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Research paper

Genome analysis of a G9P[23] group A rotavirus isolated from a dog with diarrhea in China

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ABSTRACT

Genotype G9 is an emerging genotype among species A rotavirus (RVA) circulating in humans and pigs worldwide. In this study, an RVA strain designated RVA/Dog-tc/CHN/SCCD-A/2017/G9P[23] was isolated in cell culture from a pet dog stool sample with acute diarrhea, and its whole genome was sequenced. The genotype constellation of SCCD-A was G9-P[23]-I5-R1-C1-M1-A8-N1-T1-E1-H1. All genome segments except the VP1 gene were closely related to the genes from porcine RVA strains or porcine-like human RVA strains. On the other hand, the VP1 gene clustered in a distinct lineage only with that of a G5P[6] porcine-like human RVA, preventing the identification of the exact host species origin, but very unlikely to be originated from human RVA. In addition, phylogenetic analysis showed that the G9 VP7 gene of SCCD-A clustered into a novel sublineage within the lineage III of G9. This first isolation of a G9P[23] RVA from a pet dog may justify the exploration of the role dogs play in the interaction of RVA circulating in pigs and humans.

Rotavirus A (RVA), family Reoviridae, are the major pathogens causing diarrhea in animals and children worldwide. The RVA virion encapsidates a genome of 11 dsRNA segments, encoding six structural viral proteins (VP1–VP4, VP6 and VP7) and five or six nonstructural proteins (NSP1–NSP5/6) (Vlasova et al., 2017). RVA has two outer capsid proteins, VP7 and VP4, which define the G and P genotypes, respectively. To date, at least 36 G and 51 P genotypes have been identified by the Rotavirus Classification Working Group (RCWG) (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg). For highly genetically diverse RVA strains, the dual (G/P) typing system was extended to a full-genome sequence classification system, with the notations Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx used for the genes encoding VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6, respectively (Matthijnssens et al., 2008). In dogs, RVA is usually associated with mild diarrhea in puppies (Greene, 2010). Previously, only the G3P[3] genotype had been described in dogs, until a bovine G8P[1] genotype was isolated from a young dog in 2015 (Luchs et al., 2012; Sieg et al., 2015). The isolation and characterization of G3P[3] have been extensively reported in dogs, but information about the prevalence of RVA in dogs is still limited (Otto et al., 2015; Ortega et al., 2017; Alves et al., 2018). As canine RVAs have zoonotic potential between humans and dogs, further investigation of the molecular prevalence of RVA in dogs is needed (Wu et al., 2012; Luchs et al., 2012; Papp et al., 2015).

G9 rotavirus is an emerging genotype spreading worldwide in pigs and humans (Wu et al., 2017). In mainland China, the first G9 strain was detected in 1994, and G9 strains were uncommon before 2007 (Li et al., 2009). Recently, however, G9 RVA has emerged as the predominant genotype in China (Dian et al., 2017; Yu et al., 2018). The RVA G9P[23] genotype has been detected in Thailand, the United States, Japan, South Korea, Italy, Belgium, Brazil, mainland China and Taiwan (Wang et al., 2018). Recently, a G9P[23] strain was isolated from a child with severe diarrhea in Thailand, showing that porcine G9P[23] can infect humans directly (Komoto et al., 2017).

In this study, an RVA positive sample, detected by RT-PCR as described by Ortega et al. (2017), was used to isolate the virus. Moreover, this fecal sample was detected as negative for canine parvovirus type 2, canine coronavirus and canine distemper virus by PCR assay (Decaro et al., 2004; Decaro et al., 2005; Elia et al., 2006). The sample was collected from a three-month-old Labrador with acute diarrhea in October 2017 at the animal hospital of Southwest University for Nationalities in Sichuan Province, China.

