Isolation and characterization of a novel type of rotavirus species A in sugar gliders (Petaurus breviceps)

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To estimate the risk of interspecies transmission of rotavirus species A (RVA) from exotic pets to other mammalian species, the prevalence of RVA in sugar gliders (Petaurus breviceps) was investigated. RVAs were detected in 10 of 44 sugar gliders by reverse transcription (RT)-semi-nested PCR. These viruses were classified as G27P[3] and G27P[36] genotypes, with G27 and P[36] being new genotypes as assigned by the Rotavirus Classification Working Group. To characterize sugar glider RVA in detail, one strain, RVA/SugarGlider-tc/JPN/SG385/2012/G27P[36] (SG385-tc), was isolated. All of the genes of the strain were classified as new genotypes (G27-P[36]-I19-R10-C10-M9-A20-N11-T13-E17-H12). The enterotoxin domain in NSP4, which is important for the induction of diarrhoea, was conserved between SG385-tc and previously reported mammalian strains, suggesting the potential of sugar glider RVA to cause diarrhoea in mammalian species. In fact, seven out of nine suckling mice inoculated orally with 3.9 × 10⁴ f.f.u. of strain SG385-tc had diarrhoea and the 50 % diarrhoea-inducing dose (DD₅₀) of strain SG385-tc in suckling mice was 1.2 × 10³ f.f.u. Our findings suggest that sugar glider RVA is infective to and possibly pathogenic in other mammalian species.

INTRODUCTION

Rotavirus species A (RVA), a member of the Reoviridae family, is a major cause of acute dehydrating diarrhoea in infants and young mammals and birds worldwide. Rotavirus-induced diarrhoea is a serious public health problem, responsible for about 200 000 deaths per year in children younger than 5 years of age (Lanata et al., 2013). In domestic animals, rotavirus-associated enteritis is also a major problem, particularly in calves and piglets (Martella et al., 2010).

RVA possesses a genome of 11 segments of dsRNA, which encodes six viral structural proteins (VP1–VP4, VP6 and VP7) and six non-structural proteins (NSP1–NSP6). Because of the segmented nature of the genome, reassortment events can occur in a cell co-infected with two or more different strains (Estes & Greenberg, 2013).

Today, RVA is classified by using a system established by the Rotavirus Classification Working Group (RCWG) that is based on the nucleotide sequences of all 11 genome segments (Matthijnssens et al., 2008a, b). To date, RVA has been classified into 27 G, 37 P, 17 I, 9 R, 9 C, 8 M, 18 A, 10 N, 12 T, 16 E and 11 H genotypes (relating to VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4 and NSP5 genes, respectively) (Badaracco et al., 2013; Guo et al., 2012; Jere et al., 2014; Matthijnssens et al., 2011a; Trojnar et al., 2013).

Surveillance studies of RVA have focused mainly on humans and domestic animals (Matthijnssens & Van Ranst, 2012; Matthijnssens et al., 2008a, 2011a; Papp et al., 2013a, b) and, to a lesser extent, on companion animals (German...
et al., 2015; Matthijnssens et al., 2011a). RVA has also been detected in several wild and zoo animals (Baumeister et al., 1983; Coria-Galindo et al., 2009; Eugster et al., 1978; Evans, 1984; Everest et al., 2009, 2010, 2011; Hamir et al., 1990; He et al., 2013; Kim et al., 2014; Linhares et al., 1986; Marcoppido et al., 2011; Tzipori et al., 1976), and it has been indicated that wild animals may be involved in the natural infection cycle of RVA and also represent a potential zoonotic risk (Abe et al., 2010; Esona et al., 2010; Okadera et al., 2013). Pets are considered to represent a higher potential zoonotic risk than wild animals since they share habitats with humans, thus providing a rationale to investigate the prevalence of RVA in pets to evaluate the risk of transmission of RVA from pets to humans. Indeed, a number of reports have shown that interspecies transmission of RVA has probably occurred between cats or dogs and humans (De Grazia et al., 2010; German et al., 2015; Grant et al., 2011; Khamrin et al., 2006, 2007; Martella et al., 2010; Matthijnssens et al., 2011b; Mochizuki et al., 1997; Nakagomi et al., 1999; Rahman et al., 2007; Tsugawa & Hoshino, 2008; Wang et al., 2013). However, little is known about infection of pets, such as exotic animals, with RVA.

