Molecular detection and nucleotide sequence analysis of a new Aichi virus closely related to canine kobuvirus in sewage samples

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Between 2001 and 2005, 207 raw sewage samples were collected at the inflow of a sewage treatment plant in Aichi Prefecture, Japan. Of the 207 sewage samples, 137 (66.2 %) were found to be positive for amplification of Aichi virus (AiV) nucleotide using reverse transcription (RT)-PCR with 10 forward and 10 reverse primers in the 3D region corresponding to the nucleotide sequence of all kobuviruses. AiV genotype A sequences were detected in all 137 samples. New sequences of AiV were detected in nine samples, exhibiting 83 % similarity with AiV A846/88, but 95 % similarity with canine kobuvirus (CKV) US-PC0082 in this region. The nucleotide sequences from the VP3 region to the 3’ untranslated region (UTR) of sewage sample Y12/2004 were determined. The number of nucleotides in each region was the same as that of CKV. The similarity of the nucleotide (amino acid) identity of a complete VP1 region was 90.5 % (94.8 %) between Y12/2004 and CKV US-PC0082. The phylogenetic analyses based on the nucleotide and the deduced amino acid sequences of VP1 and 3D showed that Y12/2004 was independent from AiV, but closely related to CKV. These results suggested that CKV is present in Aichi Prefecture, Japan.

INTRODUCTION

Aichi virus (AiV) was first recognized in 1989 as a pathogen of human gastroenteritis in Japan (Yamashita et al., 1991). The AiV genome comprises 8280 nt with a single ORF that encodes L protein, three structural proteins (VP0, VP3 and VP1) and seven non-structural proteins (2A–2C and 3A–3D) (Sasaki et al., 2001; Yamashita et al., 1998). AiV was classified into a new genus named Kobuvirus of the family Picornaviridae (Knowles et al., 2012). AiV consisted of a single serotype and three genotypes A, B and C, with approximately 90 % sequence homology in the VP1 and 3CD regions (Ambert-Balay et al., 2008; Yamashita et al., 2000). AiVs were detected from faecal samples of gastroenteritis patients in several countries of the world (Oh et al., 2006; Sdiri-Loulizi et al., 2008; Yamashita et al., 1993, 1995). Using reverse transcription (RT)-PCR, 32 (4.1 %) of 788 Tunisian children with diarrhoea were positive for AiV (Sdiri-Loulizi et al., 2009). However, AiV was not detected in The Netherlands (Svraka et al., 2007). The other investigations also demonstrated a low incidence (0.5–3.5 %) of AiV infection in patients with gastroenteritis (Kaikkonen et al., 2010; Pham et al., 2007; Reuter et al., 2009b; Sdiri-Loulizi et al., 2008; Yang et al., 2009).

Kobuviruses were detected not only in humans but also in cattle (bovine kobuvirus; BKV) (Khamrin et al., 2008; Mauroy et al., 2009; Reuter & Egyed, 2009; Yamashita et al., 2003) and swine (porcine kobuvirus) (Reuter et al., 2009a; Yu et al., 2011). Recently, canine kobuvirus (CKV) has been reported in stool samples from dogs with diarrhoea in the USA (Kapoor et al., 2011; Li et al., 2011). Murine kobuvirus (MKV) was also detected from the faeces of wild rodents (Peromyscus crinitus) (Phan et al., 2010). Comparative phylogenetic analysis of CKV and MKV confirmed them as picornaviruses closely related to AiV and included in the same species, with AiV renamed as Aichivirus A (Adams et al., 2013).

Human enteric viruses are known to exist in sewage. The detection of viruses in raw sewage would reflect the actual state of the circulating viruses in the population (Melnick et al., 1978; Melnic & Wenner, 1979). We detected AiV and new sequence variants of AiV related to CKV in sewage samples between 2001 and 2005. Nucleotide sequence analysis from the VP1 region to the 3’ untranslated region (UTR) of a sewage sample (Y12/2004) revealed that this...
variant was more closely related to CKV than AiV. In this study, we report on the prevalence and nucleotide analysis of AiV and a new AiV in sewage samples. To our knowledge, this paper reports the first detection of CKV in sewage samples in Japan and in the world.