RVA isolation was conducted on embryonic Rhesus monkey kidney tissue cells line (MA-104 cells, ATCC CRL-2378.1) as previously

Abbreviation: RVA, Group A rotavirus; RCWG, Rotavirus Classification Working Group; CPE, cytopathic effect; hpi, hours post-infection; ORF, open reading frame

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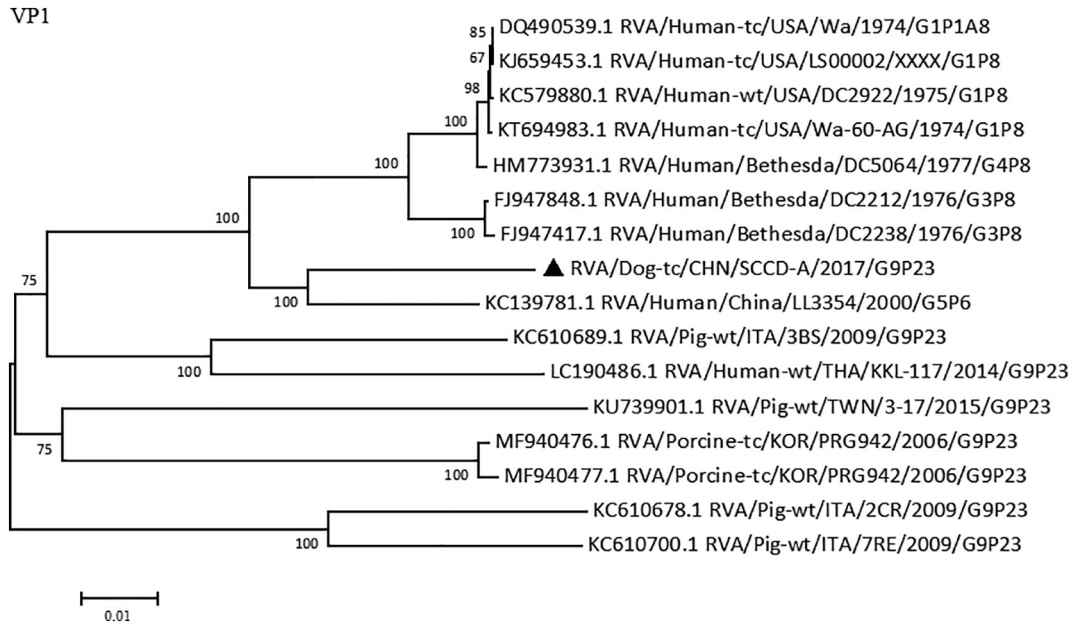

Fig. 1. Phylogenetic tree based on complete VP1 gene coding region nucleotide sequence 3267 bp, sequence alignment and clustering were performed by ClustalW using MEGA 7.0 software. The tree was constructed by the maximum likelihood method with bootstrap values calculated for 1000 replicates. ▲ marks the strain in this study.

Table 1
Closest-related strains in GenBank database.

Gene	Closely related strains	nt (%)	aa (%)	Genotype	Accession No.
VP7	RVA/pig/China/HN03/2015/G9P[23]	97.3	98.6	G9	KY649279
VP4	RVA/pig/China/NMTL/2008/G9P[23]	98.0	99.0	P[23]	JF781161
VP6	RVA/Pig/China/JN-1/2014/G5PX	96.8	98.5	I5	KT820768
VP1	RVA/Human/China//LL3354/G5P6	94.7	97.5	R1	KC139781
VP2	RVA/Pig-wt/IND/HP113/2002/G6P13	96.7	99.4	C1	KM393171
VP3	RVA/Pig/China/LNCY/2016/G3P13	97.0	97.8	M1	MF462323
NSP1	RVA/Pig/ROTA16/GXPX	94.0	95.9	A8	KJ482259
NSP2	RVA/Pig-wt/ITA/2CR/2009/G9P23	97.8	97.8	N1	KC610685
NSP3	RVA/Pig/China/TM-a/2009/G9P23	93.9	98.1	T1	GU189557
NSP4	RVA/Human/China/R479/2004/G4P6	96.6	98.3	E1	GU189558
NSP5	RVA/Human-wt/VNM/NT0073/2007/G9P19	99.0	99.5	H1	LC095909

