Journal of General Virology (2008), **89**, 1840–1848 **DOI** 10.1099/vir.0.2008/000380-0

Identification of novel porcine and bovine parvoviruses closely related to human parvovirus 4

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Human parvovirus 4 (PARV4), a recently discovered parvovirus found exclusively in human plasma and liver tissue, was considered phylogenetically distinct from other parvoviruses. Here, we report the discovery of two novel parvoviruses closely related to PARV4, porcine hokovirus (PHoV) and bovine hokovirus (BHoV), from porcine and bovine samples in Hong Kong. Their nearly full-length sequences were also analysed. PARV4-like viruses were detected by PCR among 44.4 % (148/333) of porcine samples (including lymph nodes, liver, serum, nasopharyngeal and faecal samples), 13 % (4/32) of bovine spleen samples and 2 % (7/362) of human serum samples that were sent for human immunodeficiency virus and hepatitis C virus antibody tests. Three distinct parvoviruses were identified, including two novel parvoviruses, PHoV and BHoV, from porcine and bovine samples and PARV4 from humans, respectively. Analysis of genome sequences from seven PHoV strains, from three BHoV strains and from one PARV4 strain showed that the two animal parvoviruses were most similar to PARV4 with 61.5–63 % nt identities and, together with PARV4 (HHoV), formed a distinct cluster within the family *Parvoviridae*. The three parvoviruses also differed from other parvoviruses by their relatively large predicted VP1 protein and the presence of a small unique conserved putative protein. Based on these results, we propose a separate genus, *Hokovirus*, to describe these three parvoviruses. The co-detection of porcine reproductive and respiratory syndrome virus, the agent associated with the recent 'high fever' disease outbreaks in pigs in China, from our porcine samples warrants further investigation.

Received 14 January 2008
Accepted 7 April 2008

INTRODUCTION

Many emerging infectious diseases are zoonoses that cause epidemics in humans after overcoming the inter-species barrier. The severe acute respiratory syndrome coronavirus

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The GenBank/EMBL/DDBJ sequence accession numbers for the sequences reported in this study are EU200667–EU200677.

Supplementary tables are available with the online version of this paper.

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(SARS-CoV) responsible for the SARS epidemic in 2003 originated in wild animals, with palm civets being the amplification host and horseshoe bats identified as natural reservoirs for SARS-CoV-like viruses (Lau *et al.*, 2005; Li *et al.*, 2005). Although no animal origins have been identified for other human coronaviruses, an astonishing diversity of novel coronaviruses has been identified in other bat species, suggesting that these animals play an important role in the evolution of coronaviruses (Fouchier *et al.*, 2004; Lau *et al.*, 2007b; Poon *et al.*, 2005; Tang *et al.*, 2006; van der Hoek *et al.*, 2004; Woo *et al.*, 2005, 2006). Identifying possible animal origins or counterparts of emerging viruses is important in understanding their epidemiology, evolution and potential for re-emergence.

Parvoviruses are widespread pathogens that cause a wide range of diseases in animals. They are small, non-enveloped viruses with a single-stranded DNA genome of about 5 kb that contains two open reading frames (ORFs), coding for non-structural and capsid proteins. Based on their host range, the family *Parvoviridae* was classified into two subfamilies: *Parvovirinae*, infecting vertebrates, and *Densovirinae*, infecting insects and other arthropods (van Regenmortel *et al.*, 2000). Although further classification into various genera has not been very well defined, several genera, including *Dependovirus*, *Bocavirus*, *Erythrovirus* and *Parvovirus*, have been proposed within the subfamily *Parvovirinae*. Before the discovery of several novel human parvoviruses in the past few years, only two members of the family *Parvoviridae*, parvovirus B19, belonging to the genus *Erythrovirus*, and adeno-associated viruses (AAV), belonging to the genus *Dependovirus*, were known to infect humans. Variants of parvovirus, B19, V9- and A6-related strains, were subsequently identified as two other genotypes of human erythrovirus (Hokynar *et al.*, 2002; Nguyen *et al.*, 1999, 2002; Servant *et al.*, 2002). In 2005, a new parvovirus, human bocavirus (HBoV) belonging to the genus *Bocavirus*, was identified and subsequently found to be associated with respiratory diseases in children worldwide (Allander *et al.*, 2005; Sloots *et al.*, 2006). Recently, we have also discovered its association with gastroenteritis in children (Lau *et al.*, 2007a).

