Identification and characterization of bocaviruses in cats and dogs reveals a novel feline bocavirus and a novel genetic group of canine bocavirus

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We report the identification and genome characterization of a novel bocavirus, feline bocavirus (FBoV), and novel bocaviruses closely related to canine bocavirus (CBoV) strain Con-161 in stray cats and dogs in Hong Kong, respectively. FBoV was detected by PCR in 7.2, 0.3, 1.6, 2.0 and 0.8 % of faecal, nasal, urine, kidney and blood samples, respectively, from 364 cats, while CBoV was detected in 4.6, 5.1, 6.3 and 0.3 % of faecal, nasal, urine and blood samples, respectively, from 351 dogs. Three FBoV genomes sequenced revealed the presence of three ORFs characteristic of bocaviruses. Phylogenetic analysis showed that FBoVs were related only distantly to other bocaviruses, forming a distinct cluster within the genus, with ≤59.7 % nucleotide identities to the genome of minute virus of canines. The four CBoV genomes sequenced shared 87.4–89.2 % nucleotide identities with that of CBoV strain Con-161. In addition to the three bocavirus ORFs, they encoded an additional ORF, ORF4, immediately downstream of the ORF for non-structural protein 1 (NS1), which was not found in other bocaviruses including CBoV strain Con-161. They also possessed a putative second exon encoding the C-terminal region of NS1 and conserved RNA-splicing signals, previously described in human bocaviruses. Partial VP1/VP2 sequence analysis of 23 FBoV and 25 CBoV strains demonstrated inter-host genetic diversity, with two potential genetic groups of FBoV and a novel CBoV group, CBoV-HK, distinct from the three groups, CBoV-A to -C, found in the USA. Although the pathogenicity of FBoV and CBoV remains to be determined, their presence in different host tissues suggested wide tissue tropism.

INTRODUCTION

Parvoviruses are a group of small, non-enveloped, ssDNA viruses that cause a wide range of disease in animals. Under the current International Committee on Taxonomy of Viruses (ICTV) classification system, the family Paroviridae is classified into two subfamilies based on host range: Parovirinae, infecting vertebrates, and Densovirinae, infecting insects and other arthropods. The subfamily Parovirinae is further subdivided into five genera, Amrovirus, Bocavirus, Dependovirus, Erythrovirus and Parvovirus (Fauquet et al., 2005). Several novel parvoviruses, potentially belonging to a novel genus, have been recently discovered, including human partetravirus (previously known as human parvovirus 4 or PARV4) (Jones et al., 2005; Fryer et al., 2006, 2007), porcine and bovine partetraviruses (previously known as porcine and bovine hokoviruses) (Lau et al., 2008), ovine partetraviruses (Tse et al., 2011), porcine partetravirus-like viruses in wild boar (Adlhoch et al., 2010; Cadar et al., 2011) and human partetravirus-like viruses in primates (Sharp et al., 2010).

