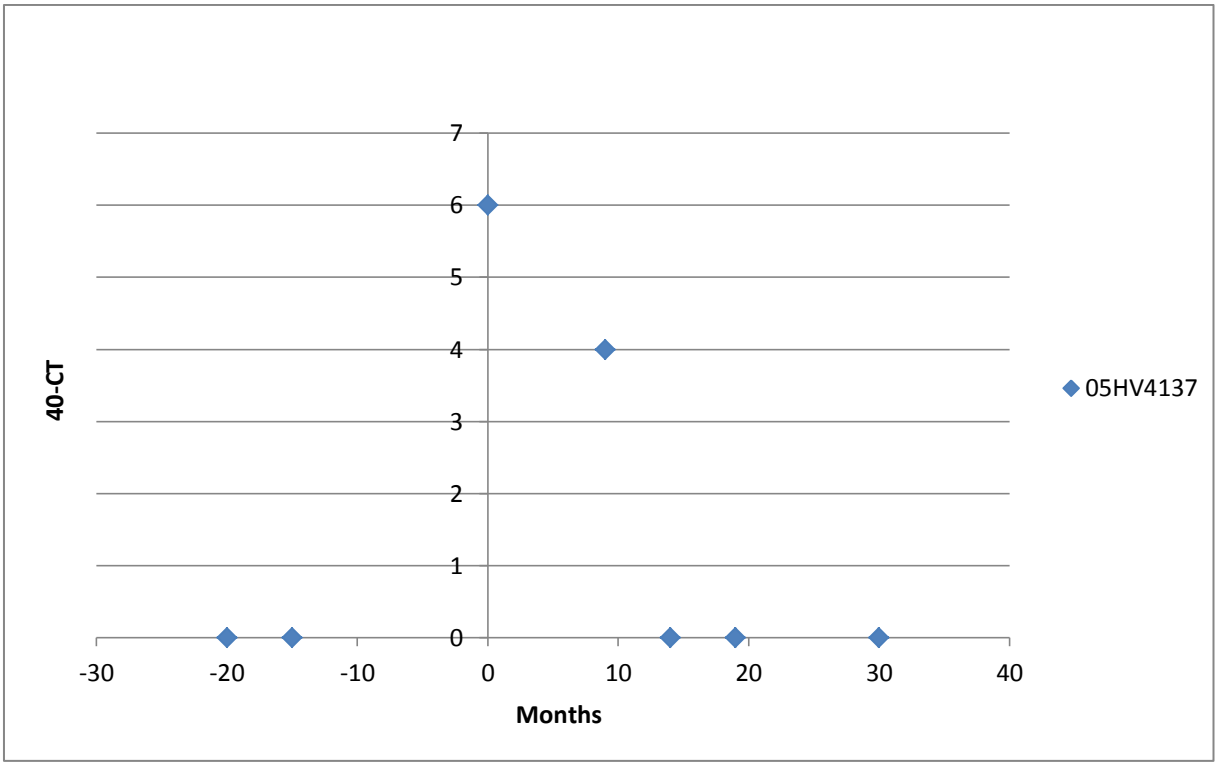
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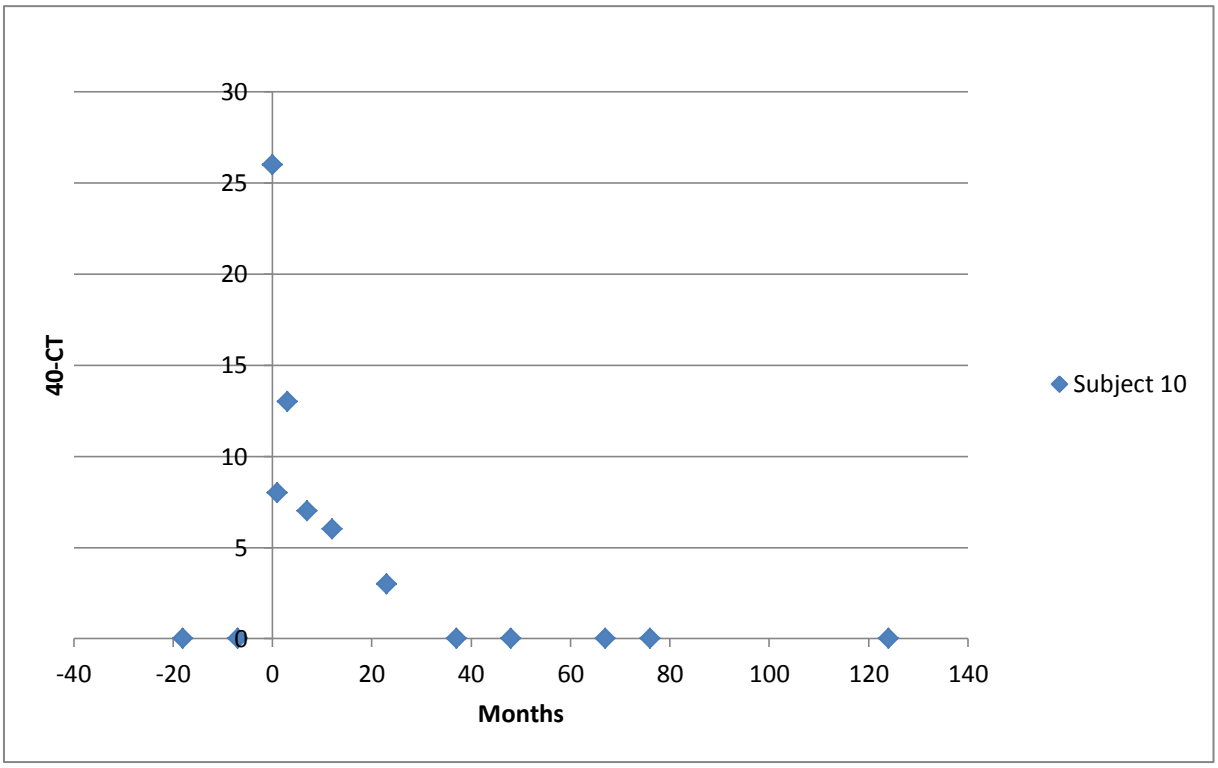
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Subject 10