Human parvovirus 4 (PARV4), another novel parvovirus named after the three human parvovirus B19 genotypes, was identified in 2005 from the plasma sample of a homeless, daily injection drug user who presented with an acute viral infection (Jones *et al.*, 2005). This virus was found to possess <30 % aa similarity to other parvoviruses and formed a distinct branch upon phylogenetic analysis. Subsequently, PARV4 and its variants were identified in 4–5 % of pooled human plasma used in the manufacture of plasma-derived medical products (Fryer *et al.*, 2006), and in individual plasma samples with an increased incidence in febrile patients and intravenous drug users (Fryer *et al.*, 2007b). However, the epidemiology, mode of transmission, clinical significance and origin of this newly identified virus are still poorly understood. In particular, unlike the other human parvoviruses with related viruses in animals, PARV4 was phylogenetically distinct without closely related animal counterparts.

To identify possible animal origins of PARV4 or related viruses, we conducted a surveillance study for PARV4-like viruses in human and animal samples. Nearly full-length sequences of the identified PARV4-related viruses (seven strains from swine, three from cattle and one from human) were also determined and analysed. Porcine reproductive and respiratory syndrome virus (PRRSV), the agent that has caused 'high fever' disease outbreaks in pigs in China in 2006, was also co-detected in porcine samples.

METHODS

Collection of animal and human samples. All specimens were collected over a 2 year period (August 2005 to July 2007). A total of 303 samples from pigs were obtained from slaughter houses and pig farms in Hong Kong with the assistance of the Veterinary Public Health Section, the Food and Environmental Hygiene Department, and the Agriculture, Fisheries and Conservation Department, the Government of Hong Kong Special Administrative Region (HKSAR). These included 89 lymph nodes, 114 serum, 50 nasopharyngeal and 50 faecal samples from 169 healthy, sick or deceased pigs, which were collected using procedures described previously (Lau *et al.*, 2005; Yob *et al.*, 2001). The health status of the pigs was determined before slaughtering and only healthy pigs were later processed for distribution to food markets for human consumption. All samples from healthy and sick pigs were collected after they were killed in the slaughter houses. In addition, 30 porcine liver and 32 bovine spleen samples were collected from food markets. Two hundred and eighty-one faecal samples were also collected from bats of seven different species as described previously (Lau *et al.*, 2005). To prevent cross contamination, dissection and collection of tissue samples were performed using disposable scalpels, samples were collected from the centre of each tissue after surface decontamination was carried out, and protective gloves were changed between each tissue sample. Three hundred and sixty-two human serum samples submitted to clinical microbiology laboratories for hepatitis C virus (HCV) or human immunodeficiency virus (HIV) antibody tests were also included in this study. Nasopharyngeal and faecal samples were placed in viral transport medium before transportation to the laboratory for nucleic acid extraction.

Detection of parvoviruses. DNA was extracted from all samples using QIAamp DNA Mini kit (Qiagen), according to the manufacturer's protocol. DNA was subjected to PCR for the detection of parvoviruses by using the forward primer 5′-CCTGGTGCNGTNTGGAA-3′ and the reverse primer 5′-AAANATNGCATACTGGTTYA-3′ targeting a 233 bp fragment within the VP2 region. The primers were designed from multiple alignments of the nucleotide sequences of VP2 regions of PARV4 and porcine parvovirus 2 (PPV2) as described previously (Lau *et al.*, 2005; Woo *et al.*, 2005). Standard precautions were taken to avoid PCR contamination and no false-positive was observed in negative controls.

The PCR products were gel-purified using the QIAquick gel extraction kit (Qiagen). Both strands of the PCR products were sequenced twice with an ABI Prism 3700 DNA Analyser (Applied Biosystems) by using the PCR primers. The sequences of the PCR products were compared with known sequences of VP2 regions of parvoviruses in the GenBank database.

Nearly full-length genome sequencing and analysis. Nearly full-length genome sequences containing the entire coding regions were