Bocaviruses are unique among parvoviruses in possessing an additional ORF, ORF3, between the non-structural and structural coding regions, ORF1 and ORF2, in their genomes. The genus was originally named according to the initial two members, bovine parvovirus (BPV) and minute virus of canines (MVC) (Binn et al., 1970; Mochizuki et al., 2002; Spahn et al., 1966; Storz et al., 1970) and later revised by ICTV to incorporate viruses infecting other animals, such as the porcine parvovirus (PPV), and characterized according to genetic relatedness (Schwartz et al., 2009; Fauquet et al., 2011). The ICTV name is Bocavirus, and the genus name is Bocavirus. The Bocavirus name is derived from the bovine and feline parvoviruses that were the first to be recognized as parvoviruses. Bocaviruses are important causes of disease in domestic animals, such as feline panleukopenia, canine distemper, and canine parvovirus enteritis. However, bocaviruses have also been recovered from other species, such as horses, sheep, and deer. Bocaviruses are also associated with disease in humans. Bocavirus infections are usually asymptomatic, but they can cause a wide range of disease, including respiratory, gastrointestinal, and central nervous system disorders. Bocavirus infections can be diagnosed by PCR, and the virus can be isolated in cell culture. The pathogenesis of bocavirus infections is not well understood, but it is likely that the virus infects cells in the epithelium of the respiratory and gastrointestinal tract, and that it can cause inflammation and cell death. Bocaviruses are transmitted by the respiratory and gastrointestinal routes, and they can be spread by contact with infected animals or by aerosolization. Bocavirus infections can be prevented by vaccination, and the vaccines are usually administered to young animals to provide lifelong immunity. Bocavirus infections can also be treated with antiviral drugs, such as ribavirin, which can be used to treat respiratory infections.
et al. 2011; McKillen et al. understood (Allander pathogenic role in human disease is still not fully
HBoV1–4, have now been described, although their
In the past 2 years, several novel animal bocaviruses have
been discovered and their genomes have been character-
ized. In particular, various groups have reported bocavirus-like
sequences in swine samples in China, Sweden and the
UK (Blomström et al., 2009; Cheng et al., 2010; Lau et al.,
2011; McKillen et al., 2011; Zeng et al., 2011; Zhai et al.,
2010). Genome-sequence studies have shown the presence
of at least nine potential novel porcine bocavirus species,
porcine bocavirus 1 (PBoV1) to PBoV5, PBoV strain
WUH1, PBoV1 H18, PBoV2 A6 and PBoV3 22 (Cheng
et al., 2010). Near-complete genome sequences were
determined for CslBoV1, CslBoV2 9822 (JN420366) and 79 % nucleotide identity to
that of PBoV2 A6 (HQ291309), suggesting that they
belonged to CBoV.

Subsequent PCR using specific primers targeted to a
133 bp fragment of the NS1 gene of FBoV was positive in
26 (7.2 %) of 363 faecal samples, one (0.3 %) of 364 nasal
samples, six (1.6 %) of 364 urine samples, one (2 %) of 51
kidney samples and three (0.8 %) of 361 blood samples
collected from cats (Table 1). PCR using specific primers
targeted to a 128 bp fragment of the NS1 gene of CBoV was
positive in 16 (4.6 %) of 351 faecal samples, 18 (5.1 %) of 351
nasal samples, 22 (6.3 %) of 351 urine samples and one
(0.3 %) of 331 blood samples collected from dogs (Table 1).
Both FBoV and CBoV were detected in various seasons
during the sampling period, although detection rates were
higher in some months than others, suggesting a seasonal
pattern of infections (Fig. 1).

Genome-sequence analysis of FBoV and CBoV
Near-complete genome sequences were determined for
FBoV detected from three feline samples (797F and 875F
from faecal samples and 797U from a urine sample) of two
cats, and CBoV from four samples (882F and 831F from
faecal samples, 880N from a nasopharyngeal sample and
882U from a urine sample) of three dogs. The sequences
obtained for FBoV were 5179–5331 bp, with a G + C
content of 49.6–50 mol%. The sequences obtained for CBoV
were 5054–5140 bp, with a G + C content of 42.8–43 mol%.
The two FBoV genomes (797F and 797U) from different
species possessed 39.5 % nucleotide identity, suggesting that
they belong to FBoV species 1 (FBoV1)