METHODS

Sewage samples. Between 2001 and 2005, raw sewage samples were collected every week at the inflow of a sewage treatment plant in Aichi Prefecture, Japan. A total of 207 sewage samples were collected. The samples were centrifuged at 1500 g for 20 min. The virus was concentrated using polyethylene glycol (PEG) precipitation as recommended by Lewis & Metcalf (1988). Briefly, each 10 ml of supernatant was adjusted to pH 7.2; PEG 6000 (Wako Pure Chemical Industries) and NaCl were then added to make a final concentration of 9% (w/v) and 0.5 M, respectively. The resulting suspensions were stirred overnight at 4°C. The suspensions (3 ml) were centrifuged at 1500 g for 10 min and the pellets were suspended in 0.2 ml of RNase-free water.

RNA extraction and RT-PCR. Nucleic acid was extracted from the 0.2 ml concentrated sample using the High Pure Viral RNA Kit (Roche) according to the manufacturer's instructions. RT-PCR for each sample was performed with the SuperScript One-Step RT-PCR for Long Templates (Invitrogen). Oligonucleotide primers (10 forward and 10 reverse) corresponding to the nucleotide sequence of the AiV polymerase gene (Yamashita et al., 2003) were used in RT-PCR for amplification of the viral genome from the sewage sample. The RT-PCR conditions were 50°C for 30 min, 95°C for 3 min, and 40 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by 10 min at 72°C.

For amplification of the VP1 region, a new set of primers was designed, based on the sequence of AiV A846/88 and bovine kobuvirus U-1, as reported in the public database as follows: KBP1 (sense: 5'-CTCYYTTCATCCTYAMAMTCTCTACTGG-3') and KBN1 (anti-sense: 5'-AAGTGGTTCAGTGGTG-3'). An internal primer set for a nested PCR was also designed as follows: KbP1N (sense: 5'-GAACCTCGACAGTTCCAT-3') and KbP1N (anti-sense: 5'-GAAGTTAGGRTGGATRGCGA-3'). The first round of RT-PCR conditions were 50°C for 30 min, 95°C for 3 min, and 35 cycles of 95°C for 20 s, 50°C for 30 s and 72°C for 1 min 30 s, followed by 10 min at 72°C. Nested PCR conditions performed with Ex Taq DNA polymerase (TakaRa Bio) were 94°C for 2 min, and 35 cycles of 94°C for 20 s, 50°C for 30 s and 72°C for 30 s, followed by 10 min at 72°C.

To amplify the other parts of the VP1 region, RT-PCR using KBP1 and KBN1 primers was also followed by semi-nested PCR using additional sets of primers whose designation was based on the AiV and the new type of AiV sequence in the Y12/2004 sewage sample, and KBN1 primers was also followed by semi-nested PCR using KBN1. Specific, non-degenerate primers were designed from preliminary sequences to close the gap between the VP1 and 3D region, as follows: Y12/10-210N for semi-nested PCR. The PCR conditions were 95°C for 3 min, and 35 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 4 min 30 s, followed by 10 min at 72°C. To close the gap between the 3D and poly-A tailing regions, two sense primers were designated as follows: Y12/12/2000 (5'-CAGTACAAAGGACATCGSGGGGATGATC-3') for the first RT-PCR, and Y12/10-1210N (5'-CAGTACAAAGGACATCGSGGGGATGATC-3') for the nested PCR. Oligo dT33 was used as an anti-sense primer with these sense primers. The PCR conditions were 95°C for 3 min, and 35 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 1 min, followed by 10 min at 72°C.

For confirmatory purposes, specific primers of Y12/2004 were designated in the VP3, VP1, and 2A regions as follows: Y12/vp3/153p (5'-CAAGCTCGCCCTCTCATGCAAACCCG-3') and Y12/2A/986n (5'-ACATGGTCATGGGTCACATCTG-3') for the first RT-PCR, and Y12/vp1/256p (5'-ACACACCGGACACCTTTGAG-3') and Y12/2A/3217n (5'-GGCCCTTGACGGTTGATGTG-3') for the first RT-PCR, and Y12/2B/1642p (5'-TACCTCCTCTCTCTCTTGTTG-3') and Y12/3B/3217n for the semi-nested PCR.