determined for seven porcine strains, three bovine strains and one human strain of PARV4-related viruses identified by using the strategy described in our previous publications (Lau *et al.*, 2007a; Woo *et al.*, 2005). The human serum sample was collected from a 40-year-old male positive for anti-HCV antibody. The DNA directly extracted from the corresponding specimens was used as template and amplified by degenerate primers designed from multiple alignment of the genomes of PARV4, bovine parvovirus 3 (BPV3) and chipmunk parvovirus, and additional primers covering the original degenerate primer sites were designed from the results of the first and subsequent rounds of sequencing. Non-overlapping regions were confirmed by independent PCR and sequencing reactions by using specific primers, and no sequence discrepancies were found between two reactions of any of the regions. Primer sequences are shown in Supplementary Table S1 (available in JGV Online). The terminal sequences were confirmed by a modified protocol for RACE (Allander *et al.*, 2005; Lau *et al.*, 2007a). Sequences were assembled and manually edited to produce final sequences of the viral genomes. The nucleotide sequences of the genomes and the predicted ORFs were compared to those of other parvoviruses (Supplementary Table S2 available in JGV Online). A maximum-likelihood (ML) phylogenetic tree was constructed using PHYML version 2.4.4 under the general time reversible (GTR) + I + Γ model of nucleotide substitution (Guindon & Gascuel, 2003). Bootstrap values were estimated by using 1000 replicates on the ML substitution model. Prediction of transmembrane domains was performed using TMHMM and TMpred (Hofmann & Stoffel, 1993; Krogh *et al.*, 2001).

Estimation of selective pressures. As a result of the uncertainty in predicting splicing sites in NS1 and VP1 genes, only VP2 genes, which are most likely unspliced, were selected for the determination of the ratio of non-synonymous to synonymous nucleotide substitutions per site (d_N/d_S) by using the Kumar method in MEGA 4 (Tamura *et al.*, 2007). Pairwise comparisons on the three PARV4-related viruses identified from porcine, bovine and human samples were performed.

Detection of PRRSV from porcine samples. In view of the recent finding that PRRSV is associated with outbreaks of devastating diseases in swine population in mainland China from the area of where pigs consumed in Hong Kong are imported from (Tian *et al.*, 2007), we also attempted to detect PRRSV from our porcine samples. Eighty-nine lymph nodes, 50 serum and 30 liver samples from swine were subjected to RNA extraction and RT-PCR for the detection of PRRSV. Viral RNA was extracted by using the QIAamp Viral RNA Mini kit (Qiagen), according to the manufacturer's protocol. RNA was eluted in 50 μl AVE buffer (Qiagen) and was used as the template for RT-PCR. Reverse transcription was performed using the SuperScript III kit (Invitrogen). PCR for the detection of PRRSV was performed by amplifying a 147 bp fragment of the RNA-dependent RNA polymerase (RdRp) gene from PRRSV by using the forward primer LPW6922 5′-CCTGTCGTCNGGNGAYCC-3′ and the reverse primer LPW6923 5′-ATGTCCTCRAAYTTNARYTG-3′ designed from multiple alignments of the nucleotide sequences of RdRp from PRRSV with available sequences from GenBank. PCR mixture (25 μl) contained cDNA, PCR buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$ and 0.01 % gelatin), 200 μM of each dNTPs and 1.0 U *Taq* polymerase (Applied Biosystems). The mixtures were amplified in 60 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 10 min in an automated thermal cycler (Applied Biosystems). PCR products were gel-purified using the QIAquick gel extraction kit (Qiagen). Both strands of the PCR products were sequenced twice with an ABI Prism 3700 DNA Analyser (Applied Biosystems) by using the PCR primers. The sequences of the PCR products were compared with known sequences of RdRp genes from PRRSV from the GenBank database. Phylogenetic tree construction was performed using the neighbour-joining method with GrowTree using Kimura's two-parameter correction (Genetics Computer Group).

Nucleotide sequence GenBank accession numbers. The nucleotide sequences of the nearly full-length genomes of the PARV4-related viruses have been lodged with the GenBank sequence database under the accession numbers EU20667–EU200677.

RESULTS

Detection of parvovirus in animal and human samples

The results of the PCR for the VP2 fragment of parvovirus in animal and human samples are summarized in Table 1. PCR was positive in 63 (71 %) of the 89 lymph nodes, 55 (48 %) of the 114 serum samples, 14 (28 %) of the 50 nasopharyngeal and five (10 %) of the 50 faecal samples from the 169 pigs collected directly from farms or slaughter houses. No relationship between the detection rates and health status of the pigs were observed. PCR was positive in 16 (53 %) of the 30 porcine liver samples and four (13 %) of the 32 bovine spleen samples collected from markets. PCR was positive in seven (2 %) of 362 human serum samples sent for HCV or HIV antibody tests. Five of the seven positive patients tested positive for anti-HCV antibody. None of the bat samples was positive. Sequencing of the PCR products of the positive porcine, bovine and human samples revealed the presence of three different parvoviruses among the three animal species, one from porcine samples, one from bovine samples and one from human samples with 74.2, 72.9 and 100 % nt identities to PARV4 (GenBank accession no.