Table 1. Detection of bocaviruses in feline and canine samples
by PCR

<table>
<thead>
<tr>
<th>Animal samples</th>
<th>No. of samples</th>
<th>Positive samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feline</td>
<td>1503</td>
<td>37 (2.5)</td>
</tr>
<tr>
<td>Faecal</td>
<td>363</td>
<td>26 (7.2)</td>
</tr>
<tr>
<td>Nasal</td>
<td>364</td>
<td>1 (0.3)</td>
</tr>
<tr>
<td>Urine</td>
<td>364</td>
<td>6 (1.6)</td>
</tr>
<tr>
<td>Kidney</td>
<td>51</td>
<td>1 (2.0)</td>
</tr>
<tr>
<td>Blood</td>
<td>361</td>
<td>3 (0.8)</td>
</tr>
<tr>
<td>Canine</td>
<td>1384</td>
<td>57 (4.1)</td>
</tr>
<tr>
<td>Faecal</td>
<td>351</td>
<td>16 (4.6)</td>
</tr>
<tr>
<td>Nasal</td>
<td>351</td>
<td>18 (5.1)</td>
</tr>
<tr>
<td>Urine</td>
<td>351</td>
<td>22 (6.3)</td>
</tr>
<tr>
<td>Blood</td>
<td>331</td>
<td>1 (0.3)</td>
</tr>
</tbody>
</table>
sampling locations of the same cat shared identical genome sequences (except for occasional degenerate bases observed in both NS1 sequences), while the other genome (875F) from a different cat possessed 142 nt differences from the former. Three CBoV genomes (880N, 882F and 882U), two from the same dog, also shared identical genome sequences with each other, while the other genome (831F) possessed 214 nt differences from the former. Similar to other bocaviruses, the three FBoV and four CBoV genomes each encode two major non-overlapping ORFs, ORF1 and ORF2, and one additional ORF, ORF3, characteristic of bocaviruses (Allander et al., 2005; Arthur et al., 2009). ORF1 encodes protein NS1 and ORF2 encodes the overlapping capsid proteins VP1/VP2, while ORF3 encodes NP1 (Fig. 2). Within NS1 of both FBoV and CBoV, conserved motifs associated with rolling-circle replication, helicase and ATPase were identified. The VP1-unique (VP1u) regions of both viruses also contained phospholipase A₂ motifs required for parvovirus infectivity, with the presence of a calcium-binding loop and catalytic residues. The function of the unique bocavirus protein, NP1, remains unclear, although it has been found to be essential for virus replication in MVC (Sun et al., 2009). In addition to the three ORFs, the three CBoV genomes encode an additional ORF, ORF4, immediately downstream of ORF1, with a predicted signal peptide. This ORF was not found in other known bocaviruses, including CBoV strain Con-161.

The three FBoV genome sequences only shared 58.6–59.7 % nucleotide identities to that of the most closely related bocavirus, MVC. The predicted NS1 protein of FBoV encodes 804 aa, while VP1/VP2 encodes 712 aa and NP1 encodes 218 aa. Phylogenetic analysis using the genome sequences of FBoV showed that they were related distantly to other bocaviruses, forming a distinct cluster within the genus Bocavirus (Fig. 3). This supported the presence of a novel bocavirus, FBoV, in the genus Bocavirus in the feline samples.

The four CBoV genome sequences shared 87.4–89.2 % nucleotide identities with that of CBoV strain Con-161. Similar to HBoV and CBoV strain Con-161, the predicted NS1 protein of the four CBoV genomes was relatively short, encoding 644 or 648 aa, compared with 664–804 aa in other bocaviruses. Recent studies have shown that the four HBoV species possess a second exon encoding the C-terminal region of NS1, and conserved RNA-splicing signals near the end of NS1 and the second exon, which may form a spliced transcript encoding 750–780 aa (Chen et al., 2010; Kapoor et al., 2010b). Similar to CBoV strain Con-161 (Kapoor et al., 2012), the four CBoV genomes also possess a putative second exon and conserved RNA-splicing signals, which may generate a longer NS1 of 764 aa (Fig. 2). The VP1/VP2 of CBoV encodes 711–712 aa, while NP1 encodes 195 aa. ORF4 encodes a 144 aa protein, which overlaps with the predicted second exon of NS1.
Phylogenetic analysis of NS1 of the present CBoV strains showed that they belonged to CBoV, with 96.5–96.8% nucleotide identities to CBoV strain Con-161 (Fig. 3).

**Genetic diversity of FBoV and CBoV**

As capsid proteins of paroviruses are responsible for determining cellular tropism and host immune response, the genetic diversity of detected FBoV and CBoV strains was studied by amplifying and sequencing of their respective partial VP1/VP2 regions. Phylogenetic analysis of the sequences from 23 FBoV strains showed that they possessed up to 6.5% nucleotide difference from each other, with 20 strains falling into two clusters, FBoV-A (14 strains) and FBoV-B (six strains) (Fig. 4a). FBoV strains from different sampling locations of the same cat were identical, while different strains were observed from different cats. Phylogenetic analysis of the sequences from 25 CBoV strains showed that they possessed up to 10.3% nucleotide difference from each other. Moreover, they appeared to fall into a separate cluster, CBoV-HK, distinct from the three genetic groups, CBoV-A to -C, described previously among strains from the USA (Fig. 4b). The 6 aa deletion found previously in CBoV-C variants, located at the variable exposed loop of VP1/VP2, was not present in the CBoV-HK strains. Interestingly, two different strains, 831F and 831N, were detected in the faecal and nasal samples, respectively, of the same dog (Fig. 4b).

