A novel dromedary camel enterovirus in the family *Picornaviridae* from dromedaries in the Middle East

Patrick C. Y. Woo,1,2,3,4† Susanna K. P. Lau,1,2,3,4† Tong Li,2† Shanty Jose,5 Cyril C. Y. Yip,2 Yi Huang,2 Emily Y. M. Wong,2 Rachel Y. Y. Fan,2 Jian-Piao Cai,2 Ulrich Wernery5 and Kwok-Yung Yuen1,2,3,4

1State Key Laboratory of Emerging Infectious Diseases, The University of Hong Kong, Hong Kong, PR China  
2Department of Microbiology, The University of Hong Kong, Hong Kong, PR China  
3Research Centre of Infection and Immunology, The University of Hong Kong, Hong Kong, PR China  
4Carol Yu Centre for Infection, The University of Hong Kong, Hong Kong, PR China  
5Central Veterinary Research Laboratory, Dubai, United Arab Emirates

The recent emergence of Middle East respiratory syndrome coronavirus from the Middle East and the discovery of the virus from dromedary camels have boosted interest in the search for novel viruses in dromedaries. Whilst picornaviruses are known to infect various animals, their existence in dromedaries was unknown. We describe the discovery of a novel picornavirus, dromedary camel enterovirus (DcEV), from dromedaries in Dubai. Among 215 dromedaries, DcEV was detected in faecal samples of four (1.9 %) dromedaries [one (0.5 %) adult dromedary and three (25 %) dromedary calves] by reverse transcription PCR. Analysis of two DcEV genomes showed that DcEV was clustered with other species of the genus *Enterovirus* and was most closely related to and possessed highest amino acid identities to the species *Enterovirus E* and *Enterovirus F* found in cattle. The G + C content of DcEV was 45 mol%, which differed from that of *Enterovirus E* and *Enterovirus F* (49–50 mol%) by 4–5 %. Similar to other members of the genus *Enterovirus*, the 5’ UTR of DcEV possessed a putative type I internal ribosome entry site. The low ratios of the number of nonsynonymous substitutions per non-synonymous site to the number of synonymous substitutions per synonymous site ($K_{s}/K_{a}$) of various coding regions suggested that dromedaries are the natural reservoir in which DcEV has been stably evolving. These results suggest that DcEV is a novel species of the genus *Enterovirus* in the family *Picornaviridae*. Western blot analysis using recombinant DcEV VP1 polypeptide showed a high seroprevalence of 52 % among serum samples from 172 dromedaries for IgG, concurring with its much higher infection rates in dromedary calves than in adults. Further studies are important to understand the pathogenicity, epidemiology and genetic evolution of DcEV in this unique group of animals.

INTRODUCTION

Picornaviruses are positive-sense, ssRNA viruses with icosahedral capsids. They are widely distributed among human and various animals in which they can cause respir-
2011, 2012; Li et al., 2009; Woo et al., 2010, 2012b). Novel human picornaviruses including the species Human rhinovirus C have also been discovered in the past few years (Drexler et al., 2008; Jones et al., 2007; Lau et al., 2007, 2009; McErlean et al., 2008). We have described the discovery of novel picornaviruses from wild dead birds as well as from bats of diverse species in Hong Kong (Lau et al., 2011; Woo et al., 2010). Recently, we also reported the first discovery of novel picornaviruses in domestic cats and dogs (Lau et al., 2012; Woo et al., 2012a, b). The identification of novel picornaviruses and previously unknown animal hosts for these viruses is crucial for a better understanding of their genetic diversity, evolution, biology, and potential for cross-species transmission and emergence.