Nucleotide sequence and analysis. Following RT-PCR, amplified products were purified and introduced into a pGEM-T vector (Promega) as described elsewhere (Yamashita et al., 1998). Subsequently, three to five clones per sample were selected for the sequence analysis. The DNA sequence was determined by using a SequiTherm Long-Read Cycle Sequencing Kit-LC (Epicentre Technologies) and a Model 4000 Automated DNA Sequencer (Li-Cor). Furthermore, the nucleotide sequences of these clones were compared with one another and with those of kobuviruses by using the Genetyx program (Genetyx Corporation). Phylogenetic analysis was performed by the neighbour joining method within the MEGA 5 analytical package with a bootstrap of 1000 replicates (Tamura et al., 2011). Reference sequences of kobuviruses were obtained from GenBank/EMBL/DDBJ: AiV genotype A A846/88 (AB040749), BAY/1/03/DEU (AY747174), D/V12287/2004 (GQ927711), kvgb99012632/2010 (JX564249), and A1156/87 (AB097164), genotype B Goiania/GO/03/01/Brazil (DQ028632), Chshc7 (F890523), D/V12169/2004 (GQ927704), M166/91 (AB097165), P766/90 (AB097166), A11277/91 (AB712380), and genotype C Rn48/2002 (EU159259); CKV US-PC0082 (JN088541), AN211D/USA/2009 (JN387133), and UK003 (KC161964); MKV M-5/USA/2010 (JF755427); BKV U-1 (AB084788).

RESULTS

Of the 207 sewage samples, 137 (66.2%) were positive for amplification of the AiV 588 bp nucleotide in the 3D region using RT-PCR with 10 forward and 10 reverse primers (Table 1). AiV nucleotides were detected throughout the years between 2001 and 2005. The annual positive rates for the AiV nucleotide ranged from 54.5 to 93.3%.

Table 1. Detection of Aichi virus RNA in sewage samples using the primer pair 10 forward and 10 reverse corresponding to the 3D region, between 2001 and 2005

<table>
<thead>
<tr>
<th>Year</th>
<th>No. tested</th>
<th>No. positive</th>
<th>% A</th>
<th>B</th>
<th>New</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>34</td>
<td>27</td>
<td>79.4</td>
<td>27</td>
<td>1</td>
</tr>
<tr>
<td>2002</td>
<td>53</td>
<td>31</td>
<td>58.5</td>
<td>31</td>
<td>1</td>
</tr>
<tr>
<td>2003</td>
<td>51</td>
<td>36</td>
<td>65.5</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>2004</td>
<td>55</td>
<td>30</td>
<td>54.5</td>
<td>30</td>
<td>3</td>
</tr>
<tr>
<td>2005</td>
<td>15</td>
<td>14</td>
<td>93.3</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>207</td>
<td>137</td>
<td>66.2</td>
<td>137</td>
<td>1</td>
</tr>
</tbody>
</table>
New Aichi virus in sewage samples
AiV genotype A sequences were detected in all 137 samples. The AiV genotype B sequence was detected in only one sample. New sequences of AiV were detected in nine samples, exhibiting 83% similarity of this region to AiV A846/88, but 95% with CKV US-PC0082. The phylogenetic tree indicated that several types of AiV genotype A sequences were detected in the same year. The AiV genotype B sequence was similar to that of Goiania/GO/03/01/Brazil. All the new sequences from the nine samples belonged to CKV (Fig. 1a). AiV genotype C and BKV were not detected in any sample.

Of the nine samples containing a CKV-related sequence, five samples were positive for the 405 bp amplicon of the VP1 region using nested RT-PCR. Of the five amplicons, three samples (J28/2002, Y12/2004 and Y23/2004) had 69–71% similarity to AiV, but 87–91% to CKV. These three samples had 90–92% similarity with each other. Phylogenetic analysis of this region revealed that J28/2002, Y12/2004 and Y23/2004 samples were clustered with CKV AN211D, US-PC0082 and UK003 (Fig. 1b). Of the three samples positive for the new sequence, Y12/2004 was used for sequence analysis of the other regions.

A total of six overlapping cDNA clones spanning from the VP3 to 3’UTR regions of the genome of sewage sample Y12/2004 were obtained and their nucleotide sequences determined. The genome consisted of 5434 nt, which encodes a potential polyprotein precursor of 1729 amino acids, and the 3’ end by 244 nt. The numbers of nucleotides in each region were the same as for CKV US-PC0082, except in the 3’UTR region. The nucleotide (amino acid) identity of the complete VP1 region was 90.5% (94.8%) between Y12/2004 and CKV US-PC0082. The nucleotide identities of Y12/2004 and US-PC0082 ranged from 93.9 to 97.9% in the 2A–2C, 3A–3D and 3’UTR regions (Table 2).