In Japan, sugar gliders (Petaurus breviceps) are popular as exotic pets. In Canada, human Salmonella infections that were found to be associated with sugar gliders have been reported (Woodward et al., 1997), indicating that sugar gliders represent a potential risk of transmission of enteric pathogens to humans. However, there are no reports about infection of sugar gliders with RVA. In this study, to estimate the risk of interspecies transmission of RVA from exotic pets to other mammalian species, the prevalence of RVA in sugar gliders was investigated. RVA isolates in sugar gliders were analysed genetically and the pathogenicity of sugar glider RVA in other mammalian species was assessed.

**RESULTS**

**Detection of RVA in faeces of sugar gliders by reverse transcription (RT)-semi-nested PCR**

Faeces or colon contents from 44 sugar gliders were examined for RVA (Table 1). Among these 44 sugar gliders, 28 were babies (2 months old) that had been imported from Thailand, Taiwan and Malaysia into Japan as pets, and 16 were adults (>1 year old) that had been reared in Japan for breeding.

RVA strains were detected in 10 of the 44 sugar gliders (Table 1). All of the sugar gliders in which RVA was detected were babies. RVA was detected in 35.7 % (10/28) of the baby sugar gliders including 16.7 % (1/6), 90.0 % (9/10) and 0.0 % (0/12) of the baby sugar gliders that had been bred in Thailand, Taiwan and Malaysia, respectively. The RVA strain that was detected in the Thai sugar glider was named strain SG33 (designated Thailand strain in this paper) and those detected in the Taiwanese sugar gliders were named strains SG385–387 and SG389–394 (designated Taiwan strains in this paper). The faeces that were confirmed to contain RVA were all of a normal consistency.

**Complete ORF analyses of VP4 and VP7 genes of RVA strains detected in faeces of sugar gliders**

The complete or nearly complete ORF sequences of VP4 and VP7 genes of the 10 RVA strains that were detected in faeces of sugar gliders were determined. The N-terminal residues (17 bp) of the ORF region of the VP4 gene of Thailand strain were not determined because of the limited amount of the faecal sample. The nucleotide identities of VP4 and VP7 genes among Taiwan strains were 98.9–100 % and 99.7–100 %, respectively. The identities of VP4 and VP7 genes between Thailand strain and the Taiwan strains ranged from 76.6 % to 76.8 % and 80.3 % to 80.6 %, respectively.

According to the genotyping recommendations of the RCWG (Matthijnssens et al., 2008b), Thailand strain and Taiwan strains were classified as G27P[3] and G27P[36] genotypes, with G27 and P[36] being new genotypes as assigned by the RCWG, respectively (Table 2). Although the VP4 gene of Thailand strain was classified as an established P[3] genotype, phylogenetic analysis revealed that Thailand strain and previously reported P[3] genotype strains were only distantly related (Fig. 1).

<table>
<thead>
<tr>
<th>Age</th>
<th>Birthplace*</th>
<th>Date of collection</th>
<th>Positive/samples</th>
<th>Detection rate (%)</th>
<th>Strain detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 months (baby)</td>
<td>Thailand</td>
<td>Dec. 2010</td>
<td>1/6</td>
<td>16.7</td>
<td>SG33</td>
</tr>
<tr>
<td></td>
<td>Taiwan</td>
<td>Feb. 2012</td>
<td>9/10</td>
<td>90.0</td>
<td>SG385, SG386, SG387, SG389, SG390, SG391, SG392, SG393 and SG394</td>
</tr>
<tr>
<td></td>
<td>Malaysia</td>
<td>Nov. 2012</td>
<td>0/12</td>
<td>0.0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>10/28</td>
<td>35.7</td>
<td>–</td>
</tr>
<tr>
<td>&gt;1 year (adult)</td>
<td>Japan</td>
<td>Jan.–Feb. 2012</td>
<td>0/6</td>
<td>0.0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Indonesia</td>
<td>Feb. 2012</td>
<td>0/10</td>
<td>0.0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>0/16</td>
<td>0.0</td>
<td>–</td>
</tr>
</tbody>
</table>

*Imported into Japan.
The nucleotide identities between Thailand strain and those strains were 81.6–88.2 %.

