A bovine G8P[1] group A rotavirus isolated from an asymptomatically infected dog

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Group A rotaviruses (RVAs) are enteric pathogens with well-documented zoonotic transmissions to humans. The segmented genome of the virus enables reassortment events which might alter host susceptibility and/or disease course. Genetic analysis of rotavirus in dogs has so far only revealed RVAs with the VP7 and VP4 genome constellation G3P[3]. RVA G3P[3] have also been found in cats, humans, monkeys and bats. In the present study, we described an unusual RVA of genotype G8P[1] which was isolated from an asymptomatically infected young dog. The dog did not show signs of diarrhoea. Analysis of full-length segments of VP2, VP6 and VP7 as well as NSP1–NSP6 revealed a typical bovine-like genotype constellation G8–P[1]–I2–Rx–C2–Mx–A3–N2–T6–E2–H3. Phylogenetic analysis supported the hypothesis of an interspecies transmission from a bovine/artiodactyl species or from humans to the young dog. The isolate was likely to represent a multiple reassortant virus.

Rotaviruses constitute one of the most important viral causes of acute gastroenteritis in humans and animals, and are estimated to cause 453 000 (95% confidence interval 420 000–494 000) deaths annually in children <5 years of age worldwide (Tate et al., 2012). Rotaviruses, belonging to the family of Reoviridae, contain 11 dsRNA genome segments encoding six structural proteins designated VP1–VP4, VP6 and VP7, as well as five to six non-structural viral proteins (NSP1–NSP6). The two outer capsid proteins VP4 and VP6 are the basis of a widely applied dual-classification system defining 35 P genotypes and 27 G genotypes, respectively (Matthijnssens et al., 2011). In dogs, the disease is usually associated with mild diarrhoea in pups <12 weeks old, although some cases of severe fatal enteritis with fatal outcome were described in pups as young as 2 weeks old (Green, 2012). McNulty et al. (1978) found a group A rotavirus (RVA) antibody prevalence of 79% in sera from adult dogs in Ireland. Additional reports from Belgium (Dagenais et al., 1980) with 62% seropositive samples and from Germany with 84% positives (Oldenburg et al., 1984) reflect the high incidence of RVA infections in dogs. To date, only rotaviruses of the G3P[3] genotype constellation have been described to infect dogs and to some extent also cats (Tsugawa & Hoshino, 2008), monkeys (Westerman et al., 2006), and bats (He et al., 2013). Although this particular genotype is rarely associated with human disease (De Grazia et al., 2007; Luchs et al., 2012), dogs are in focus for interspecies transmission of RVA as these viruses are able to infect a broad range of heterologous host species. So far, only little information is available about the different RVA genotypes able to infect dogs, but investigations have shown that rotavirus antigens were detectable in ~7% of faecal samples of young dogs with diarrhoea (Naumann, S. & Vahlenkamp, T. W., unpublished observations). This emphasizes the need for more detailed investigation of circulating RVA genotypes in dogs.

In this study, faecal samples from 15 dogs with and without diarrhoea were collected and screened for the presence of rotaviruses by reverse transcription (RT)-PCR as described by Isegawa et al. (1993) using the primer pair Bov4Com/ Bov4Com5. RNA was isolated from 50 mg faeces using the QIAamp Viral RNA Mini kit (Qiagen) following the instructions of the manufacturer. One out of 15 samples was positive for RVA RNA.

The positive sample was derived from a 4-month-old French bulldog that was admitted to the Department of Small Animal Medicine, University of Leipzig, Germany, for rectification of a portosystemic shunt. The dog showed no clinical signs of disturbance to its general condition and behaviour. Laboratory investigations showed a microcytic, non-regenerative anaemia, and elevated levels of ammonia (162 μmol L−1) and alkaline phosphatase (245 U L−1). After the surgery, the dog received permanent medication with esomeprazole (10 mg day−1) and an iron/folic acid
(Floradix; one dragée per day). The faeces was collected after surgery, and exhibited a tenacious and black appearance which was assumed to be due to the iron substitution. No signs of diarrhoea were observed at the date of sampling. These findings are in accordance with previously published data showing that dogs can be asymptomatically infected by RVA (De Grazia et al., 2007).