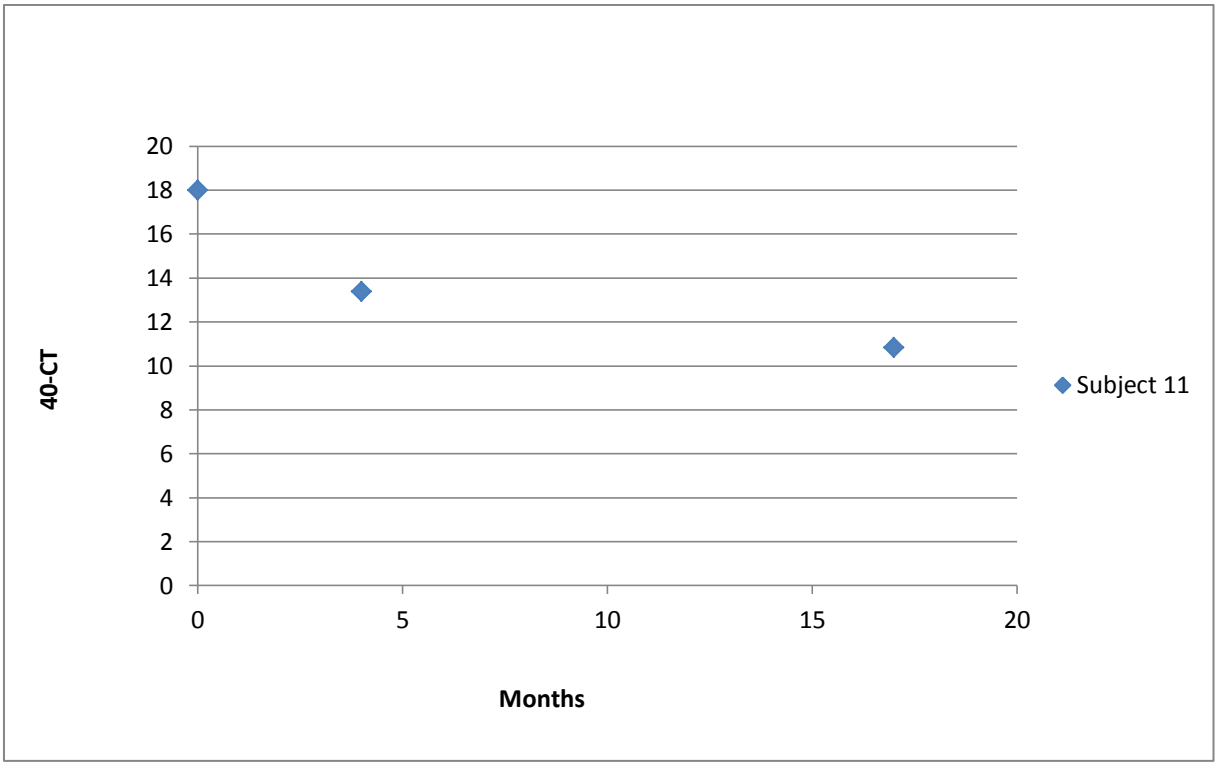

Figure 13 Graphs of longitudinal samples from subject 1-11. Parv4 CT-values on a log 2 scale minus 40 ct on the y-axis and time in months on the x-axis.