**Virus culture**

No cytopathic effect was observed in cell lines inoculated with samples that were positive for FBoV or CBoV by PCR. PCR using the culture supernatants and cell lysates for monitoring the presence of virus replication also showed negative results.

**DISCUSSION**

The present study represents the first to describe the existence of bocaviruses in domestic cats. In this study, using consensus primers for PCR of an NS1 gene fragment, bocavirus sequences were detected in various tissue samples of cats and dogs. Viral genome sequences were determined from three feline and four canine samples, which confirmed
Fig. 3. Phylogenetic analysis of near-complete genome sequences of FBoV and CBoV obtained from two feline faecal (FBoV HK797F and FBoV HK875F), one feline urine (FBoV HK797U), two canine faecal (CBoV HK831F and CBoV HK882F), one canine urine (CBoV HK882U) and one canine nasal (CBoV HK880N) specimens, and those of other parvoviruses with genome sequences available (GenBank accession numbers are in parentheses). A total of 4875 nt positions were included in the analysis. The tree was constructed by the maximum-likelihood (ML) method under the best evolutionary model (GTR + I). Bootstrap values were calculated from 100 trees. Bar, 0.2 nucleotide substitutions per site. Silkworm parvo-like virus was used as an outgroup. AAV-1, Adeno-associated virus 1; AAV-2, adeno-associated virus 2; AMDV, Aleutian mink disease virus; AAAV DA-1, avian adeno-associated virus DA-1; PBoV SX, bocavirus pig/SX/China/2010; BAAV, bovine adeno-associated virus; BHoV HK1, bovine hokovirus HK1; BPV, bovine parvovirus; BPV2, bovine parvovirus 2; BPV3, bovine parvovirus 3; CsAAV1 1136, California sea lion adeno-associated virus 1 1136; CsBoV1 1136, California sea lion bocavirus 1 1136; CsBoV2 9822, California sea lion bocavirus 2 9822; CsBoV3 9805, California sea lion bocavirus 3 9805; CBoV Con-161, canine bocavirus Con-161; MVC, minute virus of canines; CPV, canine parvovirus; ChpPV, chipmunk parvovirus; FPV, feline panleukopenia virus; GPV, goose parvovirus; GBoV1, gorilla bocavirus 1; HaPV, hamster parvovirus; HBoV, human bocavirus; HBoV3, human bocavirus 3; HBoV4, human bocavirus 4; PARV4 HK1, human parvovirus 4 HK1; PARV5 BR10627-5, human parvovirus 5 BR10627-5; B19 V9, human parvovirus B19 V9; KRV, Kilham rat virus; LuIII, LuIII virus; MVM, minute virus of mice; MEV, mink enteritis virus; MPV-1, mouse parvovirus 1; MPV-2, mouse parvovirus 2; MPV-3, mouse parvovirus 3; MDPV, Muscovy duck parvovirus; H1, parvovirus H1; PmPV, pig-tailed macaque parvovirus; PBoV1, porcine bocavirus 1 pig/ZJD/China/2006; PBoV1 H18, porcine bocavirus 1 H18; PBoV2, porcine bocavirus 2 pig/ZJD/China/2006; PBoV2 A6, porcine bocavirus 2 A6; PBoV3 22, porcine bocavirus 3 22; PBoV3, porcine bocavirus 3 SH20F; PBoV4, porcine bocavirus 4-1 SH17F-1; PHoV HK1, porcine hokovirus HK1; PPV, porcine parvovirus; PPV2, porcine parvovirus 2; RMV-2a, rat minute virus 2a; RPV-1a, rat parvovirus 1a; RmPV, rhesus macaque parvovirus; SPV, simian parvovirus.
the presence of two different bocaviruses, FBoV and CBoV, respectively. Phylogenetic analysis revealed that the different strains of FBoV and CBoV each formed a distinct cluster among known bocaviruses. While CBoV is phylogenetically closely related to the recently discovered CBoV strain Con-161, FBoV represents a novel bocavirus identified in cats. The FBoV genomes possessed $\leq 59.7\%$ nucleotide identities to those of other bocaviruses, being only distantly related to CslBoV, CBoV and MVC. Under ICTV criteria for species classification of the genus Bocavirus, members of each species are defined as: (i) probably antigenically distinct; (ii) natural infection is confined to a single host species; and (iii) having $< 95\%$ homologous NS gene DNA sequence (http://www.ictvdb.org/). Although the antigenic properties of FBoV and CBoV were not studied, the NS1 of FBoV possessed $\leq 56.7\%$ nucleotide identities to those of other known bocaviruses, supporting its classification as a separate bocavirus species. Analysis of partial VP1/VP2 sequences demonstrated genetic diversity among different strains of FBoV, with most strains belonging to two potential genetic groups.