Table 1. Detection of parvovirus in animal and human samples by PCR

Animal/ human	Health status	Type of tissue or sample	No. positive/ no. tested samples (%)
Pigs	Healthy	Lymph node	24/30 (80)
		Serum	9/20 (45)
		Nasopharyngeal	8/20 (40)
		Faecal	2/20 (10)
	Sick	Lymph node	19/29 (66)
		Serum	2/4 (50)
	Deceased	Lymph node	20/30 (67)
		Serum	25/60 (42)
		Nasopharyngeal	6/30 (20)
		Faecal	3/30 (10)
		Liver	16/30 (53)
	Unknown	Serum	19/30 (63)
Cows	Unknown	Spleen	4/32 (13)
Bats	Healthy	Faecal	0/281 (0)
Human	Serum sent for HIV or HCV serology	Serum	7/362 (2)

NC_007018), respectively. This suggested the presence of two novel parvoviruses in porcine and bovine samples, respectively. Attempts to stably passage the porcine parvovirus in PK15 (swine kidney), Vero E6 (African green monkey kidney), MA-104 (fetal rhesus monkey kidney), tRhk-4, THP1 (human monocyte) or HEL (primary embryonic human lung fibroblast) cells were unsuccessful (data not shown).

Genome organization and coding potential

To characterize further the identified parvoviruses from animals and humans, the nearly full-length genomes of seven strains from porcine, three strains from bovine and one strain from human parvovirus were determined, with obtained sequence lengths of 4898–5043, 5088–5105 and 5160 bp, respectively. Their genome sizes are expected to be larger, as further sequencing of the ends was hampered by hairpin structures. The genome sequences from the seven porcine samples exhibited limited variations with $\leqslant$4 % nt differences, also the three bovine samples exhibited $\leqslant$0.4 % nt differences. The genomes of the seven strains of parvovirus from porcine samples exhibited 61.5–62.3 % nt identities to that of PARV4, whereas the genomes of the three strains of parvovirus from bovine samples possessed 62.9–63 % nt identities to that of PARV4. In contrast, the genome of the human strain was highly similar to that of PARV4, with 98.2 % nt identity. Their genome organization was typical of a parvovirus. Similar to PARV4, all the 11 genomes encode two large non-overlapping ORFs, ORF1 and ORF2, separated by a small non-coding gap (Jones *et al.*, 2005). ORF1 encodes non-structural protein and ORF2 encodes overlapping VP1/VP2 capsid proteins and small conserved putative protein.

Within ORF1, conserved motifs associated with rolling-circle replication, helicase and ATPase were identified. Within ORF2, the phospholipase A_2 motifs required for parvovirus infectivity situated within the VP1-unique (VP1u) region were also found in all 11 genomes, with the presence of the calcium-binding loop and catalytic residues. The predicted sizes of VP1 were 101–102 kDa, as compared with $<$90 kDa observed for most other parvoviruses, except BPV2 and BPV3 with VP1 of about 105 kDa. A unique small putative protein was also observed in ORF2 of all 11 parvovirus strains and PARV4, consisting of 67 aa in PARV4 and the human strain and 84 aa in porcine and bovine strains. This putative protein, which overlaps with the phospholipase domain within VP1u and is encoded in a different reading frame, displayed no significant similarity to any known proteins or other small conserved proteins of other parvoviruses. There were 47.6–71.8 % aa identities in this putative protein among the three parvoviruses from porcine, bovine and human strains including PARV4. A transmembrane helix was predicted in the central region of this putative protein. Similar to PARV4, inverted terminal repeats that connect the two ends of parvovirus DNA were observed near the 5′ and 3′ ends of the genomes. However, further sequencing of both ends has not been successful, which could be due to the presence of hairpin structures.

Comparison of the nucleotide and predicted amino acid sequences of ORF1 and ORF2 of the 11 genomes with those of other parvovirus genomes showed that they were most closely related to PARV4. In ORF1, the porcine and bovine strains possessed 59.7 and 60.1 % nt identities to that of PARV4, respectively. In ORF2, the porcine and bovine strains possessed 64.6 and 65.8 % nt identities to that of PARV4, respectively (Table 2). Nucleotide similarity plots along the nearly full-length genomes of PARV4 and the porcine and bovine strains showed that ORF1 encoding the NS1 was generally more divergent than ORF2 encoding the capsid proteins (data not shown), a phenomenon also observed in human erythroviruses and different strains of PARV4 (Hokynar *et al.*, 2002; Fryer *et al.*, 2007a). The highly conserved region previously recognized among PARV4 and its variants (between nt 2955 and 3420; GenBank accession no. AY622943) located at the phospholipase A_2 motifs of ORF2 was also found to be the most conserved region among the PARV4-related viruses (Fryer *et al.*, 2006).