The number of samples available for each subject differed greatly and at which time points the samples were collected. The number of available samples from subjects ranged from two additional samples (Subject 11) to 14 additional samples (subject 2) (see Figure 13). Parv4 viremia was detected in subjects for at least for 9 months and up to 38 months, the mean duration was 20 months. Parv4 viremia remained undetectable for up to 101 months (see Figure 13) after the last detection of Parv4 DNA.

5 DISCUSSION

5.1 Parvovirus 4 prevalence in Iceland

The main aim of the study was to estimate the prevalence of Parv4 in Iceland. The virus was only detected in IVDUs, HBV and/or HCV positive as 9.4% of the samples from that group were positive for Parv4 DNA.

The control group was composed of low risk subjects, blood donors and University students. The average age of the control group was significantly lower than the average age of the HBV/HCV infected group as most of the University students were in their early twenties. The average age of the Parv4 positive IVDUs was significantly lower than the average age of the rest of that group. In the early part of the last decade there was a great increase in intravenous drug use in individuals younger than 25 years of age, (78) which possibly explains the lower age of the Parv4 positive. The gender ratios in the control group and hepatitis group were both skewed. The control group was 60% female because of their majority among the University students. The hepatitis group was 63% male which was expected as the majority of IVDUs in Iceland are male (78).

The STD clinic group was composed of anonymous samples from the STD outpatient clinic in Reykjavik. These subjects could have engaged in high-risk behavior, usually sexual and possibly drug use related. Parv4 DNA was not detected in this group. None of these subjects was HCV or HBV infected. Their possible parenteral exposure was evidently not sufficient to expose them to Parv4 infection.

The results from this study are very similar to results on prevalence in Malmö, where no Parv4 DNA was found in 249 blood donors, but 12% of a 100 HCV positive IDVUs tested positive (unpublished data, Anders Widell, Viktor Dahl). The populations in Iceland and Malmö are of similar size but they are different demographically. Iceland is fairly homogenous. In 2009 immigrants made up 7,6% of the population (92) while Malmö has a very large immigrant population with 30% of the inhabitants having been born outside of Sweden (93). These results agree with data from other countries in the northern hemisphere where Parv4 DNA and Parv4 antibodies are rarely found in blood donors or other low risk subjects (16, 49, 52, 53). Some studies have found low frequencies of Parv4 DNA in blood donors, 2% in US blood donors (9) and 4% in Thai blood donors (50). Possibly the difference between these studies, and others that were conducted in Europe, could be explained by differences between these donors and volunteer blood donor populations in Europe.

The Swedish group contained only HCV infected IVDUs whereas the Icelandic group contained HCV and/or HBV infected subjects. In spite of this difference, the frequencies of Parv4 in IVDUs in Malmö and Iceland are similar, 12% and 9,4% respectively. Other studies also show similar levels of Parv4 DNA in blood in high-risk subjects, e.g. 8% of IVDUs in Thailand (50) and 6% of highly exposed, symptomatic individuals in the US (9). Parenteral transmission is the main, if not the exclusive, route of transmission in the Northern Hemisphere. The same applies for Iceland where only parenterally exposed individuals were detected with Parv4 viremia. However, some other route of transmission must exist to account for the high Parv4 prevalence found in Africa.

None of the six smaller patient groups tested yielded any Parv4 DNA. Dialysis patients are at risk for healthcare associated infections due to parenteral exposure (94) however no Parv4 DNA samples were detected in the dialysis patient group. While this study failed to discover a link between Parv4 and neurological symptoms, recently Parv4 has been linked to neurological symptoms in children (67). Neither the serum samples from patients with respiratory symptoms nor the NPS samples from children were found to be Parv4 positive. This is supported by two other studies that have also tested for Parv4 in respiratory samples (45, 57) however Parv4 has been detected at low frequencies in nasal samples from children in Ghana (62). The six patient groups were small, so the result of Parv4 not being detected in those groups does not preclude a connection with Parv4.

5.2 Parvovirus 4 persistence

Parv4 DNA was not detected in the cancer group composed of immunosuppressed patients. Parv4 has been linked with HIV infection (16, 49, 60) raising the question of Parv4 association with immunodeficiency. However, this could be explained by parenteral exposure and Parv4 viral replication does not seem to increase with a greater degree of immunosuppression (16).

This study found that Parv4 can persist in blood for up to 38 months. In cases where samples were available for more than 3 years, Parv4 DNA always became undetectable at some point. This is because either the immune system managed to clear out Parv4 viremia or the viral load was too low to be detected by the PCR assay. Simmonds et al. 2011 found that CD8+ T cell responses against Parv4 persisted for up to 3 years which could be attributed to persistent Parv4 viremia (73). Possibly, Parv4 is persistent in both blood and tissue.