A novel genetic group of CBoVs was identified among the present dogs, suggesting that diverse CBoVs may be distributed in different geographical areas. As the NS1 genes of the present CBoV strains exhibited $\geq 96.5\%$ nucleotide identities to CBoV strain Con-161, they should belong to the same bocavirus species. However, a unique ORF4, absent in CBoV strain Con-161 and other bocaviruses, was found in the present CBoV strains. As only one genome sequence of CBoV from strain Con-161 was available among strains detected in a previous study (Kapoor et al., 2012), it remains to be determined whether this ORF is functional and can be found in CBoV strains from other countries. Moreover, phylogenetic analysis of partial VP1/VP2 sequences revealed a distinct group, CBoV-HK, among the present CBoV strains.
strains, different from the three genetic groups, CBoV-A to –C, detected in dogs from the USA (Kapoor et al., 2012). In the present study, we detected only CBoV, but not MVC, in our canine samples, although the consensus primers were theoretically able to detect MVC. This suggests that CBoV may be more prevalent than MVC among our canine population. Further studies are required to better define the epidemiology and genetic diversity of canine bocaviruses in different countries.

Despite having DNA genomes with the use of cellular replication machinery, increasing studies have shown that parvoviruses can undergo rapid evolution to generate new genotypes or species and may possess substitution rates close to those of RNA viruses (Duffy et al., 2008; Hokynar et al., 2002; Nguyen et al., 2002; Servant et al., 2002; Shackelton et al., 2005; Shade et al., 1986). Moreover, parvoviruses such as HBoV and PBoV can undergo genetic rearrangement and recombination similar to that which has occurred in RNA viruses (Hoeltzer et al., 2008; Hogan & Faust, 1986; Kapoor et al., 2009, 2010b; Lau et al., 2011). We have previously described marked inter- and intra-host genetic diversity and co-infection of two different porcine bocaviruses in the same host (Lau et al., 2011). The presence of within-host genetic diversity has also been described in other parvoviruses including canine parvovirus (CPV) and feline panleukopenia virus (FPV), although their degree of sequence diversity was relatively low compared with that in porcine bocaviruses (Battilani et al., 2006; Hoeltzer et al., 2008). In the present study, although no recombination event was detected among the present FBoV and CBoV strains (data not shown), both viruses were found to exhibit inter-host genetic diversity. While intra-host genetic diversity was also observed in VP1/VP2 of CBoV, the degenerate bases observed in the NS1 of FBoV may suggest the presence of variant strains or quasispecies in the same host.

Cats and dogs are the commonest domestic pets worldwide that share a close habitat with humans. They are increasingly known to carry a large diversity of viruses, many of which are closely related between these two animals, with interspecies transmission having been described between feline and canine coronaviruses, herpesviruses, papillomaviruses and parvoviruses (Bernard et al., 2012; Herrewegh et al., 1998; Lau et al., 2012; Pellett et al., 2012; Shackelton et al., 2005; Siegl et al., 1985; Truyen, 2006; Woo et al., 2012a, b). In particular, CPV (with the first known strain designated CPV2), belonging to the genus Parvovirus, emerged in 1978 from a feline parvovirus, FPV, differing at several key amino acid residues (Shackelton et al., 2005; Steinel et al., 2000). Although CPV2 can infect feline cells in vitro, it did not infect cats. However, CPV2 was subsequently replaced by a new lineage, CPV2a, and its variants, which can infect both dogs and cats (Truyen et al., 1996). It has been shown that, since its emergence in the 1970s, CPV has undergone an epidemic-like pattern of exponential growth, which was associated with a lineage that acquired a broader host range and greater infectivity (Shackelton et al., 2005).