**Virus isolation and complete ORF analysis of all gene segments of the isolated virus**

To characterize sugar glider RVA in detail, one strain, RVA/SugarGlider-tc/JPN/SG385/2012/G27P[36] (designated SG385-tc in this paper), was isolated from a Taiwanese sugar glider. Briefly, the faecal suspension that was confirmed to contain strain SG385 was inoculated onto rhesus monkey kidney (MA-104) cells after trypsin treatment, and a cytopathic effect was observed at 3 days post-inoculation (p.i.). The culture supernatant at 5 days p.i. was collected and the presence of RVA in the supernatant was confirmed by RT-semi-nested PCR. The virus titre of strain SG385-tc at passage 3, which was used for the complete ORF analyses and animal inoculation, was $7.7 \times 10^5$ f.f.u. ml$^{-1}$.

To analyse strain SG385-tc genetically, the complete ORF sequences of all gene segments of the strain were determined. All the genes of the strain were assigned to be new genotypes by the RCWG; the complete genotype constellation was found to be G27-P[36]-I19-R10-C10-M9-A20-N11-T13-E17-H12 (relating to VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 genes) (Table 3). This finding shows that strain SG385-tc is a novel RVA.

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**Table 2. Genotypes of VP4 and VP7 genes of strain SG33 (Thailand) and strains SG385–387 and SG389–394 (Taiwan) and strains exhibiting the highest nucleotide identities**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gene</th>
<th>Genotype</th>
<th>Strain exhibiting highest identity</th>
<th>Nucleotide identity (%)</th>
<th>Cut-off value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VP7</td>
<td>G27</td>
<td>RVA/Pig-wt/BRA/PGRV12/2011/G3P[X]</td>
<td>79.8</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>VP7</td>
<td>G27</td>
<td>RVA/Human-wt/THA/CMH222/2001/G3P[3]</td>
<td>79.6–79.7</td>
<td>80</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Phylogenetic tree based on the nucleotide sequences of VP4 genes (nucleotides 27–2340 of the NCDV strain) of Thailand strain (SG33) that was detected in this study and previously reported P[3] genotype strains. The tree was generated by the neighbour-joining method using MEGA version 5.0.5. Bootstrap values (1000 replicates) above 70 are shown. Strain SG33 is indicated by bold type. GenBank accession numbers of VP4 genes compared: FRV64 (D14723), Ro1845 (EU708893), Cat97 (EU708948), RV198-95 (HQ661137), K9 (EU708926), HCR3A (EU708904), PA260-97 (HQ661115), RV52-96 (HQ661126), A79-10 (EU708937), CU-1 (EU708915), RRV (AY033150), MSLH14 (KC960622), SA11 (DQ841262). Bar, 0.02 substitutions per nucleotide position.
Comparison of deduced amino acid sequences of strain SG385-tc and previously reported strains

It is known that the pathogenesis of RVA infection is multifactorial, and genetic analysis of selected virus reassortants revealed several viral proteins that are involved in virulence (VP7, VP4, VP3, NSP1, NSP2 and NSP4) (Burke & Desselberger, 1996; Mori et al., 2003). Among these proteins, NSP4 plays a role in the induction of diarrhea in suckling mice, acting as an enterotoxin (Ball et al., 1996; Estes & Greenberg, 2013; Horie et al., 1999; Mori et al., 2002; Morris et al., 1999; Zhang et al., 1998). Since Ball et al. (1996) showed that the enterotoxin domain in NSP4 of SA11 strain is located in the aa 114 to 135 sequence, this region of strain SG385-tc was compared with that of other E genotype strains detected in mammals, including SA11 (SA11-H96) strain. The amino acid sequence of NSP4 residues 114 to 130 of strain SG385-tc was completely identical to that of strain SA11-H96, and this region was fully conserved in RVA strains of genotypes E1–E3, E8, E9, E12 and E13 (Fig. 2).