Camels are one of the most unique mammals on earth that have shown perfect adaptation to desert life. There are two surviving Old World camel species, Camelus dromedarius (dromedary or one-humped camel), which inhabits the Middle East and North and North-east Africa, and Camelus bactrianus (Bactrian or two-humped camel), which inhabits Central Asia. Among the 20 million camels on earth, 90 % are dromedaries. Before 2013, viruses of at least eight families had been found to infect camels. The recent emergence of Middle East respiratory syndrome coronavirus (MERS-CoV) from the Middle East and the discovery of the virus from nasal samples of dromedaries have boosted interest in the search for novel viruses in dromedaries (de Groot et al., 2013; Lau et al., 2013a; Perera et al., 2013; Reusken et al., 2013; Zaki et al., 2012). Recently, we reported the discovery of a novel dromedary coronavirus, UAE-HKU23, and a novel genotype of hepatitis E virus (HEV) from dromedaries in the Middle East (Woo et al., 2014a, b). As dromedaries are not known as either a reservoir or as being susceptible to any known picornviruses, we hypothesized that previously unrecognized picornviruses may be present in dromedaries. In this article, we described the discovery of a novel picornavirus, dromedary camel enterovirus (DcEV), in dromedaries from the Middle East. Comparative genomic and phylogenetic analyses showed that the virus belonged to the genus Enterovirus in the family Picornaviridae. The virus was detected in 1.9 % of faecal samples from dromedaries included in this study, with a seroprevalence of 52 % for IgG by Western blot analysis. Based on our results, we propose that DcEV represents a novel species in the genus Enterovirus in the family Picornaviridae.

**RESULTS**

**Dromedary surveillance and identification of a novel picornavirus**

Reverse transcription PCR (RT-PCR) for a 112 bp fragment in the 5’ UTR of picornviruses was positive in specimens from the faecal samples of four (1.9 %) of 215 dromedaries [one (0.5 %) adult dromedary and three (25 %) dromedary calves]. The sequences from these positive samples had <80 % nucleotide identity to the corresponding parts of the 5’ UTR in all other known picornviruses, suggesting the presence of a novel picornavirus (Fig. 1a).

**Genome organization and coding potential of DcEV**

The size of the two genomes of DcEV sequenced (19CC and 20CC) was 7426 bases, after excluding the polyadenylated tract, and the G+C content was 45 mol%. Each genome contained a large ORF of 6507 bases, encoding a potential polyprotein precursor of 2168 aa. The genome organization was similar to other picornviruses, with the characteristic gene order 5’-VP4-VP2-VP3-VP1-2A-2B-2C-3A-3B-3Cpro-3Dpol-3’. The hypothetical protease cleavage sites of the polyprotein, as determined by multiple alignments with other enteroviruses, were analysed. The amino acids at the cleavage sites at the junctions between VP2/VP3, 2A/2B, 2C/3A, 3A/3B, 3B/3C and 3C/3D were identical compared to most or all known enteroviruses, but those at the cleavage sites at the junctions between VP4/VP2, VP3/VP1, VP1/2A and 2B/2C were more variable among the different members of the genus Enterovirus.

**Phylogenetic analyses**

The phylogenetic trees were constructed using the amino acid sequences of P1, P2 (excluding 2A) and P3 (excluding 3A) of DcEV and other picornviruses are shown in Fig. 1(b–d), and the corresponding pairwise amino acid identities are shown in Table 1. The 2A and 3A regions were excluded to avoid bias due to poor sequence alignment. For all three regions, DcEV possessed higher amino acid identities to the corresponding regions or genes of other members of the genus Enterovirus, especially members of the species Enterovirus E and Enterovirus F, than to those of other picornviruses (Table 1). Phylogenetic analyses also showed that the two DcEV strains were clustered with other members of the genus Enterovirus and were most closely related to bovine enterovirus group A (Enterovirus E) and bovine enterovirus group B (Enterovirus F) in all three trees (Fig. 1b–d).

**Genome analyses**

The 5’ UTR of DcEV (strain 19CC) contained seven domains (Fig. 2) and shared 63–66 % nucleotide identity to those of porcine enteroviruses 9 and 10 (GenBank accession nos AF363453 and AF363455), bovine enterovirus I (D00214), human enterovirus B (AY466029) and human coxsackievirus B3 (AY752944). Domain I contained 86 nt (nt 1–86) and formed a cloverleaf structure, whereas domains II, III, IV, V and VI were the main domains of the internal ribosome entry site (IRES) element, responsible for directing the initiation of translation in a cap-independent manner, which requires both canonical translation initiation and IRES trans-acting factors (Shih et al., 2011). Between domains I and II, an additional region of about 131 nt, previously only recognized in bovine enteroviruses (Zell et al., 1999), was also observed in DcEV. This
insertion possessed the potential to form a stem–loop (domain I**) and second cloverleaf structure (domain I*). Similar to other members of the genus Enterovirus, the IRES of DcEV is a putative type I element (Fig. 2). The putative translation initiation site of DcEV was contained by an optimal Kozak context AAGAUGG, with an in-frame AUG at position 848. Upstream of the AUG start codon, the Yn-Xm-AUG motif was present at domain V (nt 667–695). Similar to other enteroviruses, there was no L protein in the polyprotein of DcEV.