described (Wang et al., 2018). Characterized by cell shrinking, cell layer splitting, lysis, detachment, and shedding, was observed at 48 h post-infection (hpi). After four continuous passages, virus cytopathic effect (CPE) was more visible, and the time of occurrence of CPE was stable. The fourth-generation cultures were used to plaque purification the virus. In passages 5–10, the time of occurrence of CPE was stable at 24 hpi. The VP7, VP4 and VP6 genes of the purified virus were sequenced at passages 4, 6, and 8, and they shared 100% nucleotide sequence identity between each passage, which indicated that the virus stock contained only a single virus strain. This RVA strain was designated RVA/Dog-tc/CHN/SCCD-A/2017/G9P[23]. At passage 10, virus titration was performed in 96-well plates with tenfold serial dilutions with eight replicates per dilution. The virus titer was determined by the Reed–Muench method, and endpoints were expressed as 50% tissue culture infective dose (TCID50)/ml, the virus titer was $10^{6.2}$ TCID50/ml.

To determine the RVA genome sequence, 27 pairs of primers were designed (Table S1, available in the online Supplementary Material). Viral RNA was extracted from culture-adapted virus using RNAiso Plus® (TaKaRa Bio Inc., Japan) and then reverse transcribed into cDNA using the PrimeScript™ RT Reagent Kit (TaKaRa Bio Inc., Japan) according to the manufacturer's instructions. The resulting cDNA was stored at −20 °C for PCR amplification. The PCR products were purified and cloned into the pMD19-T simple vector prior to sequencing, and the sequences were assembled using SeqMan software (version 7.0; DNASTAR). The open reading frame (ORF) was identified by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Genotype assignments were carried out using the RotaC v2.0 online tool according to the genotyping recommendations of the RCWG (Maes et al., 2009). Sequence identity analyses were performed with aligned nucleotide and amino acid sequences by the ClustalW method using the MegAlign 7.0 program (DNASTAR). Phylogenetic trees were constructed using the maximum likelihood method with the Jukes-Cantor model, 1000 bootstrap replicates and default parameters in MEGA 7. Recombination events were assessed using Simplot software (version 3.5.1) and RDP 4.0 with RDP, GeneConv, Chimaera, MaxChi, BootScan, SiScan, 3Seq methods and LARD.

The genome of RVA/Dog-tc/CHN/SCCD-A/2017/G9P[23] was successfully determined, and the constellation of this strain was G9-P[23]-I5-R1-C1-M1-A8-N1-T1-E1-H1. To exclude the possibility of virus reassortment between RVA strains that might have been presented in the clinical sample during the cell culture adaptation, we amplified fragments of all 11 genome segments from the original fecal sample. We confirmed that the nucleotide sequences were 100% identical between the virus genome present in the original fecal sample and the culture-adapted strain. The accession numbers for the 11 segments for VP1–VP4, VP6, VP7 and NSP1–NSP5 were deposited in GenBank (MH910063–MH910073). According to nucleotide identity of the