5.3 Phylogenetic analysis

All seven sequences were identified as genotype 1 and were most similar to the subgroup containing the original Parv4 isolate. The sequences detected were short but the region amplified differs between Parv4 genotypes. All the Icelandic Parv4 positive subjects were born after 1969, which fits with the temporal succession of Parv4 genotype 1 replacing genotype 2 around the 1960's, and that genotype 1 is the Parv4 genotype currently circulating in the Northern Hemisphere. Intravenous drug use in Iceland probably did not start until the 1980's so it is possible that genotype 2 was never introduced there.

In conclusion, this study has shown that Parv4 is present among IVDUs in Iceland, suggesting a parenteral transmission route for Parv4 in Iceland. These results are comparable to observations on Parv4 epidemiology from other European countries. Longditutional data from this study suggests Parv4 viremia can persist for up to 3 years. This study adds to the accumulating knowledge of the epidemiology and biology of a newly discovered human virus.

6 REFERENCES

1. Jones MS, Kapoor A, Lukashov VV, Simmonds P, Hecht F, Delwart E. New DNA viruses identified in patients with acute viral infection syndrome. J Virol. 2005;79(13):8230-6.
2. King AMQ, Lefkowitz E, Adams MJ, Carstens EB. Virus taxonomy: ninth report of the International Committee on Taxonomy of Viruses: Elsevier; 2011.
3. Fields BN, Knipe DM, Howley PM. Fields' virology: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2007. 3117 p.
4. Brown KE. The expanding range of parvoviruses which infect humans. Rev Med Virol. 2010;20(4):231-44.
5. Brown KE, Simmonds P. Parvoviruses and blood transfusion. Transfusion. 2007;47(10):1745-50.
6. Tattersall P, Bergoin, M., Bloom, M.E., et al. Virus taxonomy: classification and nomenclature of viruses: eighth report of the International Committee on the Taxonomy of Viruses. Fauquet CM, Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (eds). editor. New York: Academic Press; 2005.
7. Lukashov VV, Goudsmit J. Evolutionary relationships among parvoviruses: virus-host coevolution among autonomous primate parvoviruses and links between adeno-associated and avian parvoviruses. J Virol. 2001;75(6):2729-40.
8. Bando H, Kusuda J, Gojobori T, Maruyama T, Kawase S. Organization and nucleotide sequence of a densovirus genome imply a host-dependent evolution of the parvoviruses. J Virol. 1987;61(2):553-60.
9. Fryer JF, Delwart E, Hecht FM, Bernardin F, Jones MS, Shah N, Baylis SA. Frequent detection of the parvoviruses, PARV4 and PARV5, in plasma from blood donors and symptomatic individuals. Transfusion. 2007;47(6):1054-61.
10. Fryer JF, Delwart E, Bernardin F, Tuke PW, Lukashov VV, Baylis SA. Analysis of two human parvovirus PARV4 genotypes identified in human plasma for fractionation. J Gen Virol. 2007;88(Pt 8):2162-7.
11. Qiu J, Cheng F, Burger LR, Pintel D. The transcription profile of Aleutian mink disease virus in CRFK cells is generated by alternative processing of pre-mRNAs produced from a single promoter. J Virol. 2006;80(2):654-62.
12. Heegaard ED, Brown KE. Human parvovirus B19. Clin Microbiol Rev. 2002;15(3):485-505.
13. Zhi N, Mills IP, Lu J, Wong S, Filippone C, Brown KE. Molecular and functional analyses of a human parvovirus B19 infectious clone demonstrates essential roles for NS1, VP1, and the 11-kilodalton protein in virus replication and infectivity. J Virol. 2006;80(12):5941-50.
14. Cossart YE, Field AM, Cant B, Widdows D. Parvovirus-like particles in human sera. Lancet. 1975;1(7898):72-3.
15. Norja P, Hokynar K, Aaltonen LM, Chen R, Ranki A, Partio EK, Kiviluoto O, Davidkin I, Leivo T, Eis-Hubinger AM, Schneider B, Fischer HP, Tolba R, Vapalahti O, Vaheri A, Soderlund-Venermo M, Hedman K. Bioportfolio: lifelong persistence of variant and prototypic erythrovirus DNA genomes in human tissue. Proc Natl Acad Sci U S A. 2006;103(19):7450-3.
16. Manning A, Willey SJ, Bell JE, Simmonds P. Comparison of tissue distribution, persistence, and molecular epidemiology of parvovirus B19 and novel human parvoviruses PARV4 and human bocavirus. J Infect Dis. 2007;195(9):1345-52.
17. Ragni MV, Sherman KE, Jordan JA. Viral pathogens. Haemophilia. 2010;16 Suppl 5:40-6.