**Fig. 1.** (a) Phylogenetic analysis of partial 5′ UTR sequences of dromedary picornaviruses detected in this study. The strains in the present study are in bold, with ‘C’ representing adult dromedary samples and ‘CC’ representing dromedary calf samples. Asterisks indicate the strains for which complete genome sequencing was performed in this study. Bootstrap values expressed as percentages are shown at nodes and only those >70% are shown. Bar, estimated number of substitutions per 20 nt. EV, enterovirus; HRV, human rhinovirus; FMDV-O, foot-and-mouth disease virus type O (GenBank accession no. NC_004004). (b–d) Phylogenetic analyses of the P1 (b), P2 (excluding 2A) (c) and P3 (excluding 3A) (d) regions of two strains of DcEV (shown in bold). Bootstrap values expressed as percentages are shown at nodes and only those >70% are shown. Bars, estimated number of substitutions per 50 aa.
Table 1. Comparison of genomic features of DcEV and other enteroviruses, and amino acid identities between the predicted P1, P2 (excluding 2A) and P3 (excluding 3A) regions of DcEV and the corresponding proteins of other enteroviruses

<table>
<thead>
<tr>
<th>Enterovirus species</th>
<th>Representative member</th>
<th>GenBank accession no.</th>
<th>Size (nt)</th>
<th>G + C content (mol%)</th>
<th>P1</th>
<th>P2*</th>
<th>P3†</th>
<th>P1</th>
<th>P2*</th>
<th>P3†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterovirus A</td>
<td>Human enterovirus A</td>
<td>NC_001612</td>
<td>7413</td>
<td>48</td>
<td>55.0</td>
<td>58.0</td>
<td>60.0</td>
<td>54.9</td>
<td>57.8</td>
<td>59.9</td>
</tr>
<tr>
<td>Enterovirus B</td>
<td>Human enterovirus B</td>
<td>NC_001472</td>
<td>7389</td>
<td>47</td>
<td>51.0</td>
<td>61.1</td>
<td>67.2</td>
<td>51.2</td>
<td>61.3</td>
<td>66.9</td>
</tr>
<tr>
<td>Enterovirus C</td>
<td>Poliovirus</td>
<td>NC_002058</td>
<td>7440</td>
<td>46</td>
<td>45.3</td>
<td>54.9</td>
<td>64.8</td>
<td>45.3</td>
<td>54.9</td>
<td>64.9</td>
</tr>
<tr>
<td>Enterovirus D</td>
<td>Human enterovirus D</td>
<td>NC_001430</td>
<td>7390</td>
<td>43</td>
<td>51.6</td>
<td>57.4</td>
<td>64.5</td>
<td>51.3</td>
<td>57.7</td>
<td>64.5</td>
</tr>
<tr>
<td>Enterovirus E</td>
<td>Bovine enterovirus</td>
<td>NC_001859</td>
<td>7414</td>
<td>49</td>
<td>71.1</td>
<td>68.8</td>
<td>80.7</td>
<td>71.0</td>
<td>69.0</td>
<td>80.1</td>
</tr>
<tr>
<td>Enterovirus F</td>
<td>Enterovirus F</td>
<td>KC748420</td>
<td>7433</td>
<td>50</td>
<td>72.4</td>
<td>75.2</td>
<td>89.2</td>
<td>72.2</td>
<td>75.5</td>
<td>88.9</td>
</tr>
<tr>
<td>Enterovirus G</td>
<td>Porcine enterovirus B</td>
<td>NC_004441</td>
<td>7388</td>
<td>46</td>
<td>59.6</td>
<td>73.4</td>
<td>72.3</td>
<td>59.8</td>
<td>73.6</td>
<td>72.1</td>
</tr>
<tr>
<td>Enterovirus H</td>
<td>Simian enterovirus A</td>
<td>NC_003988</td>
<td>7374</td>
<td>43</td>
<td>50.4</td>
<td>56.4</td>
<td>64.4</td>
<td>50.6</td>
<td>56.6</td>
<td>63.9</td>
</tr>
<tr>
<td>Enterovirus J</td>
<td>Enterovirus J</td>
<td>NC_013695</td>
<td>7379</td>
<td>43</td>
<td>56.7</td>
<td>60.7</td>
<td>66.6</td>
<td>57.0</td>
<td>60.5</td>
<td>66.3</td>
</tr>
<tr>
<td>Rhinovirus A</td>
<td>Human rhinovirus A</td>
<td>NC_001617</td>
<td>7152</td>
<td>39</td>
<td>46.0</td>
<td>47.3</td>
<td>54.1</td>
<td>45.9</td>
<td>47.6</td>
<td>54.1</td>
</tr>
<tr>
<td>Rhinovirus B</td>
<td>Human rhinovirus B</td>
<td>NC_001490</td>
<td>7212</td>
<td>40</td>
<td>45.9</td>
<td>50.7</td>
<td>57.3</td>
<td>46.0</td>
<td>51.2</td>
<td>57.0</td>
</tr>
<tr>
<td>Rhinovirus C</td>
<td>Human rhinovirus C</td>
<td>NC_000996</td>
<td>7099</td>
<td>43</td>
<td>44.0</td>
<td>45.6</td>
<td>53.9</td>
<td>44.0</td>
<td>45.8</td>
<td>53.9</td>
</tr>
<tr>
<td>Unclassified</td>
<td>DcEV_19CC</td>
<td>KP345887</td>
<td>7426</td>
<td>45</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>99.0</td>
<td>99.3</td>
<td>98.7</td>
</tr>
<tr>
<td>Unclassified</td>
<td>DcEV_20CC</td>
<td>KP345888</td>
<td>7426</td>
<td>45</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>99.0</td>
<td>99.3</td>
<td>98.7</td>
</tr>
</tbody>
</table>