Table 3. Genotypes of all genes of strain RVA/SugarGlider-tc/JPN/SG385/2012/G27P[36] and strains exhibiting the highest nucleotide identities to each gene segment

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Strain name_genotype</th>
<th>Nucleotide identity (%)</th>
<th>Cut-off value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1</td>
<td>R10</td>
<td>RVA/Human-tc/THA/T152/1998/G12P[9]_R3</td>
<td>80.1</td>
<td>83</td>
</tr>
<tr>
<td>VP2</td>
<td>C10</td>
<td>RVA/Human-tc/THA/T152/1998/G12P[9]_C3</td>
<td>81.3</td>
<td>84</td>
</tr>
<tr>
<td>VP3</td>
<td>M9</td>
<td>RVA/Horse-tc/JPN/CH-3/1987/G14P[12]_M3</td>
<td>73.2</td>
<td>81</td>
</tr>
<tr>
<td>VP6</td>
<td>I19</td>
<td>RVA/Rhesus-tc/USA/TUCH/2002/G3P[24]_I9</td>
<td>80.6</td>
<td>85</td>
</tr>
<tr>
<td>VP7</td>
<td>G27</td>
<td>RVA/Human-wt/THA/CMH222/2001/G3P[3]_G3*</td>
<td>79.6</td>
<td>80</td>
</tr>
<tr>
<td>NSP1</td>
<td>A20</td>
<td>RVA/Human-tc/ITA/PA260-97/1997/G3P[3]_A15</td>
<td>61.9</td>
<td>79</td>
</tr>
<tr>
<td>NSP2</td>
<td>N11</td>
<td>RVA/Cow-tc/JPN/DAO-10/2007/G24P[33]_N2</td>
<td>80.3</td>
<td>85</td>
</tr>
<tr>
<td>NSP3</td>
<td>T13</td>
<td>RVA/Human-tc/ITA/PA260-97/1997/G3P[3]_T3</td>
<td>80.5</td>
<td>85</td>
</tr>
<tr>
<td>NSP4</td>
<td>E17</td>
<td>RVA/Human-wt/THA/CMH222/2001/G3P[3]_E3</td>
<td>81.8</td>
<td>85</td>
</tr>
<tr>
<td>NSP5</td>
<td>H12</td>
<td>RVA/Human-wt/AUS/V585/2011/G10P[14]_H3</td>
<td>86.8</td>
<td>91</td>
</tr>
</tbody>
</table>

*Except for strains SG33, SG386, SG387, SG389, SG390, SG391, SG392, SG393 and SG394 that were detected in this study.

Fig. 2. Comparison of the amino acid sequence of NSP4 of strain SG385-tc with those of other E genotype strains detected in mammals. The amino acid sequences of NSP4 residues 114 to 135 in which the enterotoxin domain of strain SA11-H96 existed are shown. Strain name and genotype of NSP4 gene (strain name_E genotype) are indicated on the left side. Strains SG385-tc and SA11-H96 are in bold type. Only amino acids that differ from the sequence of strain SA11-H96 are shown.
In contrast, the amino acid sequence of NSP4 residues 131 to 135 varied among the different genotype strains. Similarly, VP4 and NSP1 of strain SG385-tc were partially conserved. In VP4, trypsin cleavage sites at aa 231, 241 and 247, which are involved in protease-enhanced infectivity of RVA (Estes & Greenberg, 2013), were conserved between strain SG385-tc and other established P genotype strains detected in mammals (Fig. 3a). Also, the RING finger domain in NSP1 (residues 42 to 72), motif C-X$_2$-C-X$_2$-C-X$_2$-H-X-C-X$_2$-C-X$_5$-C (where X is any amino acid) (Arnold & Patton, 2009; Hua et al., 1993), which is thought to be important for IFN antagonist activity of RVA (Estes & Greenberg, 2013; Graff et al., 2007), was conserved between strain SG385-tc and other established A genotype strains detected in mammals (Fig. 3b).