To further analyse the genotype of the detected rotavirus, RT-PCR-based genotyping of all 11 rotavirus segments was performed using gene-specific primers as previously described (Fuji et al., 2012; Gentsch et al., 1992; Honma et al., 2007) with some modifications (Table S1, available in the online Supplementary Material). Briefly, 5 μl isolated RNA was mixed with 1 μl 10 mM dNTPs (Thermo Scientific), and 1 μl each forward and reverse segment-specific primer (10 μM); DEPC-treated water was then added to a volume of 13 μl. After denaturation at 97 °C for 5 min the mixture was placed on ice. Then 4 μl 5 × First Strand Buffer (250 mM Tris/HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂), 1 μl DTT (0.1 M), 40 U RiboLock RNase Inhibitor (Thermo Scientific) and 200 U Superscript III transcriptase (Life Technologies) were added. cDNA synthesis was performed at 55 °C for 60 min and reverse transcriptase was inactivated at 85 °C for 5 min. RNA was degraded by adding 2 U RNase H (NEB) and incubating at 37 °C for an additional 20 min.

PCR was performed in 25 μl reaction buffer (50 mM KCl, 10 mM Tris/HCl, pH 8.5) containing 2.0 mM MgCl₂, 500 nM each primer, 200 μM each of the four dNTPs, 0.5 U Q5 Hot Start High-Fidelity DNA Polymerase (NEB) and 1 μl reverse-transcribed cDNA product. Amplification of the 11 full-length rotavirus segments was carried out under the following conditions: initial denaturation at 98 °C for 30 s; 40 cycles at 98 °C for 10 s, 61 °C for 20 s and 72 °C for 2 min; and a final extension at 72 °C for 5 min. PCR products were visualized by agarose gel electrophoresis with 1 × Tris/acetate-EDTA buffer (40 mM Tris/acetate, 1 mM EDTA, pH 8.2) containing 0.2 μg ethidium bromide ml⁻¹.

Using this approach, near-full-length segments of all non-structural proteins as well as the structural proteins VP2, VP6 and VP7 were amplified (Fig. S1a). In the case of VP4, a partial sequence using the RT-PCR approach described by Gentsch et al. (1992) was amplified (Fig. S1b). All amplification attempts failed for the VP1 and VP3 genes. Primer sequences applied in this study are listed in Table S2. For nucleotide sequence analysis and determination of the genotype constellation, the PCR products were cloned into the pJET1.2/blunt vector (Thermo Scientific) according to the manufacturer’s instructions. Recombinant plasmids were transformed into competent *Escherichia coli* preparation (Floradix; one dragée per day). The faeces was collected after surgery, and exhibited a tenacious and black appearance which was assumed to be due to the iron substitution. No signs of diarrhoea were observed at the date of sampling. These findings are in accordance with previously published data showing that dogs can be asymptomatically infected by RVA (De Grazia et al., 2007).

**Table 1. Genotype constellation of selected rotavirus strains**

Typical bovine-like, dog/cat-like, human DS-1-like and human AU-1-like genotypes are shown in blue, green, red and yellow, respectively.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype constellation</th>
<th>VP7</th>
<th>VP4</th>
<th>VP6</th>
<th>VP1</th>
<th>VP2</th>
<th>VP3</th>
<th>NSP1</th>
<th>NSP2</th>
<th>NSP3</th>
<th>NSP4</th>
<th>NSP5</th>
</tr>
</thead>
</table>
Fig. 1. Phylogenetic tree reconstructed from nucleotide sequences of near-full-length genome segments from non-structural proteins NSP1–NSP5. Bootstrap values (1000 replicates) are shown. The canine RVA identified in this study is highlighted in a black box. Bar, 0.05 substitutions or 0.02 substitutions per site as indicated in the figure.
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TOP10 cells (Life Technologies) using the heat-shock method described previously by Maniatis et al. (1982).

Sequence analysis of the recombinant plasmids was performed with pJET1.2 forward primer (5'-CGACTCAC-TATAGGAGAGCGGC-3') and pJET1.2 reverse primer (5'-AAGAACATCGATTTTCCATGCG-3'). The chromatogram sequencing files were analysed with BioEdit version 7.2.4 and edited sequences were screened using BLAST. Each plasmid was sequenced twice. The nucleotide sequences obtained have been submitted to the GenBank nucleotide database, and the accession numbers for nucleotide sequences for the genes for the non-structural proteins NSP1–NSP5 and the structural proteins VP2, VP4, VP6 and VP7 genes are KJ940157–KJ940161, and KJ940162, KJ940163, KJ940164 and KJ940165, respectively.