*P2 region excluding 2A.
†P3 region excluding 3A.

B. Enterovirus G and Enterovirus J but different from those of Enterovirus A, Enterovirus C, Enterovirus D, Enterovirus E, Enterovirus F, Enterovirus H, Rhinovirus A, Rhinovirus B and Rhinovirus C, the cleavage site at the junction of VP1/2A in the genome of DcEV was Thr/Gly. Similar to most other enteroviruses, the VP1 of DcEV possessed the [PS]ALXAXETG motif.

The P2 regions in the genomes of DcEV encode the non-structural proteins 2A, 2B and 2C. Similar to members of the species Enterovirus A, Enterovirus D, Enterovirus E, Enterovirus F, Enterovirus H, Rhinovirus A, Rhinovirus B and Rhinovirus C, but different from those of Enterovirus B, Enterovirus C, Enterovirus F, Enterovirus G, Rhinovirus B and Rhinovirus C, the cleavage site at the junction of 2B/2C in the genome of DcEV was Gln/Ser. The 2A protein of picornaviruses is a highly variable region (9–305 aa). The 2A protein of DcEV (150 aa) exhibited only 37–82 % (median 62 %) amino acid identity to those of other members of the genus Enterovirus. This 2A protein shared 98.7 % amino acid identity between the two DcEV strains. The 2A of DcEV possessed the characteristic chymotrypsin-like structures with cysteine-reactive catalytic sites. A putative catalytic triad of His-Asp-Cys found in the 2A proteins of enteroviruses and rhinoviruses was also identified (Hughes & Stanway, 2000; Ryan & Flint, 1997). The conserved GXCXG motif in the chymotrypsin-like protease, present in the 2A proteins of other enteroviruses, was also identified in the 2A protein of DcEV as GDCG. Similar to other enteroviruses, the Asn-Pro-Gly-Pro (NPGP) motif found in 2A and 2B of avihepatoviruses and avisiviruses, required for co-translational cleavage (Ryan & Flint, 1997), was absent in DcEV. The conserved H-box/NC motif involved in cell proliferation control was also absent (Hughes & Stanway, 2000; Tseng et al., 2007; Woo et al., 2010). Similar to other picornaviruses, 2C of DcEV possessed the GXXGXGKS motif for NTP binding (Gorbalenya et al., 1989b).