Phylogenetic analyses

To determine the phylogenetic relationship of the parvovirus strains, their complete genome sequences were aligned with those of other members of the family *Parvoviridae* with near-full-length genome sequences available in GenBank. Phylogenetic analysis showed that the parvovirus strain from the human sample clustered with PARV4 with a very short branch length, supporting that it is a strain of PARV4. However, the seven strains of parvovirus from porcine samples and the three strains of parvovirus from bovine samples formed two distinct clusters closely related to PARV4, respectively (Fig. 1). The topologies of the trees performed separately for ORF1 and ORF2 were similar to that of the nearly full-length genome tree (data not shown). These data supported the fact that the porcine and bovine strains represented two different novel parvoviruses most closely related to PARV4. On the other hand, these two novel parvoviruses and PARV4 formed a distinct cluster among parvoviruses, distantly related to PPV2. Based on these results, we proposed a new genus, *Hokovirus* (HoV) (for Hong Kong where the two novel parvoviruses were identified), to describe this distinct parvovirus clade. The two novel porcine and bovine parvoviruses are proposed to be named porcine hokovirus (PHoV) and bovine hokovirus (BHoV), respectively, under the genus *Hokovirus*.

Estimation of selective pressures

The d_N/d_S ratios within and between different hokovirus species were low (range: 0–0.107) (Table 3), suggesting that most amino acid residues in VP2 genes have been subjected to purifying selection.

Table 2. Pairwise nucleotide and amino acid identities of ORF1 and ORF2 of PARV4 and the 11 strains of PARV4-related viruses

Porcine strain, PHoV HK7; bovine strain, BHoV HK1; human strain, PARV4 HK1. Percentages in bold indicate nucleotide identities and non-bold percentages indicate amino acid identities.

Virus/strain	Identity (%)											
	ORF1				ORF2 (VP1)				ORF2 (VP2)			
	Porcine strain (PHoV)	Bovine strain (BHoV)	Human strain (PARV4 HK1)	PARV4	Porcine strain (PHoV)	Bovine strain (BHoV)	Human strain (PARV4 HK1)	PARV4	Porcine strain (PHoV)	Bovine strain (BHoV)	Human strain (PARV4 HK1)	PARV4
Porcine strain (PHoV)	–	**63.2**	**59.7**	**59.7**	–	**65.7**	**64.7**	**64.6**	–	**68.2**	**68.0**	**67.9**
Bovine strain (BHoV)	68.1	–	**60.6**	**60.1**	66.7	–	**65.8**	**65.8**	76.3	–	**69.7**	**69.5**
Human strain (PARV4 HK1)	56.6	58.3	–	**97.9**	65.4	66.1	–	**98.5**	76.2	78.0	–	**98.3**
PARV4	56.2	57.6	98.6	–	65.4	65.8	99.8	–	76.2	78.0	100	–

Detection of PRRSV in porcine samples

Two (2 %) of the 89 lymph nodes, two (4 %) of the 50 serum and 25 (83 %) of the 30 liver samples from swine were positive for PRRSV detected by RT-PCR. One of the two lymph nodes and the two serum samples positive for PRRSV were also positive for PhoV, which was detected by PCR, while 16 (64 %) of the 25 positive liver samples were positive for PHoV, suggesting that coinfection by PRRSV and PHoV was not uncommon. There was also association between the detection rates of PRRSV and PHoV among the liver samples ($P<0.05$ by χ-square test). Sequencing of the PCR products showed 0–7 nt difference from the corresponding region of RdRp of type II PRRSV strain GD (GenBank accession no. EU109503) (Fig. 2).