18. Boschetti N, Niederhauser I, Kempf C, Stuhler A, Lower J, Blumel J. Different susceptibility of B19 virus and mice minute virus to low pH treatment. Transfusion. 2004;44(7):1079-86.
19. Blumel J, Schmidt I, Willkommen H, Lower J. Inactivation of parvovirus B19 during pasteurization of human serum albumin. Transfusion. 2002;42(8):1011-8.
20. Mani B, Gerber M, Lieby P, Boschetti N, Kempf C, Ros C. Molecular mechanism underlying B19 virus inactivation and comparison to other parvoviruses. Transfusion. 2007;47(10):1765-74.
21. Young NS, Brown KE. Parvovirus B19. N Engl J Med. 2004;350(6):586-97.
22. Blacklow NR, Hoggan MD, Rowe WP. Isolation of adenovirus-associated viruses from man. Proc Natl Acad Sci U S A. 1967;58(4):1410-5.
23. Calcedo R, Vandenberghe LH, Gao G, Lin J, Wilson JM. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. J Infect Dis. 2009;199(3):381-90.
24. Gao G, Vandenberghe LH, Alvira MR, Lu Y, Calcedo R, Zhou X, Wilson JM. Clades of Adeno-associated viruses are widely disseminated in human tissues. J Virol. 2004;78(12):6381-8.
25. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A. 2005;102(36):12891-6.
26. Allander T, Jartti T, Gupta S, Niesters HG, Lehtinen P, Osterback R, Vuorinen T, Waris M, Bjerkner A, Tiveljung-Lindell A, van den Hoogen BG, Hyypia T, Ruuskanen O. Human bocavirus and acute wheezing in children. Clin Infect Dis. 2007;44(7):904-10.
27. Kantola K, Hedman L, Allander T, Jartti T, Lehtinen P, Ruuskanen O, Hedman K, Soderlund-Venermo M. Serodiagnosis of human bocavirus infection. Clin Infect Dis. 2008;46(4):540-6.
28. Lindner J, Modrow S. Human bocavirus--a novel parvovirus to infect humans. Intervirology. 2008;51(2):116-22.
29. Cecchini S, Negrete A, Virag T, Graham BS, Cohen JI, Kotin RM. Evidence of prior exposure to human bocavirus as determined by a retrospective serological study of 404 serum samples from adults in the United States. Clin Vaccine Immunol. 2009;16(5):597-604.
30. Sharp CP, LeBreton M, Kantola K, Nana A, Diffo Jle D, Djoko CF, Tamoufe U, Kiyang JA, Babila TG, Ngole EM, Pybus OG, Delwart E, Delaporte E, Peeters M, Soderlund-Venermo M, Hedman K, Wolfe ND, Simmonds P. Widespread infection with homologues of human parvoviruses B19, PARV4, and human bocavirus of chimpanzees and gorillas in the wild. J Virol. 2010;84(19):10289-96.
31. Allander T, Emerson SU, Engle RE, Purcell RH, Bukh J. A virus discovery method incorporating DNase treatment and its application to the identification of two bovine parvovirus species. Proc Natl Acad Sci U S A. 2001;98(20):11609-14.
32. Servant A, Laperche S, Lallemand F, Marinho V, De Saint Maur G, Meritet JF, Garbarg-Chenon A. Genetic diversity within human erythroviruses: identification of three genotypes. J Virol. 2002;76(18):9124-34.
33. Fryer JF, Kapoor A, Minor PD, Delwart E, Baylis SA. Novel parvovirus and related variant in human plasma. Emerg Infect Dis. 2006;12(1):151-4.
34. Simmonds P, Douglas J, Bestetti G, Longhi E, Antinori S, Parravicini C, Corbellino M. A third genotype of the human parvovirus PARV4 in sub-Saharan Africa. J Gen Virol. 2008;89(Pt 9):2299-302.
35. Tuke PW, Parry RP, Appleton H. Parvovirus PARV4 visualization and detection. J Gen Virol. 2010;91(Pt 2):541-4.