The P3 region in the genome of DcEV encodes 3A, 3B (VPg, small genome-linked protein), 3Cpro (protease) and 3Dpol (RNA-dependent RNA polymerase). Similar to the 3Cpro of other enteroviruses, the catalytic triad of the 3Cpro of DcEV was His-Glu-Cys. This is different from the 3Cpro of picornaviruses of some other genera, such as Avihepatovirus, Hepatovirus, Megrivivirus, Parechovirus, Tremovirus and Avisivirus, which have catalytic triads of His-Asp-Cys. Similar to all other picornaviruses, 3Cpro also contained the conserved GXXG motif, which forms part of the active site of the protease and the conserved GXH motif. Similar to all other enteroviruses as well as hepatoviruses and tremoviruses, 3Dpol also contained the conserved RNA-binding motif, KPRD I (Gorbalenya et al., 1989a; Hämmerle et al., 1992). The 3Dpol of DcEV contained the conserved KDE[LIR], GG[LMM]PSG, YGDD and FLKR motifs (Kamer & Argos, 1984).

Using the two DcEV genome sequences for analysis, the ratio of the number of nonsynonymous substitutions per nonsynonymous site to the number of synonymous substitutions per synonymous site (Ks/Ka) for the various coding regions...
was calculated (Table 2). All $K_s/K_a$ ratios were generally low, suggesting that DcEV is stably evolving in dromedaries.

**Seroprevalence of DcEV in dromedaries**

To determine the seroprevalence of DcEV in dromedaries, Western blot analysis using the recombinant DcEV VP1 polypeptide was performed. For IgG detection, prominent immunoreactive protein bands of about 36 kDa were observed in 89 (52 %) of the tested serum samples from 172 dromedaries in a recombinant DcEV VP1 protein-based Western blot assay (Fig. 3).

**Virus culture**

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

---

<table>
<thead>
<tr>
<th>Putative protein</th>
<th>No. of amino acids</th>
<th>$K_s$</th>
<th>$K_a$</th>
<th>$K_s/K_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP4</td>
<td>69</td>
<td>0.0064</td>
<td>Not calculable*</td>
<td>–</td>
</tr>
<tr>
<td>VP2</td>
<td>246</td>
<td>0.0042</td>
<td>0.0572</td>
<td>0.0741</td>
</tr>
<tr>
<td>VP3</td>
<td>242</td>
<td>0.0023</td>
<td>0.0625</td>
<td>0.0364</td>
</tr>
<tr>
<td>VP1</td>
<td>277</td>
<td>0.0075</td>
<td>0.0651</td>
<td>0.1154</td>
</tr>
<tr>
<td>2A</td>
<td>150</td>
<td>0.0075</td>
<td>0.0851</td>
<td>0.0881</td>
</tr>
<tr>
<td>2B</td>
<td>99</td>
<td>0.0058</td>
<td>0.2871</td>
<td>0.0202</td>
</tr>
<tr>
<td>2C</td>
<td>329</td>
<td>0.0035</td>
<td>0.1727</td>
<td>0.0201</td>
</tr>
<tr>
<td>3A</td>
<td>89</td>
<td>0.0000</td>
<td>0.0133</td>
<td>0.0000</td>
</tr>
<tr>
<td>3B</td>
<td>23</td>
<td>0.0000</td>
<td>0.0719</td>
<td>0.0000</td>
</tr>
<tr>
<td>3C</td>
<td>183</td>
<td>0.0142</td>
<td>0.0832</td>
<td>0.1709</td>
</tr>
<tr>
<td>3D</td>
<td>456</td>
<td>0.0051</td>
<td>0.0446</td>
<td>0.1147</td>
</tr>
</tbody>
</table>