### Assessment of pathogenicity of strain SG385-tc in suckling mice

To assess the infectivity and pathogenicity of sugar glider RVA in other mammalian species, suckling mice were

### Table 3

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>RVA/Human-tc/ITA/160-01/2001/G3P[18]</td>
<td>A2</td>
</tr>
<tr>
<td>RVA/Human-tc/ indicted on the left side. Only amino acids that differ from the sequence of strain SG385-tc are shown.</td>
<td></td>
</tr>
</tbody>
</table>
inoculated experimentally with strain SG385-tc that was isolated from a Taiwanese sugar glider. Suckling mice were inoculated orally with $3.9 \times 10^4$ f.f.u. per 50 μl of strain SG385-tc ($n=9$) and uninfected culture supernatant (mock) ($n=9$). None of the mock-inoculated mice showed symptoms, whereas all of the mice that had been inoculated with strain SG385-tc defecated loose or liquid stools (Table 4). Diarrhoea was observed in three, seven and four suckling mice at 2, 3 and 4 days p.i., respectively. In addition, VP4 genes of RVA were detected by RT-semi-nested PCR in all stools collected from mice inoculated with strain SG385-tc, whereas no genes of RVA were detected in stools collected from mock-inoculated mice. To determine the 50 % diarrhoea-inducing dose (DD$_{50}$), suckling mice were inoculated orally with serial 10-fold dilutions of strain SG385-tc ($n=4$, per dilution). Diarrhoea was observed in three suckling mice and one suckling mouse that had been inoculated with $3.9 \times 10^4$ f.f.u. and $3.9 \times 10^3$ f.f.u. of strain SG385-tc, respectively. None of the mice that had been inoculated with $3.9 \times 10^2$ f.f.u. of the strain had diarrhoea. These data indicated that the DD$_{50}$ of strain SG385-tc in suckling mice was $1.2 \times 10^6$ f.f.u.

**DISCUSSION**

The prevalence of RVA in sugar gliders and the pathogenicity of sugar glider RVA in other mammalian species were investigated to estimate the risk of interspecies transmission of RVA from exotic pets to other mammalian species. In this study, RVAs were detected in 10 of 44 sugar gliders (one Thai and nine Taiwanese) that had been bred as pets (Table 1). The nucleotide identities of VP4 and VP7 genes among Taiwan strains were 98.9–100 % and 99.7–100 %, respectively. This indicates that the Taiwanese strains were genetically very similar to each other. The nucleotide identities of VP4 and VP7 genes between the Thailand strain and Taiwan strains ranged from 76.6 to 76.8 % and 80.3 to 80.6 %, respectively, suggesting that a variety of RVA strains are present in sugar gliders.

The Thailand strain and Taiwan strains were classified as G27P[3] and G27P[36] genotypes, respectively (Table 2). The G27 and P[36] genotypes were new genotypes, which were assigned by the RCWG. In addition, the SG385-tc strain, which was isolated as a representative strain of the Taiwan strains, contained new genotypes in all of the gene segments (G27-P[36]-I19-R10-C10-M9-A20-N11-T13-E17-H12 genotypes) (Table 3). In addition, considering that the VP4 gene of the Thailand strain was only distantly related to previously reported P[3] genotype strains (Fig. 1), it is thought that RVA strains in sugar gliders have evolved uniquely.

The sugar glider is a member of the order Diprotodontia (marsupial species) and is native to eastern and northern mainland Australia and various islands in the region. There are only two reports on detection of RVA in marsupial species: a red kangaroo (*Megaleia rufa*) (Baumeister *et al.*, 1983) and a common opossum (*Didelphis marsupialis*) (Linhares *et al.*, 1986), but these strains have not been characterized genetically. Considering that novel RVA strains were detected in sugar gliders, RVA might have evolved uniquely not only in sugar gliders but also in other marsupial species. However, since the sugar gliders in which RVA was detected were animals bred as pets, which are often in contact with other animals and humans and are not native, it is possible that RVA strains detected in the sugar gliders were transmitted from other animals. However, despite the fact that RVA strains were detected in both Thai and Taiwanese sugar gliders (Table 1), the VP7 genes of these were all classified as the same new (G27) genotype (Table 2 and Fig. 4). To clarify the

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**Table 4.** State of the stool in suckling mice that had been inoculated orally with strain SG385-tc or mock and detection of VP4 gene of RVA by RT-semi-nested PCR in the stool collected from suckling mice