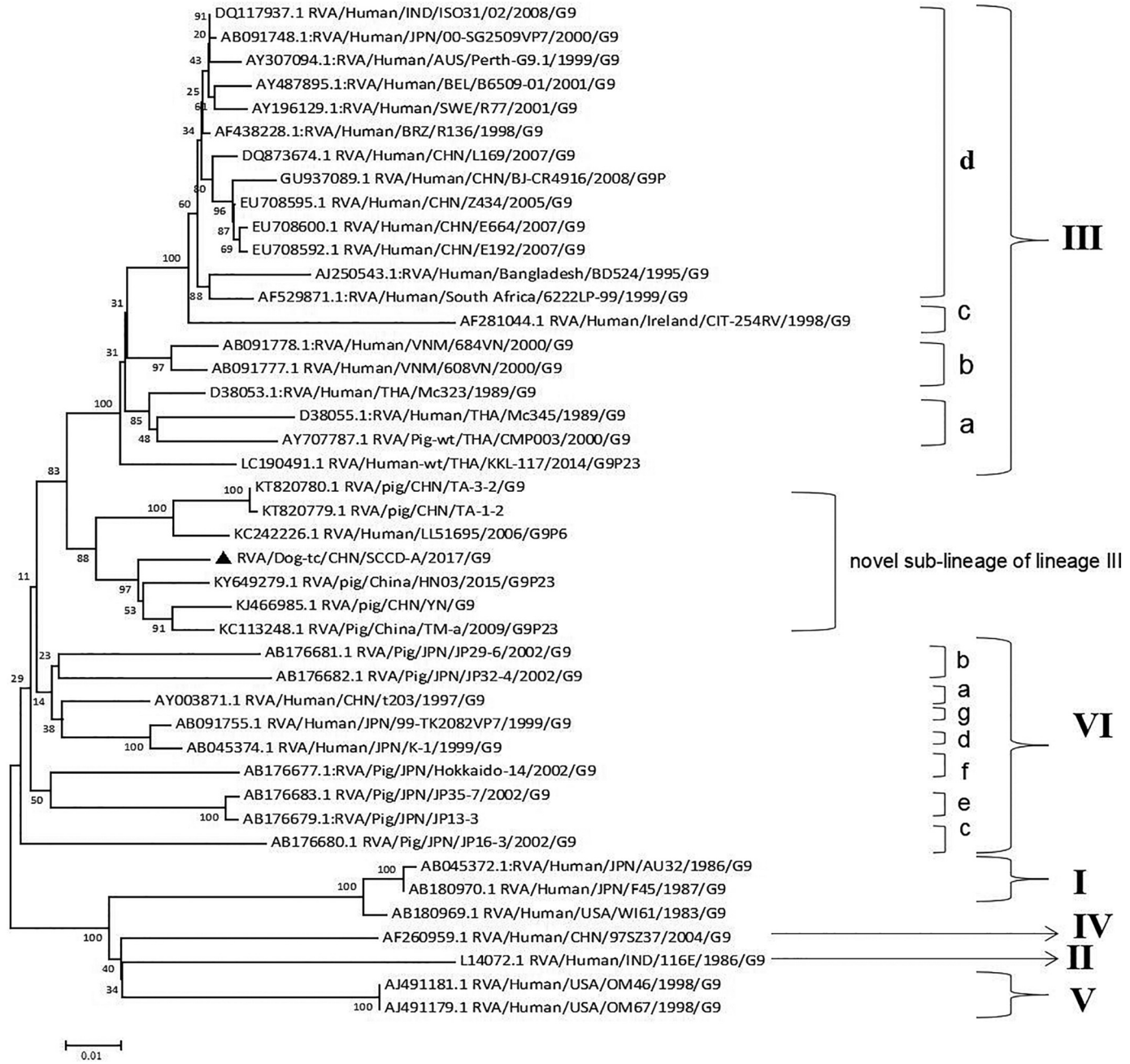

Fig. 2. Lineage classification of G9 rotavirus strains according to previous reports (Phan et al., 2007; Shi et al., 2012; Esona et al., 2013). Phylogenetic tree based on complete VP7 gene coding region nucleotide sequence 981 bp, sequence alignment and clustering were performed by ClustalW using MEGA 7.0 software. The tree was constructed by the maximum likelihood method with bootstrap values calculated for 1000 replicates. ▲ marks the strain in this study.