While recombination played no role in the emergence of CPV, its rapid adaptation to dogs was found to be dependent on a high rate of mutation and the positive selection of mutations in the major capsid gene (Shackelton et al., 2005). In the present study, FBoV was also related more closely to MVC, CBoV and CsBoV than to other bocaviruses, although the genetic difference was much larger than that between FPV and CPV. This suggests that feline and canine bocaviruses may share a common ancestor and have co-evolved among cats and dogs, although interspecies transmission is unlikely to have occurred recently.

The pathogenicity of the novel FBoVs and CBoVs remains to be determined. The first bocavirus, BPV, is known to be associated with diarrhoea and to cause mild respiratory symptoms in experimentally infected calves (Spahn et al., 1966; Storz et al., 1978). Initially isolated from apparently normal dogs in a canine cell line in 1970, MVC was later found to be a respiratory pathogen of neonatal puppies and to cause fetal deaths in infected dogs (Binn et al., 1970; Mochizuki et al., 2002). However, MVC infections are mostly subclinical in adult animals (Carmichael et al., 1991). Although HBoVs have been detected in respiratory and faecal samples of children worldwide, their pathogenicity in respiratory or enteric disease is still in debate because of the frequent co-detection of other pathogens and their presence in healthy children (Allander et al., 2005; Lau et al., 2007; Sloots et al., 2006; Söderlund-Venermo et al., 2009). The recently discovered novel porcine bocaviruses have been identified in both healthy and diseased pigs, including those with clinical post-weaning multisystemic wasting syndrome (Blomström et al., 2009; Cheng et al., 2010; Lau et al., 2011; McKillen et al., 2011; Zeng et al., 2011; Zhai et al., 2010). Although these viruses were most prevalent in porcine faecal samples, their presence in other tissues, including lymph node, serum and nasopharyngeal samples, suggested a wide tissue tropism (Lau et al., 2011). Whilst GBoV1 was associated with acute enteritis in western gorillas (Kapoor et al., 2010a), CBoV group C variants were found to be more prevalent in dogs with respiratory diseases than in healthy animals (Kapoor et al., 2012). However, other bocaviruses from primates and California sea lions detected in faecal samples were not known to cause diseases in these mammals (Li et al., 2011; Sharp et al., 2010). In this study, both FBoV and CBoV were detected in different samples from the respective animals, suggesting a wide tissue tropism. While FBoV was mainly detected in faecal samples in cats, CBoV demonstrated the highest detection rate in urine samples (6.3 %), suggesting that the latter virus may cause more systemic infections in dogs rather than respiratory disease alone. Interestingly, HBoV, still of unresolved clinical significance in humans, has also been occasionally detected in serum and urine samples of infected children (Pozo et al., 2007; Wang et al., 2010). Further studies are required to better understand the epidemiology, evolution, pathogenicity and diversity of bocaviruses in various mammals including humans.
METHODS

Collection of animal specimens. In total, 1503 feline and 1384 canine samples from 364 cats and 351 dogs, respectively, collected over a 21 month period (December 2009–August 2011), were provided by the Agriculture, Fisheries and Conservation Department (AFCD) of the Government of Hong Kong Special Administrative Region (HKSAR), as part of a surveillance programme on stray cats and dogs. Nasopharyngeal, faecal, urine, kidney and/or blood samples were collected from the stray cats and dogs by AFCD Animal Management Centres, using procedures described previously (Table 1) (Lau et al., 2005, 2008). To prevent cross-contamination, dissections and collection of tissues were performed using disposable scalpels and from the centre of each tissue after surface decontamination and with protective gloves changed for each tissue sample. All samples were collected immediately after euthanasia as routine policies for disposal of locally captured stray cats and dogs. The study was approved by the Committee on the Use of Live Animals in Teaching and Research at the University of Hong Kong.