DISCUSSION

The present study represents the first report describing the existence of animal parvoviruses closely related to PARV4, which has been exclusively found in human plasma. Human parvoviruses other than PARV4 are known to be related to viruses found in animals especially mammals. In particular, human erythrovirus was very closely related to simian parvovirus and Rhesus macaque parvovirus. Viruses closely related to AAVs from humans have also been identified in simian species and cattle. HBoV was related to bovine parvovirus from cattle and canine minute virus from dogs. In contrast, the previously unclassified PARV4 was thought to be phylogenetically distinct from other parvoviruses, with the highest amino acid similarity in ORF1 with AAV-4 (23.9–28.6 %) and in ORF2 with human erythrovirus B19 (23.0–29.5 %) (Jones *et al.*, 2005). In this study, we detected PARV4 or related viruses in 148 (44.4 %) of 333 porcine samples, four (13 %) of 32 bovine samples and seven (2 %) of 362 human samples. Three distinct parvoviruses were identified from the three mammalian species, including two novel parvoviruses, PHoV and BHoV from porcine and bovine samples, respectively, and PARV4 from human samples. Comparative sequence analysis showed that the genomes of PHoV and BHoV were most similar to PARV4. Based on phylogenetic analysis, PARV4 (HHoV), PHoV and BHoV formed a distinct cluster within parvoviruses (Fig. 1). They also differed from other parvoviruses by their relatively large predicted VP1 protein and the presence of a small conserved putative protein with transmembrane domain. Based on these results, we propose a separate genus, *Hokovirus*, to describe this distinct group of parvoviruses.

The pathogenicity of members of the genus *Hokovirus* remains to be determined. Two porcine parvoviruses, PPV and PPV2, have been described previously. PPV is ubiquitous in the swine population worldwide, causing reproductive failure in pregnant females and fetal death. PPV DNA has also been detected in porcine factor VIII concentrates intended for use by patients with haemophilia (Soucie *et al.*, 2000). PPV2 is a parvovirus identified in

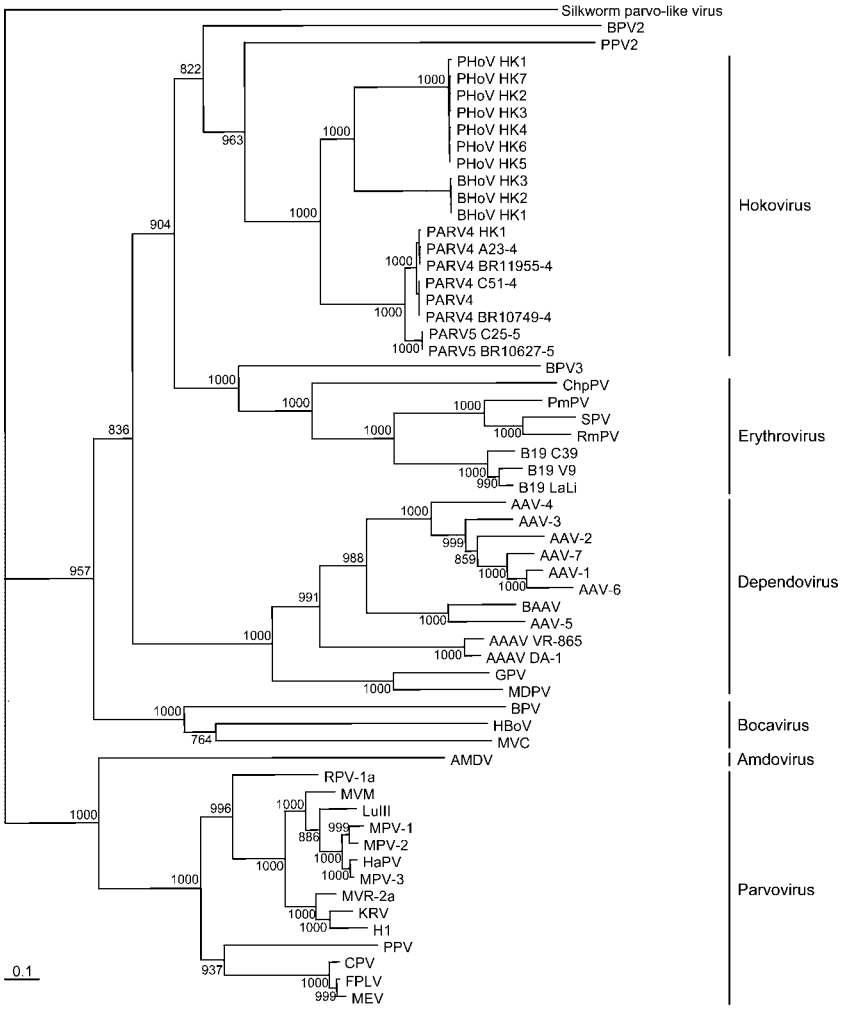

Fig. 1. Phylogenetic analysis of the nearly full-length genome sequences of PHoV, BHoV and PARV4 identified in the present study. Bar indicates the estimated number of substitutions per 10 nt. Silkworm parvo-like virus (GenBank accession no. S78547) was used as the outgroup.