coding region sequences, the NSP1, NSP2, NSP3, VP2, VP3, VP4, VP6 and VP7 genes were closely related to cognate genes of porcine rotavirus strains. The sequence closest to the NSP4 gene of RVA/Dog-tc/CHN/SCCD-A/2017/G9P[23] was that of human strain R479 which was previously shown to be of porcine rotavirus origin (Wang et al., 2010). Similarly, the sequence closest to the NSP5 gene of SCCD-A was that of human strain NT0073 which was previously shown to be of porcine rotavirus origin (Do et al., 2017). On the other hand, the sequence closest to the VP1 gene of SCCD-A was that of strain LL3354 which was reported to be the result of a porcine rotavirus having transmitted to a human, but the phylogenetic tree for the VP1 genes showed that strains SCCD-A and LL3354 clustered together in an independent group that was distinct from any of the previously established lineages (Fig. 1). As the exact host species origin of the VP1 gene of strain LL3354 was reported to be indeterminable (Li et al., 2008), so was the origin of the VP1gene of SCCD-A. The nucleotide and amino acid identity of 11genes in strain SCCD-A with the closest sequences was shown in Table 1, the phylogenetic tree of VP7 was shown in Fig. 2. The phylogenetic tree of VP4 and VP6 genes were shown in Fig. S1 and Fig. S2 (available in the online Supplementary Material), the table of listing G9P[23] strains for which the whole genotype constellation was shown in Table S2 (available in the online Supplementary Material). No recombination event was identified in the RVA/Dog-tc/CHN/SCCD-A/2017/G9P[23] strain. Therefore, strain RVA/Dog-tc/CHN/SCCD-A/2017/G9P[23] was considered as a porcine origin of RVA strain. The dog from which the sample was obtained is a pet dog, and had no contact with pigs. Maybe the dog was not true host, and it was probable that the dog was just a incidental host, further investigation on

prevalence of the G9P[23] strain in dogs is required. Due to rotavirus is transmitted via fecal-oral transmission, whether the infectious source of this virus in the dog was from dietary contamination needed to be further investigated.

RVA VP7 defines the G genotype and induces neutralizing antibodies (Aoki et al., 2009). To accurately understand the antigenicity of G9 RVA, it was proposed that G9 can be divided into six distinct lineages (I–VI) based on the VP7 nucleotide sequence (Phan et al., 2007). Lineages I, II, IV and V are found only in humans, while lineages III (with sublineages a–d) and VI (with sublineages a–g) are found in both humans and pigs (Shi et al., 2012; Esona et al., 2013). According to previous reports about the division of G9 lineages (Phan et al., 2007; Shi et al., 2012; Esona et al., 2013), all strains in the three papers which represented every lineages and sublineages in G9 and 30 G9 sequences which had the highest similarity with strain SCCD-A in the GenBank were used for phylogenetic analysis. VP7 of strain RVA/Dog-tc/CHN/SCCD-A/2017/G9P[23] clustered into lineage III, but was located at a unique sub-branch with six G9 strains (KC242226.1, KY649279.1, KT820780.1, KT820779.1, KJ466985.1 and KC113248.1), which were distinct from the other known sublineages in lineage III, indicating that these seven strains may represent a novel sublineage of lineage III (Fig. 2). Only representative strains and strains of potential novel sublineage were retained in Fig. 2. The table of amino acids comparison between the potential novel lineage and other lineages was shown in Table S3 (available in the online Supplementary Material). No recombination event was identified in these strains, G9 lineage III is an emerging genotype, and both porcine and human strains of lineage III might have a common progenitor (Phan et al., 2007).

In conclusion, a G9P[23] RVA strain, named RVA/Dog-tc/CHN/SCCD-A/2017/G9P[23], was isolated from a young pet dog with acute diarrhea, and the genome constellation of this strain was G9-P[23]-I5-R1-C1-M1-A8-N1-T1-E1-H1. This strain was considered as a porcine origin of RVA strain, which indicated that dogs may play a role in RVA G9P[23] circulating in pigs and humans. The potential public health significance this poses, because of close contact between dogs and humans, highlights the necessity of further surveillance for this virus in dogs. To our knowledge, this is the first isolation of the G9P[23] genotype from dogs.

Depositories

The full-length genome of RVA/Dog-tc/CHN/SCCD-A/2017/G9P[23] has been deposited in GenBank under accession no. MH910063-MH910073.

Conflict of interest

The authors declare that they have no conflicts of interest.

Ethical statement

This study did not involve animal experiments besides the fecal sampling of diarrhea dogs that visited animal hospitals for clinical treatment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.02.020.

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