Genotype assignments were carried out using the RotaC v2.0 online tool (Maes et al., 2009) according to the genotyping recommendations of the Rotavirus Classification Working Group (Matthijnssens et al., 2008b).

Using this approach, we found a genotype constellation of G8–P[1]–I2–Rx–C2–Mx–A3–N2–T6–E2–H3, which is characteristic for artiodactyl bovine-like rotavirus strains (Matthijnssens & Van Ranst, 2012; Matthijnssens et al., 2008a).

Comparisons with other available G8P[1] genotype constellations from different human, artiodactyl and canine rotaviruses (Table 1) with the RVA isolate presented here revealed that VP6, VP2, NSP2 and NSP4 belong to DS-1-like genotypes, whereas NSP1 and NSP5 reflect AU-1-like strains, both representing typical human rotavirus isolates. The VP4, VP7 and NSP3 genotypes were most closely related to those of bovine RVAs.

These data clearly show that the obtained canine RVA has high homology to bovine-like human and artiodactyl strains, but virtually no homology with any other canine or feline rotavirus genotypes described so far (Tsugawa & Hoshino, 2008), suggesting that the canine RVA investigated is the result of reassortment events between artiodactyl/bovine and human rotavirus strains. This finding is in accordance with previously published data that G8P[1] strains are typical bovine isolates (Papp et al., 2013), and that they are able to cross species barriers and also infect humans (Ghosh & Kobayashi, 2011).
**Fig. 2.** Phylogenetic tree reconstructed from nucleotide sequences of near-full-length genome segments encoding structural proteins VP2, VP6 and VP7, and a partial sequence from VP4. Bootstrap values (1000 replicates) are shown. The canine RVA identified in this study is highlighted in a black box. Bar, 0.05 substitutions or 0.02 substitutions per site as indicated in the figure.
For phylogenetic analysis of the canine RVA, we calculated genetic distances employing the Tamura–Nei model at the nucleotide level. Dendrograms were reconstructed by the maximum-likelihood method with 1000 bootstrap replicates (Kumar et al., 2004).

In the case of the non-structural proteins NSP2, NSP3 and NSP4, the analysis revealed close relatedness to the goat rotavirus strain OVR762 which was isolated in Spain in 2002. For NSP1 and NSP5, the phylogenetic investigation showed that these two segments evolved from another source, as they were most closely related to cogent genes of human rotavirus strains (Fig. 1). Thus, it is likely that the NSP1 and NSP5 genes had been reassorted from a double infection with human and artiodactyl rotavirus strains. The four amplified structural proteins VP2, VP4, VP6 and VP7 showed a distinct phylogenetic pattern, very closely matching those of isolated bovine rotavirus strains, with the exception of VP6, which showed the highest homology with the VP6 gene of the human strain 111-05-27 isolated in Italy in 2005 (Fig. 2).

Our results strongly suggest that the canine RVA described in this study may have evolved from a human rotavirus strain through multiple reassortment events with artiodactyl and/or bovine RVA strains.

However, the issue of whether the source of this particular strain was really bovine, artiodactyl or human cannot be resolved definitely as we were not able to amplify VP1 and VP3 sequences. Nevertheless, the infection of a dog with a RVA strain as described here is unusual.

Reassortment and interspecies transmission incidents have been shown previously for other canine and feline rotavirus strains (Martella et al., 2010; Matthijnssens et al., 2011; Wang et al., 2013). Interestingly, rotavirus infections of human patients with strains of G8P[1] genotypes have been described (Ghosh et al., 2011). Therefore, the zoonotic potential needs to be further assessed. So far, the RVA carrier status of young dogs has not been analysed in detail, mainly because all isolated canine rotavirus strains belong to the G3P[3] group, which only rarely causes human infections (De Grazia et al., 2007). Thus, the zoonotic potential of rotavirus strains from dogs was believed to be of minor significance. The current investigation raises the question whether this assumption has to be revised, as we were able to demonstrate that dogs are susceptible to rotavirus strains with an artiodactyl/bovine ancestor and that a RVA originating from a possible reassortment event with a human strain can infect young dogs. Due to the fact that dogs can have contact with bovine as well to human faeces, it is possible that they may act as a mediator of RVA transmission between these two species.

This highlights the need for further investigations of RVA genotype distribution within the dog population, and the possible spread of these strains to livestock and humans.

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References


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