*VP4 is short and there is only one D/E substitution.
DISCUSSION

We have reported the discovery of a novel picornavirus from dromedaries in the Middle East. Although some picornaviruses are able to infect Bactrians and cause diseases such as foot-and-mouth disease, dromedaries have not been known to be susceptible to these agents as demonstrated by four independent infection trials, and camels are not the reservoir of these picornaviruses (Wernery et al., 2014). In this study, a novel picornavirus, DcEV, was detected in 25% of dromedary calves but only 0.5% of adult dromedaries. This was in line with the high seroprevalence of 52% among tested dromedaries for IgG against recombinant DcEV VP1 polypeptide detected by Western blot analysis, suggesting that DcEV infection is common in the dromedary population studied. Interestingly, this much higher detection rate of acute DcEV infection in dromedary calves compared with adult dromedaries and a high seroprevalence rate is very similar to the situation observed in dromedary camel coronavirus UAE-HKU23 infection, which also showed a high detection rate (>20%) of acute infection in dromedary calves compared with adult dromedaries (0.4%) and a high seroprevalence rate of >50% (Woo et al., 2014b). The low $K_p/K_s$ ratio of all coding regions of the DcEV genome showed that the virus is stably evolving in dromedaries. All these results suggest that dromedaries are the natural reservoir of DcEV, rather than animals that are just occasionally infected by the virus. Further molecular epidemiological and serological studies on Bactrians as well as other camels will be important to understand the host specificity of DcEV.

Phylogenetic and genomic analyses showed that DcEV belongs to the genus Enterovirus in the family Picornaviridae. Phylogenetic analysis of P1, P2 (excluding 2A) and P3 (excluding 3A) using two complete genomes of DcEV showed unambiguously that DcEV clusters with other members of the genus Enterovirus. Comparative genome analysis also showed that there were higher amino acid identities between DcEV and members of the genus Enterovirus than with members of the other genera in the family Picornaviridae (data not shown). Moreover, DcEV possessed genomic features similar to other members of the genus Enterovirus. Similar to other enteroviruses and rhinoviruses, DcEV has a type I IRES. Its genome does not encode an L protein. In the P1 region, VP0 is cleaved into VP4 and VP2 with the cleavage site at Lys/Ser, and VP1 possesses the [PS]ALXAXETG motif. In the P2 region, 2A has the putative catalytic triad of His-Asp-Cys and conserved CXCG motif that enables it to function as a chymotrypsin-like protease, and 2C has the NTPase motif GXXGXGXS. In the P3 region, 3Cpro has the putative catalytic triad of His-Glu-Cys and has a genome composition that varies by ≤2.5% (King et al., 2011). Although DcEV is most closely related to members of the species Enterovirus E and Enterovirus F with >70% amino acid identity in their polyproteins, >60% amino acid identity in their P1 and >70% amino acid identity in their 2C + 3CD, the G + C contents of DcEV (45 mol%) differed from those of members of the species Enterovirus E and Enterovirus F (49–50%) by 4–5%. Moreover, the natural host of DcEV is dromedaries, whereas that for members of the species Enterovirus E and Enterovirus F is cattle. Therefore, DcEV should be a distinct species in the genus Enterovirus.

Recent studies have revealed a previously unrecognized spectrum of viruses in dromedaries. Dromedaries are known to be reservoirs of viruses in the families Flaviviridae, Herpesviridae, Papillomaviridae, Paramyxoviridae, Poxviridae, Reoviridae and Rhabdoviridae. In the last 2 years, studies have shown that dromedaries are the hosts of at least two distinct coronaviruses. MERS-CoV is a lineage C betacoronavirus closely related to bat coronaviruses discovered by us (Lau et al., 2013b; Memish et al., 2013; Yang et al., 2014), whereas DcCoV UAE-HKU23 is a lineage A betacoronavirus closely related to other members of the species Betacoronavirus 1 found in humans and at least 15 other mammalian species (Woo et al., 2014b). Serological studies have shown that minimal cross-reactivity exists between MERS-CoV and DcCoV UAE-HKU23 (Woo et al., 2014b). Recently, we also described the discovery of HEV in dromedaries (Woo et al., 2014a). This has not only led to the discovery of a novel genotype of HEV but is also the first description of different genomic features in different strains of the same HEV (Woo et al., 2014a). Although
a study has shown that HEV is the commonest cause of acute hepatitis in Dubai (Abro et al., 2009), no sequence information from the HEV strains involved is available. Further genomic studies on HEV from Dubai patients should reveal the importance of dromedaries as a possible source of HEV in the Middle East. Recently, our metagenomics study has shown that dromedaries harbour a large number and wide variety of circovirus-like viruses and picobirnaviruses (Woo et al., 2014c). Including the present study, dromedaries have been shown to be the reservoirs of five additional families of viruses: Circoviridae, Coronaviridae, Hepeviridae, Picobirnaviridae and Picornaviridae (Woo et al., 2014; Woo et al., 2014a, b, c). Further studies should reveal the roles of these viruses in dromedaries, as well as their possible transmission to other animals and humans.