<table>
<thead>
<tr>
<th>Sample inoculated</th>
<th>Mouse</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG385-tc</td>
<td>1</td>
<td>(+)†</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(N)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(N)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>(N)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(N)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>(N)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(N)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>(N)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(N)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(N)</td>
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<td></td>
<td>8</td>
<td>(N)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(N)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>(N)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(N)</td>
</tr>
<tr>
<td>Mock</td>
<td>1–9</td>
<td>(N)−</td>
<td>(N)−</td>
<td>(N)−</td>
<td>(N)−</td>
<td>(N)−</td>
</tr>
</tbody>
</table>

*State of the stool: 0, ordinary stool or no stool; 1, loose orange or yellow stool; 2, liquid yellow stool.
†Detection of VP4 gene: +, detected; N, stool was not collected; −, not detected.
prevalence and characteristics of RVA in marsupial species, there is a need for further surveillance of RVA in wild marsupial species.

The enterotoxin domain in NSP4, trypsin cleavage sites in VP4, and RING finger domain in NSP1 were conserved between strain SG385-tc and strains of other established genotypes detected in mammals (Figs 2 and 3). NSP4, VP4 and NSP1 are involved in virulence, and particular domains and sites in these proteins are thought to be important for the induction of diarrhoea, virus entry into cells and regulation of the induction of IFN of RVA, respectively (Ball et al., 1996; Estes & Greenberg, 2013; Graff et al., 2007). Our results, together with these findings, suggest that NSP4 in strain SG385-tc has enterotoxin activity and that RVA in sugar gliders would be infective to and pathogenic in mammals. To assess the infectivity and pathogenicity of sugar glider RVA in other mammalian species, suckling mice were inoculated experimentally with strain SG385-tc. All of the suckling mice that had been inoculated orally with the strain defecated loose or liquid stools, and seven of the nine mice had diarrhoea (Table 4), indicating that RVA in sugar gliders is infective to and pathogenic in suckling mice. In addition, the DD50 of strain SG385-tc in suckling mice (1.2 × 10^4 f.f.u.) was similar to that of a simian RVA, strain SA11 (1 × 10^4 p.f.u., 2.6 × 10^4 f.f.u. and 1 × 10^5 p.f.u.) (Bell et al., 1987; Mori et al., 2001; Offit et al., 1986). These findings suggested that RVA in sugar gliders is infective to and possibly pathogenic in other mammalian species, implying that exotic pets represent a risk of transmission of RVA to humans.

In this study, RVA strains were detected in sugar gliders and characterized. To understand the risk of interspecies transmission of RVA from exotic pets to other mammalian species and consequently prevent zoonotic infection with RVA, the pathogenicity of RVA, which exists in exotic pets, should be investigated. Moreover, to clarify the diversity, evolution and ecology of RVA, RVA strains prevalent in exotic pets should be characterized.

**METHODS**

**Faecal samples.** Faeces or colon contents were collected from 44 sugar gliders in Japan between December 2010 and November 2012. Twenty-eight of the 44 sugar gliders were babies and 16 were adults (Table 1). The baby sugar gliders had been imported from Thailand, Taiwan and Malaysia into Japan as pets, and the adult sugar gliders had been reared in Japan for breeding. The faecal samples were diluted with PBS to 20% suspensions and clarified by centrifugation at 750 g for 10 min. The supernatants were collected and stored at −80 °C until use.

**Detection of RVA in faecal samples by reverse transcription-semi-nested PCR and direct sequencing.** Detection of RVA (Abe et al., 2010) and direct sequencing (Abe et al., 2009) were performed as described previously. Briefly, to detect the RVA VP4 gene, the cDNA was amplified by an outer PCR with the primers VP4-HeadF and VP4-1094R2 and by a semi-nested PCR with the primers VP4-HeadF and VP4-887R, which were designed in the conserved regions of the VP4 gene (Table S1 available in the online Supplementary Material) (Abe et al., 2009). The VP7 gene was then partially amplified by nested or semi-nested PCR with the outer primer pair Beg9 (Gouvea et al., 1990) and VP7-W998-2 (Abe et al., 2010) and the inner primer pair VP7-up2-2 (Abe et al., 2011) and VP7-945R-3 (Abe et al., 2010) or the outer primer pair VP7_headF and VP7-932R and the inner primer pair VP7_headF and VP7_611R (Table S1). Outer PCR was performed with an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of 95 °C for 45 s, 45 °C for 45 s, 45 °C for 1 min, and a final extension at 72 °C for 5 min. The cycle conditions for inner PCR were 30 cycles of 95 °C for 45 s, 45 °C for 45 s and 68 °C for 1 min. The inner PCR primers (VP4-HeadF, VP4-887R, VP7-up2-2, VP7-945R-3, VP7_headF and VP7_611R) were also used as sequencing primers.