36. Zadori Z, Szelei J, Lacoste MC, Li Y, Gariepy S, Raymond P, Allaire M, Nabi IR, Tijssen P. A viral phospholipase A2 is required for parvovirus infectivity. Dev Cell. 2001;1(2):291-302.
37. Hijikata M, Abe K, Win KM, Shimizu YK, Keicho N, Yoshikura H. Identification of new parvovirus DNA sequence in swine sera from Myanmar. Jpn J Infect Dis. 2001;54(6):244-5.
38. Besselsen DG, Gibson SV, Besch-Williford CL, Purdy GA, Knowles RL, Wagner JE, Pintel DJ, Franklin CL, Hook RR, Jr., Riley LK. Natural and experimentally induced infection of Syrian hamsters with a newly recognized parvovirus. Lab Anim Sci. 1999;49(3):308-12.
39. Lau SK, Woo PC, Tse H, Fu CT, Au WK, Chen XC, Tsoi HW, Tsang TH, Chan JS, Tsang DN, Li KS, Tse CW, Ng TK, Tsang OT, Zheng BJ, Tam S, Chan KH, Zhou B, Yuen KY. Identification of novel porcine and bovine parvoviruses closely related to human parvovirus 4. J Gen Virol. 2008;89(Pt 8):1840-8.
40. Szelei J, Liu K, Li Y, Fernandes S, Tijssen P. Parvovirus 4-like virus in blood products. Emerg Infect Dis. 2010;16(3):561-4.
41. Canuti M, Eis-Huebinger AM, Deijs M, de Vries M, Drexler JF, Oppong SK, Muller MA, Klose SM, Wellinghausen N, Cottontail VM, Kalko EK, Drosten C, van der Hoek L. Two novel parvoviruses in frugivorous New and Old World bats. PLoS One. 2011;6(12):e29140.
42. Shackelton LA, Holmes EC. Phylogenetic evidence for the rapid evolution of human B19 erythrovirus. J Virol. 2006;80(7):3666-9.
43. Shackelton LA, Parrish CR, Truyen U, Holmes EC. High rate of viral evolution associated with the emergence of carnivore parvovirus. Proc Natl Acad Sci U S A. 2005;102(2):379-84.
44. Adlhoch C, Kaiser M, Loewa A, Ulrich M, Forbrig C, Adjogoua EV, Akoua-Koffi C, Couacy-Hymann E, Leendertz SA, Rietschel W, Boesch C, Ellerbrok H, Schneider BS, Leendertz FH. Diversity of parvovirus 4-like viruses in humans, chimpanzees, and monkeys in hunter-prey relationships. Emerg Infect Dis. 2012;18(5):859-62.
45. Botto S, Bergallo M, Sidoti F, Terlizzi ME, Astegiano S, Ponti R, Costa C, Cavallo R. Detection of PARV4, genotypes 1 and 2, in healthy and pathological clinical specimens. New Microbiol. 2009;32(2):189-92.
46. Bergallo M, Costa C, Sidoti F, Novelli M, Ponti R, Castagnoli C, Merlino C, Bernengo MG, Cavallo R. Variants of parvovirus B19: bioinformatical evaluation of nested PCR assays. Intervirology. 2008;51(2):75-80.
47. Schneider B, Fryer JF, Reber U, Fischer HP, Tolba RH, Baylis SA, Eis-Hubinger AM. Persistence of novel human parvovirus PARV4 in liver tissue of adults. J Med Virol. 2008;80(2):345-51.
48. Corcioli F, Zakrzewska K, Fanci R, De Giorgi V, Innocenti M, Rotellini M, Di Lollo S, Azzi A. Human parvovirus PARV4 DNA in tissues from adult individuals: a comparison with human parvovirus B19 (B19V). Virol J. 2010;7:272.
49. Simmonds P, Manning A, Kenneil R, Carnie FW, Bell JE. Parenteral transmission of the novel human parvovirus PARV4. Emerg Infect Dis. 2007;13(9):1386-8.
50. Lurcharchaiwong W, Chieochansin T, Payungporn S, Theamboonlers A, Poovorawan Y. Parvovirus 4 (PARV4) in serum of intravenous drug users and blood donors. Infection. 2008;36(5):488-91.
51. Yu X, Zhang J, Hong L, Wang J, Yuan Z, Zhang X, Ghildyal R. High prevalence of human parvovirus 4 infection in HBV and HCV infected individuals in shanghai. PLoS One. 2012;7(1):e29474.
52. Sharp CP, Lail A, Donfield S, Simmons R, Leen C, Klenerman P, Delwart E, Gomperts ED, Simmonds P. High frequencies of exposure to the novel human parvovirus