swine sera of unknown significance from Myanmar in 2001 (Hijikata *et al.*, 2001). In this study, a third parvovirus from swine, PHoV, was identified, which was present in all five different porcine tissues sampled, with the highest detection rates in lymph nodes, followed by spleen and serum samples, suggesting that this virus can infect a wide variety of porcine tissues. As no apparent difference was observed between the detection rates in healthy, sick or deceased pigs, it is yet to be determined if PHoV causes disease. Nevertheless, the high frequency of detection of PHoV in tissues from healthy, sick and deceased pigs suggests that the infection may be persistent, a feature also described in human PARV4 (HHoV) and parvovirus B19 infections (LaMonte *et al.*, 2004; Simmonds *et al.*, 2007). As for bovine parvoviruses, BPV, belonging to the genus *Bocavirus*, they cause diarrhoea and mild respiratory symptoms in calves (Spahn *et al.*, 1966). Two additional bovine parvoviruses, BPV2 and BPV3, have recently been identified as frequent contaminants of commercial bovine serum (Allander *et al.*, 2001). Examination of commercial sera suggests that infection by both viruses is frequent, with BPV2 occurring in calves soon after birth and BPV3 possibly transmitted *in utero*. Although the presence of BHoV in bovine spleen samples in the present study may indicate viraemia, more data are required to know if the virus causes disease in cattle. Similarly, little is known about the role of PARV4 viruses in human disease and their mode of transmission. While some patients infected with PARV4 presented with febrile illness, the presence of the virus in healthy individuals may represent subclinical infection. Its higher prevalence in injection drug users and

Table 3. Estimation of d_N/d_S ratios in the VP2 genes of hokoviruses

Virus	d_N/d_S
PHoV	0.029
BHoV	0
PARV4	0.006
PHoV versus BHoV	0.098
PHoV versus PARV4	0.102
BHoV versus PARV4	0.107

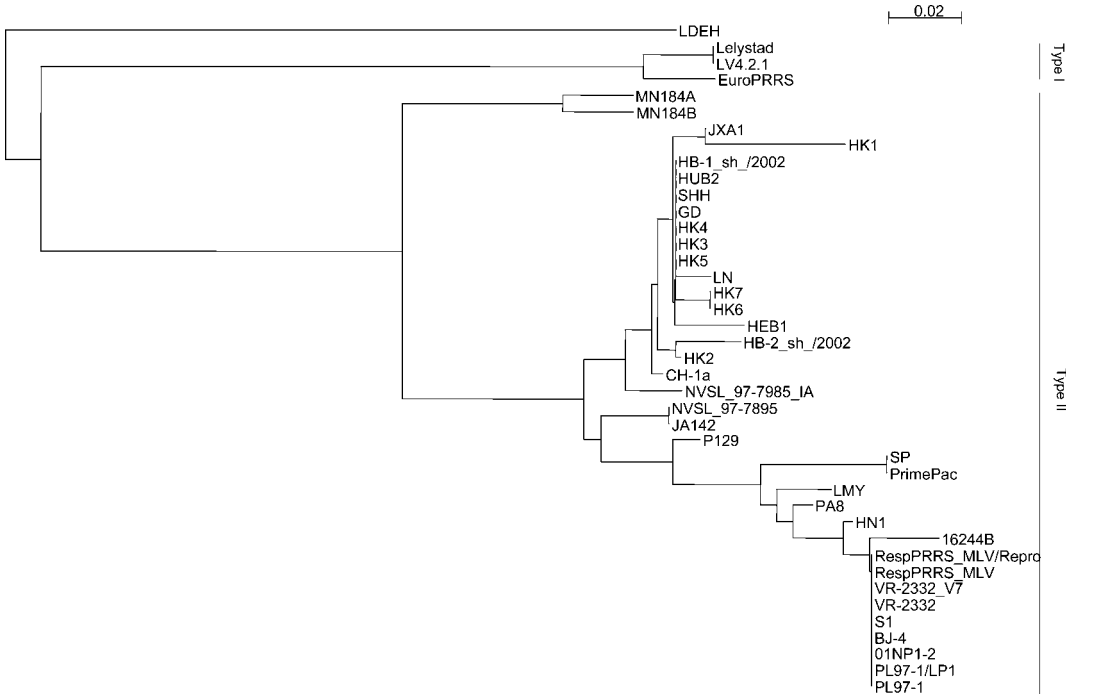

Fig. 2. Phylogenetic analysis of the RdRp gene fragment of the seven PRRSV (HK1–HK7) strains identified from porcine samples. Bar indicates the estimated number of substitutions per 50 nt. Lactate dehydrogenase-elevating virus (LDEH) (GenBank accession no. NC_001639) was used as the outgroup.