Detection of bocaviruses. DNA was extracted from all samples using a QIAamp DNA Mini kit (Qiagen), according to the manufacturer’s protocol. DNA was subject to PCR for bocaviruses, using forward primer 5’-GCCGCAGCGNCCCAAMCMAA-3’ and reverse primer 5’-CATNAGNCAYTGYCCACCA-3’ targeted to a 141 bp fragment of the NS1 gene, designed by multiple alignments of the nucleotide sequences of NS1 regions of known bocaviruses including HBoV, BPV, and MVC. As potentially novel bocaviruses were detected, subsequent bocavirus detection was performed using specific primers (forward primer 5’-TCTACAAATGGGCACATTGGA-3’ and reverse primer 5’-GAGCTTGGATTTGACATTCAGA-3’) for FBoV, which targeted a 133 bp fragment of NS1; forward primer 5’-AGGTGGGCGACCTGGCTGT-3’ and reverse primer 5’-CAGCCTAAGGGCCATCCTAA-3’ for CBoV, which targeted a 128 bp fragment of NS1) designed based on the obtained genome sequences. The PCR mixture (25 µl) contained DNA, PCR buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl, 2 mM MgCl₂, and 0.01% gelatin), 200 µM of each dNTP and 0.625 U Taq polymerase (Applied Biosystems). The mixtures were amplified by 40 cycles of 94 °C for 1 min, 50 °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). Standard precautions were taken to avoid PCR contamination and no false positives were observed in negative controls. PCR products were gel-purified using the QIAquick gel extraction kit (Qiagen). Both strands of the PCR products were sequenced twice with the centre of each tissue after surface decontamination and with protective gloves changed for each tissue sample. All samples were collected immediately after euthanasia as routine policies for disposal of locally captured stray cats and dogs. The study was approved by the Committee on the Use of Live Animals in Teaching and Research at the University of Hong Kong.

Genome sequencing and analysis. Near-complete genome sequences were determined for the potentially novel feline bocavirus, FBoV, from three samples (875F, 797F and 797U) of two cats and CBoV from four canine samples (880N, 882U, 882F and 831F) of three dogs, using the strategy described in our previous publications (Lau et al., 2008, 2011; Tse et al., 2011). DNA extracted directly from the specimens was used as template and amplified by degenerate primers designed from multiple alignment of the genomes of HBoV, BPV, MVC and PBoV, and additional primers covering the original degenerate primer sites were designed from the results of the first and subsequent rounds of sequencing. Primer sequences are available on request. The terminal sequences were confirmed by a modified protocol for RACE (Allander et al., 2005; Lau et al., 2011). Sequences were assembled and edited manually to produce final sequences of the viral genome. All assembled sequences were confirmed by independent PCR using specific primers across overlapping regions to ensure accuracy of the assembled sequences. The nucleotide sequences of the genomes and the predicted ORFs were compared with those of other bocaviruses. A maximum-likelihood (ML) phylogenetic tree was constructed using PhyML version 3.0 (Guindon & Gascuel, 2003) under the best evolutionary model (GTR + I) determined by MODELEGENERATOR (Keane et al., 2006). Bootstrap values were estimated by using 100 replications on the ML substitution model. Protein domain and family analysis was performed using InterProScan (Apweiler et al., 2001) and/or multiple sequence alignment.

Sequencing of partial VP1/VP2 sequences. The genetic diversity of FBoV and CBoV strains was studied by amplifying and sequencing of their partial capsid protein sequences, using primers (forward primer 5’-AAAAATCTTAAACACAAAA-3’ and reverse primer 5’-TATGGCC-AATTCTGGCATT-3’ for FBoV; and forward primer 5’-GGAGGGAGG-TGGAGGACAT-3’ and reverse primer 5’-CGTCCGTCAGGTCAGATT-3’ for CBoV) targeted to a 562 bp and a 526 bp region of their VP1/VP2 genes, respectively, designed by multiple alignments of the obtained genome sequences, and the above PCR conditions.

Virus culture. Four faecal and three urine samples positive for FBoV were cultured in Vero E6 (African green monkey kidney; ATCC CRL-1586), CrFK (Crandell feline kidney; ATCC CCL-94), HFL (human embryonic lung fibroblast), CaCo-2 (human colorectal adenocarcinoma; ATCC HTB-37), and primary feline lung and kidney cells. Two faecal, urine and nasal samples positive for CBoV were cultured in MDCK (Madin–Darby canine kidney; ATCC CCL-34), and primary canine lung and kidney cells.

Nucleotide sequence accession numbers. The nucleotide sequences of the genomes of FBoVs and CBoVs have been deposited in GenBank under accession numbers JQ692585–JQ692591.

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REFERENCES


Novel feline and canine bocaviruses