**METHODS**

**Dromedary surveillance and sample collection.** All dromedary faecal samples were left-over specimens submitted for pathogen screening to the Central Veterinary Research Laboratory in Dubai, United Arab Emirates (UAE), from January to February 2013. A total of 215 faecal samples from 203 adult and teenager (≥1-year-old) dromedaries (C. dromedarius) and 12 dromedary calves (<1-year-old) had been tested. All 172 serum samples of dromedaries were also left-over specimens submitted for routine checks to the Central Veterinary Research Laboratory in Dubai in the same period. The serum samples were not collected from the same dromedaries as the faecal samples.

**RNA extraction.** Viral RNA was extracted from the faecal samples using an EZI Virus Mini kit v.2.0 (Qiagen). The RNA was eluted in 60 μl AVE buffer (Qiagen) and was used as the template for RT-PCR.

**RT-PCR of the 5’ UTR of picornaviruses using conserved primers and DNA sequencing.** Initial picornavirus screening was performed by amplifying a 112 bp fragment of the 5’ UTR of picornaviruses using primers (5’-GGGCCCCYGAATGYGGCTAA-3’ and 5’-ACACCGGACACCCAAAAAGTGT-3’) targeting conserved sequences as published previously (Lau et al., 2011; Woo et al., 2010; Yip et al., 2010). Reverse transcription was performed using a SuperScript III kit (Invitrogen) and the reaction mixture (10 μl) contained RNA, first-strand buffer [50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2], 5 mM dithiothreitol, 50 ng random hexamers, 500 μM each dNTP and 100 U SuperScript III reverse transcriptase. The mixtures were incubated at 25 °C for 5 min, followed by 50 °C for 60 min and 70 °C for 15 min. The PCR mixture (25 μl) contained cDNA, PCR buffer [10 mM Tris/HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 0.01% gelatin], 200 μM each dNTP and 1.0 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, with 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-positive result was observed in negative controls.

All PCR products were gel purified using a QIAquick gel extraction kit (Qiagen). Both strands of the PCR products were sequenced twice with an ABI Prism 3730xl DNA Analyser (Applied Biosystems) using the two PCR primers. The sequences of the PCR products were compared with known sequences of the 5’ UTR of picornaviruses in GenBank.

**Genome sequencing.** Two complete genomes of DcEV, including the full 5’ UTR regions, were amplified and sequenced using strategies that we used previously for complete genome sequencing of other picornaviruses, with the RNA extracted from the faecal samples as templates (Lau et al., 2011; Woo et al., 2010; Yip et al., 2010). The RNA was converted to cDNA by a combined random-priming and oligo(dT) priming strategy. As initial results showed that DcEV was most closely related to members of the species Enterovirus E, Enterovirus F and Enterovirus G in the genus Enterovirus, the cDNA was amplified by degenerate primers designed by multiple alignment of the genomes of bovine enteroviruses and porcine enteroviruses (GenBank accession nos Y14459.1, HQ702854.1, AF363455.1, AY508697.1, DQ092770.1 and HQ663846.1), and additional primers designed from the results of the first and subsequent rounds of sequencing. The 5’ ends of the viral genomes were confirmed by rapid amplification of cDNA ends using a SMARTer RACE cDNA Amplification kit (Clontech). Sequences were checked manually and assembled to produce the final sequences of the full viral genomes.

**Genome analysis.** The nucleotide sequences of the genomes and the deduced amino acid sequences of the encoded polyproteins were compared to those of other picornaviruses. The unrooted phylogenetic tree of the 5’ UTR was constructed using the neighbour-joining method for aligned nucleotide sequences in CLUSTAL W v.2.1. The maximum-likelihood phylogenetic tree of P1, P2 (excluding 2A) and P3 (excluding 3A) were constructed using the PhyML 3.0 program (Guindon & Gascuel, 2003), with bootstrap values calculated from 1000 trees. Secondary structure prediction in the 5’ UTR was performed using the RNAstructure Web Server on strain 19CC (Reuter & Mathews, 2010).