PARV4 in hemophiliacs and injection drug users, as detected by a serological assay for PARV4 antibodies. J Infect Dis. 2009;200(7):1119-25.
53. Lahtinen A, Kivela P, Hedman L, Kumar A, Kantele A, Lappalainen M, Liitsola K, Ristola M, Delwart E, Sharp C, Simmonds P, Soderlund-Venermo M, Hedman K. Serodiagnosis of primary infections with human parvovirus 4, Finland. Emerg Infect Dis. 2011;17(1):79-82.
54. Simmons R, Sharp C, McClure CP, Rohrbach J, Kovari H, Frangou E, Simmonds P, Irving W, Rauch A, Bowness P, Klenerman P. Parvovirus 4 Infection and Clinical Outcome in High-Risk Populations. J Infect Dis. 2012;205(12):1816-20.
55. Panning M, Kobbe R, Vollbach S, Drexler JF, Adjei S, Adjei O, Drosten C, May J, Eis-Hubinger AM. Novel human parvovirus 4 genotype 3 in infants, Ghana. Emerg Infect Dis. 2010;16(7):1143-6.
56. Vallerini D, Barozzi P, Quadrelli C, Bosco R, Potenza L, Riva G, Gregorini G, Sandrini S, Tironi A, Montagnani G, De Palma M, Torelli G, Delwart E, Luppi M. Parvoviruses in blood donors and transplant patients, Italy. Emerg Infect Dis. 2008;14(1):185-6.
57. Manning A, Russell V, Eastick K, Leadbetter GH, Hallam N, Templeton K, Simmonds P. Epidemiological profile and clinical associations of human bocavirus and other human parvoviruses. J Infect Dis. 2006;194(9):1283-90.
58. Hagan H, Des Jarlais DC. HIV and HCV infection among injecting drug users. Mt Sinai J Med. 2000;67(5-6):423-8.
59. Gerberding JL. Management of occupational exposures to blood-borne viruses. N Engl J Med. 1995;332(7):444-51.
60. Sharp CP, Vermeulen M, Nebie Y, Djoko CF, LeBreton M, Tamoufe U, Rimoin AW, Kayembe PK, Carr JK, Servant-Delmas A, Laperche S, Harrison GL, Pybus OG, Delwart E, Wolfe ND, Saville A, Lefrere JJ, Simmonds P. Changing epidemiology of human parvovirus 4 infection in sub-Saharan Africa. Emerg Infect Dis. 2010;16(10):1605-7.
61. Yang SJ, Hung CC, Chang SY, Lee KL, Chen MY. Immunoglobulin G and M antibodies to human parvovirus 4 (PARV4) are frequently detected in patients with HIV-1 infection. J Clin Virol. 2011;51(1):64-7.
62. Drexler JF, Reber U, Muth D, Herzog P, Annan A, Ebach F, Sarpong N, Acquah S, Adlkofer J, Adu-Sarkodie Y, Panning M, Tannich E, May J, Drosten C, Eis-Hubinger AM. Human parvovirus 4 in nasal and fecal specimens from children, ghana. Emerg Infect Dis. 2012;18(10):1650-3.
63. Chen MY, Yang SJ, Hung CC. Placental transmission of human parvovirus 4 in newborns with hydrops, Taiwan. Emerg Infect Dis. 2011;17(10):1954-6.
64. Longhi E, Bestetti G, Acquaviva V, Foschi A, Piolini R, Meroni L, Magni C, Antinori S, Parravicini C, Corbellino M. Human parvovirus 4 in the bone marrow of Italian patients with AIDS. AIDS. 2007;21(11):1481-3.
65. Fryer JF, Hubbard AR, Baylis SA. Human parvovirus PARV4 in clotting factor VIII concentrates. Vox Sang. 2007;93(4):341-7.
66. Schneider B, Fryer JF, Oldenburg J, Brackmann HH, Baylis SA, Eis-Hubinger AM. Frequency of contamination of coagulation factor concentrates with novel human parvovirus PARV4. Haemophilia. 2008;14(5):978-86.
67. Benjamin LA, Lewthwaite P, Vasanthapuram R, Zhao G, Sharp C, Simmonds P, Wang D, Solomon T. Human parvovirus 4 as potential cause of encephalitis in children, India. Emerg Infect Dis. 2011;17(8):1484-7.
68. Ma YY, Guo Y, Zhao X, Wang Z, Lv MM, Yan QP, Zhang JG. Human parvovirus PARV4 in plasma pools of Chinese origin. Vox Sang. 2012.