**Complete ORF analyses.** The VP4 and VP7 genes of RVA strains that were detected in faeces and all of the genes of strain SG385-tc were amplified by PCR, nested PCR or semi-nested PCR and sequenced directly (Tables S2 and S3). The C-terminal residue of the
ORF region of the VP4 gene of Thailand strain was sequenced with a BigDye Direct Cycle Sequencing kit (Applied Biosystems). The entire or nearly entire coding regions were assembled from the sequences and analysed using A plasmid Editor v2.0.36 (http://biologylabs.utah.edu/jorgensen/wayned/apel/). Searches for similar sequences were conducted using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The genotypes were determined according to the genotyping recommendations of the RCWG (Matthijnssens et al., 2008b).

**Phylogenetic analysis.** Sequence alignments and reconstruction of phylogenetic trees were performed using CLUSTAL W (Thompson et al., 1994) and MEGA version 5.0.5 software (Tamura et al., 2011) by the neighbour-joining method with 1000 bootstrap replicates.

**Virus isolation.** Virus isolation was performed as described by Minamoto et al. (1993). Virus isolation was performed as described by Fukusho et al. (1981) with modification. Briefly, first, a 20 % faecal suspension of a Taiwanese sugar glider (SG385) was passed through a 0.45 μm syringe filter (Foyo Roshi Kaisha). The filtrate was then treated with an equal volume of 20 μg trypsin ml⁻¹ (from bovine pancreas; Sigma) for 30 min at 37 °C and inoculated onto a confluent monolayer of MA-104 cells. After adsorption, the cells were maintained in serum-free Eagle’s minimal essential medium (Nissui Pharmaceutical) supplemented with 0.5 μg trypsin ml⁻¹, 400 μg gentamicin ml⁻¹ (Life Technologies) and 5 μg amphotericin B ml⁻¹ (Sigma) using a roller drum apparatus for 5 days. The presence of RVA was confirmed by RT-semi-nested PCR as described above. For complete ORF analyses and animal inoculation, the virus culture at passage 3 was used. The virus titre (f.f.u. ml⁻¹) was determined by an indirect immuno-fluorescence assay in MA-104 cells using anti-VP6 monoclonal antibody as described previously (Minamoto et al., 1993).

**Animal inoculation.** Experimental infection of mice was performed as described by Inagaki et al. (2013) with modification. Briefly, pregnant ddY mice were obtained from Japan SLC (Shizuoka, Japan) and housed individually in cages. After delivery, blood samples were taken from each dam, and anti-SG385-tc serum antibody was checked by a neutralization test. All of the dams that were checked were seronegative (titre, <2). Litters of four-day-old suckling mice were inoculated orally with 3.9 × 10⁴ f.f.u. per 50 μl of strain SG385-tc (n=9) or mock (n=9) by gavage. The stools were examined daily, every 24 h after virus inoculation, for the development of diarrhoea beginning at 1 day p.i. for 5 days p.i. State of the stool was classified into three categories: ordinary stool or no stool, loose orange or yellow stool, and liquid yellow stool. Liquid stool was considered to be diarrhoea, but loose stool was not. To determine the DDₐₐₐₐₐ₁ of strain SG385-tc, litters of four-day-old suckling mice were inoculated with 50 μl of serial 10-fold dilutions of strain SG385-tc (n=4, per dilution). The DDₐₐₐₐₐ₁ was calculated by the method of Reed & Muench (1938). The animal inoculation in this study was conducted in accordance with the Regulations for Animal Experiments in Gifu University, and the protocols were approved by the Committee for Animal Research and Welfare of Gifu University (approval no. 12074).

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