**DISCUSSION**

Recent developments in molecular detection technology and available reference nucleotide sequence data make it possible for the existence of AiV to be confirmed by molecular detection and identification in sewage samples (Alcalá et al., 2010; Kitajima et al., 2011, 2013; Sdiri-Loulizi et al., 2010). In this study, we used a primer set (10 forward and 10 reverse) which was designated for amplifying a 588 bp region of the 3D protein of AiV (A846/88) and BKV (U-1) (Yamashita et al., 2003). The primer set was useful for amplifying the AiV nucleotide in sewage samples only by the first round of RT-PCR.

AiV genotype A sequences were detected in all 137 samples but the genotype B sequence was detected in only one sample. A seroprevalence study conducted in Japan showed that this virus is quite prevalent (Yamashita et al., 1993).

Table 2. Comparison of RNA and amino acid between Y12/2004 and kobuviruses

<table>
<thead>
<tr>
<th>Region</th>
<th>No. of Y12/2004 RNA residues</th>
<th>canine kobuvirus</th>
<th>Aichi virus</th>
<th>murine kobuvirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP3</td>
<td>183</td>
<td>183 (92.3/100)</td>
<td>183 (74.3/82.0)</td>
<td>183 (81.4/90.2)</td>
</tr>
<tr>
<td>VP1</td>
<td>759</td>
<td>759 (90.5/94.8)</td>
<td>759 (69.1/64.9)</td>
<td>756 (72.7/74.1)</td>
</tr>
<tr>
<td>2A</td>
<td>408</td>
<td>408 (96.8/99.3)</td>
<td>408 (73.6/72.1)</td>
<td>408 (77.9/78.7)</td>
</tr>
<tr>
<td>2B</td>
<td>495</td>
<td>495 (93.9/98.8)</td>
<td>495 (76.8/78.8)</td>
<td>495 (77.7/81.2)</td>
</tr>
<tr>
<td>2C</td>
<td>1005</td>
<td>1005 (94.4/100)</td>
<td>1005 (80.0/87.5)</td>
<td>1005 (83.1/91.6)</td>
</tr>
<tr>
<td>3A</td>
<td>282</td>
<td>282 (96.8/98.9)</td>
<td>285 (67.4/67.7)</td>
<td>285 (72.0/78.5)</td>
</tr>
<tr>
<td>3B</td>
<td>81</td>
<td>81 (95.4/100)</td>
<td>81 (72.5/81.5)</td>
<td>81 (81.2/81.5)</td>
</tr>
<tr>
<td>3C</td>
<td>570</td>
<td>570 (95.4/98.9)</td>
<td>570 (81.0/83.7)</td>
<td>570 (81.6/85.8)</td>
</tr>
<tr>
<td>3D</td>
<td>1407</td>
<td>1407 (95.3/99.4)</td>
<td>1407 (82.8/89.5)</td>
<td>1407 (85.8/91.9)</td>
</tr>
<tr>
<td>3’UTR</td>
<td>244</td>
<td>242 (97.9/-)</td>
<td>237 (80.6/-)</td>
<td>241 (88.9/-)</td>
</tr>
</tbody>
</table>

* Not applicable.
On the other hand, the incidence of AiV infection in patients was very rare (Pham et al., 2007; Yamashita et al., 1993). These findings suggest that AiV genotype A is prevalent but usually asymptomatic in Japan.

The nucleotide identity of Y12/2004 with AiV A846/88 in the VP1 region was lower than 70%; such a value is proposed to indicate a new type of enterovirus (Oberste et al., 1999). According to this criterion, Y12/2004 represents a new AiV member, and we have tentatively designated it as a new type of AiV. On the other hand, Kapoor et al. (2011) and Li et al. (2011) reported a CKV which is closely related to human AiV and had 90% identity to Y12/2004 in the VP1 region. Hence, Y12/2004 is the first example of CKV RNA in the inflow of a sewage treatment plant.

To our knowledge, there is nothing known about the transmission of this virus from dogs to humans or about a recombination between these viruses in the gut (Lukashev et al., 2012). Of the 137 samples, nine were closely related to CKV in the 3D region; it was also confirmed in three samples in the VP1 region, and one sample (Y12/2004) in the VP1, 2A, 2B, 2C, 3AB, 3C, 3D and 3′UTR regions. These results suggested that CKVs are present in Aichi Prefecture, Japan.

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