persons with haemophilia suggests that the virus may be transmitted predominantly by parenteral routes (Simmonds *et al.*, 2007). In a recent study, PARV4 has been identified from bone marrow and lymphoid tissues, but not brain tissues, of HIV-infected individuals (Manning *et al.*, 2007). As PHoV can be found in various porcine tissues, further studies are warranted to investigate the tissue distribution of PARV4. At the moment, no culture system is available for studying the tissue tropism of PARV4. More data are required to elucidate the significance and pathogenicity of this novel parvovirus genus. Nevertheless, in view of the substantial nucleotide sequence differences between human and animal hokoviruses and the absence of animal hokoviruses in our human samples, the risk of the novel animal hokoviruses to human health is likely to be minimal unless further mutation and/or recombination occurs.

The increasingly recognized diversity of parvoviruses in a single host species is intriguing. Since parvoviruses utilize host DNA polymerase, they are generally considered to be relatively stable. Parvovirus B19 genome was once regarded to undergo little genetic variation, with <2 % nt divergence (Shade *et al.*, 1986). It was not until recently that its variants, V9-, LaLi- and A6-related viruses with up to >11 % nt divergence, were identified (Hokynar *et al.*, 2002; Nguyen *et al.*, 1999, 2002; Servant *et al.*, 2002). The discovery of these novel parvovirus genotypes and various genotypes of anelloviruses with extensive genetic diversity suggests that these structurally simple single-stranded DNA viruses can undergo rapid evolution to generate new genotypes or species. This is also supported by the recent finding that carnivore parvoviruses may possess nucleotide substitution rates similar to that of RNA viruses, which has led to the emergence of canine parvovirus (Shackelton *et al.*, 2005). Before the discovery of the different porcine and bovine parvoviruses, mainly one parvovirus per host species with virus–host co-evolution, with the exception of humans, has been described (Lukashov & Goudsmit, 2001). The diversity of parvoviruses subsequently identified in swine and cattle suggests that the evolution of parvoviruses is far more complicated. In the present study, the d_N/d_S ratios for all pairwise comparisons were $<<1$, which is in line with previous results showing that positive selection was extremely limited in parvoviruses, even during recent cross-species transmission (Lukashov & Goudsmit, 2001; Shackelton *et al.*, 2005). Further studies are required to understand the evolution and mechanisms of host adaptation in parvoviruses.

In addition to the finding of PARV4-related viruses, PRRSV was also identified in our porcine samples. PRRSV, a member of the family *Arteriviridae*, is known to cause disease and chronic immunosuppression in pigs, predisposing them to other infections. In 2005, the largest outbreak of *Streptococcus suis* infecting both pigs and humans emerged in China, which was believed to be related to the emergence of a variant of type II PRRSV,

causing the subsequent 'high fever' disease outbreaks in 2006 (Tian *et al.*, 2007; Yu *et al.*, 2006). Unlike the typical PRRS, numerous adult sows were affected in the 'high fever' disease outbreaks with high morbidity and mortality, suggesting that superimposed infections may have played a role. In the present study, association was found between rates of infection by PRRSV and PHoV among the porcine liver samples. However, given the high detection rate of PHoV, such association remains to be ascertained with more data. Further studies are also required to investigate the presence of PHoV in pigs involved and its possible role in these unusual outbreaks.

ACKNOWLEDGEMENTS

We would like to thank Professor York Y. N. Chow, Secretary of Health, Welfare and Food, HKSAR, The Peoples' Republic of China; Mr Eddy Chan and Dr Constance Chan of the Food and Environmental Hygiene Department; and Ms Siu-Hing Cheung and Mr Thomas Sit of the Agriculture, Fisheries and Conservation Department, the Government of the HKSAR; for their facilitation and support. We are grateful to the generous support of Mr Hui Hoy and Mr Hui Ming in the genomic sequencing platform. This work is partly supported by the HKSAR Research Fund for the Control of Infectious Diseases of the Health, Welfare and Food Bureau; Research Grant Council Grant; University Development Fund, HKU Special Research Achievement Award, The Croucher Senior Medical Research Fellowship 2006–2007, and Outstanding Young Researcher Award, The University of Hong Kong; The Tung Wah Group of Hospitals Fund for Research in Infectious Diseases; and the Providence Foundation Limited in memory of the late Dr Lui Hac Minh.

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