69. Doyle S, Corcoran A. The immune response to parvovirus B19 exposure in previously seronegative and seropositive individuals. J Infect Dis. 2006;194(2):154-8.
70. Kreil TR, Wieser A, Berting A, Spruth M, Medek C, Polsler G, Gaida T, Hammerle T, Teschner W, Schwarz HP, Barrett PN. Removal of small nonenveloped viruses by antibody-enhanced nanofiltration during the manufacture of plasma derivatives. Transfusion. 2006;46(7):1143-51.
71. Sharp CP, Lail A, Donfield S, Gomperts ED, Simmonds P. Virologic and clinical features of primary infection with human parvovirus 4 in subjects with hemophilia: frequent transmission by virally inactivated clotting factor concentrates. Transfusion. 2011.
72. Parrish CR. Emergence, natural history, and variation of canine, mink, and feline parvoviruses. Adv Virus Res. 1990;38:403-50.
73. Simmons R, Sharp C, Sims S, Kloverpris H, Goulder P, Simmonds P, Bowness P, Klenerman P. High frequency, sustained T cell responses to PARV4 suggest viral persistence in vivo. J Infect Dis. 2011;203(10):1378-87.
74. Gillespie GM, Wills MR, Appay V, O'Callaghan C, Murphy M, Smith N, Sissons P, Rowland-Jones S, Bell JI, Moss PA. Functional heterogeneity and high frequencies of cytomegalovirus-specific CD8(+) T lymphocytes in healthy seropositive donors. J Virol. 2000;74(17):8140-50.
75. Callan MF. The evolution of antigen-specific CD8+ T cell responses after natural primary infection of humans with Epstein-Barr virus. Viral Immunol. 2003;16(1):3-16.
76. Isa A, Kasprowicz V, Norbeck O, Loughry A, Jeffery K, Broliden K, Klenerman P, Tolfvenstam T, Bowness P. Prolonged activation of virus-specific CD8+T cells after acute B19 infection. PLoS Med. 2005;2(12):e343.
77. Lefrere JJ, Servant-Delmas A, Candotti D, Mariotti M, Thomas I, Brossard Y, Lefrere F, Girot R, Allain JP, Laperche S. Persistent B19 infection in immunocompetent individuals: implications for transfusion safety. Blood. 2005;106(8):2890-5.
78. SÁÁ, Annual Report 2007-2011. 2011.
79. Mathers BM, Degenhardt L, Phillips B, Wiessing L, Hickman M, Strathdee SA, Wodak A, Panda S, Tyndall M, Toufik A, Mattick RP. Global epidemiology of injecting drug use and HIV among people who inject drugs: a systematic review. Lancet. 2008;372(9651):1733-45.
80. Garfein RS, Vlahov D, Galai N, Doherty MC, Nelson KE. Viral infections in short-term injection drug users: the prevalence of the hepatitis C, hepatitis B, human immunodeficiency, and human T-lymphotropic viruses. Am J Public Health. 1996;86(5):655-61.
81. Van Ameijden EJ, Van den Hoek JA, Mientjes GH, Coutinho RA. A longitudinal study on the incidence and transmission patterns of HIV, HBV and HCV infection among drug users in Amsterdam. Eur J Epidemiol. 1993;9(3):255-62.
82. Alter MJ, Kruszon-Moran D, Nainan OV, McQuillan GM, Gao F, Moyer LA, Kaslow RA, Margolis HS. The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. N Engl J Med. 1999;341(8):556-62.
83. WHO. Hepatitis C fact sheet. [cited 2012 08.01]; Available from: http://www.who.int/mediacentre/factsheets/fs164/en/index.html.
84. Love A, Stanzeit B. Hepatitis C virus infection in Iceland: a recently introduced blood-borne disease. Epidemiol Infect. 1994;113(3):529-36.
85. Nelson PK, Mathers BM, Cowie B, Hagan H, Des Jarlais D, Horyniak D, Degenhardt L. Global epidemiology of hepatitis B and hepatitis C in people who inject drugs: results of systematic reviews. Lancet. 2011;378(9791):571-83.
86. WHO. Hepatitis B fact sheet. [cited 2012 07.01]; Available from: http://www.who.int/mediacentre/factsheets/fs204/en/.

87. Lai CL, Ratziu V, Yuen MF, Poynard T. Viral hepatitis B. Lancet. 2003;362(9401):2089-94.
88. Jónsdóttir G, Briem H, Blöndal, Pálsson G, Guðnason, Ólafsson S. Viral hepatitis B and C among immigrants in Iceland. Læknablaðið. 2006;92:669-73.
89. Högnadottir H, Tyrfingsson, Löve A. Greining lifrarbólguveiru B: Faraldur meðal fíkniefnaneytenda. Læknablaðið. 1993;79:227-31.
90. Briem H, Atladóttir ÁS, Sigmundsdóttir G, Hauksdóttir S, Guðnason. HIV infection and hepatitis B in Iceland in 2007. EPI - ICE [Internet]. 2008; 4(2). Available from: http://www.landlaeknir.is/servlet/file/store93/item15742/version6/feb%202008.pdf.
91. WHO. HIV/AIDS Fact Sheet. WHO; [updated November 2011; cited 2012 19.01.12]; Available from: http://www.who.int/mediacentre/factsheets/fs360/en/index.html.
92. Statistics Iceland: Foreign citizens 1950-2010. [cited 2011 02.11.]; Available from: http://statice.is/Statistics/Population/Citizenship-and-country-of-birth.
93. Malmö Stad Statistik. [cited 2011 02.11]; Available from: http://malmo.se/Kommun--politik/Om-oss/Statistik.html.
94. CDC. Dialysis safety. [cited 2013 14.05.]; Available from: http://www.cdc.gov/dialysis/clinician/index.html.