**Estimation of non-synonymous and synonymous substitution rates.** The ratio of Ks/Ka for each coding region among the two strains of DcEV was calculated using the KaKs_Calculator Toolbox v.2.0 (Wang et al., 2010).

**Cloning and purification of 6 X His-tagged recombinant DcEV VP1 polypeptide.** As VP1 is the most surface-exposed virion protein and contains most of the motifs important for interaction with neutralizing antibodies and cellular receptors in picornaviruses (Racaniello, 2007), the sequence encoding the VP1 polypeptide of DcEV was cloned and the expressed recombinant protein was purified according to previously described strategies (Francis et al., 1987; Lau et al., 2005, 2010). To produce plasmid for expression of fusion protein containing affinity tags needed for its purification, primers 5’-CTAGCTAGCCACACAAAAAGTAGCTTGTCAG-3’ and 5’-GGCTCTTCTATAACAAACGCGTACGTAGCATGACGC-3’ were used to amplify the VP1 gene of DcEV by RT-PCR. The sequence coding for a total of 278 aa was amplified and cloned into the NdeI site of expression vector pET-28b(+ ) (Novagen) in-frame and downstream of the series of six His codons. The 6 X His-tagged recombinant VP1 polypeptide was expressed and purified using a Ni2+ -loaded HitTrap Chelating System (GE Healthcare) according to the manufacturer’s instructions.

**Western blot analysis.** To determine the seroprevalence of DcEV, Western blot analysis, using purified DcEV VP1 polypeptide and serum samples from dromedaries, was performed as described previously (Lau et al., 2005, 2010). Briefly, 100 ng purified 6 X His-tagged recombinant VP1 polypeptide of DcEV was analysed by SDS-PAGE (10 % acrylamide) and subsequently electroblotted onto a nitrocellulose membrane (Bio-Rad). The blot was cut into strips and the strips were incubated separately with 1:1000 dilutions of sera collected from dromedaries. Antigen–antibody interaction was detected with 1:4000 diluted HRP-conjugated goat anti-llama IgG (Novex) and an enhanced chemiluminescence fluorescence system (GE Healthcare). The intensity of the band was measured using ImageJ. A serum sample was regarded as positive if the intensity of the band generated was more than 10 times that of the background intensity.
**Virus culture.** The four faecal samples positive for DcEV were cultured in Vero (African green monkey kidney; ATCC CCL-81), MDBK (Madin–Darby bovine kidney; ATCC CCL-22), Dubca (Arabian camel fibroblast, ATCC CRL-2276) and Caki-3-R (Arabian camel kidney, CCLV-RIE 1284) cells. After centrifugation, the faecal samples were diluted fivefold with virus transport medium and filtered. Two hundred microlitres of the filtrate was inoculated into 200 μl MEM, and 400 μl mixture was added to 24-well tissue culture plates, with each of the cell lines, by adsorption inoculation. After 1 h of adsorption, the wells were washed twice with PBS solution, and the medium was replaced with 1 ml serum-free MEM. The cultures were incubated at 37 °C with 5% CO₂ and inspected daily by inverted microscopy for cytopathic effects. After 1 week of incubation, sub-culturing to a fresh cell line was performed, even if there were no cytopathic effects, and culture lysates were collected for RT-PCR for DcEV. Three blind passages were carried out for each sample.

**ACKNOWLEDGEMENTS**

We thank Dr Wing-Man Ko, Secretary for Food and Health, and Dr Constance Chan, Director of the Department of Health, Hong Kong Special Administrative Region (HKSAR), PR China, for their continuous support. This work is partly supported by the HKSAR Health and Medical Research Fund, Strategic Research Theme Fund, University of Hong Kong, Research Council Grant, University Grant Council and Consultancy Service for Enhancing Laboratory Surveillance of Emerging Infectious Disease for the HKSAR Department of Health.

**REFERENCES**


Novel dromedary camel enterovirus


