First identification and characterization of rotavirus H in swine in Europe

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Abstract

Rotaviruses (RVs) are a major cause of viral gastroenteritis in both animals and humans worldwide. According to the molecular and serological properties of Viral Protein 6 (VP6), RVs are classified into nine species or groups (RVA-RVD and RVF-RVJ). RVA, RVB and RVC are well-recognized as etiological agents of enteric disease on swine farms and have been identified in all countries with a relevant pork production. Contrarily, RVH has only been identified on swine farms from Japan and more recently from Brazil, USA, South Africa and Vietnam but not yet in Europe. The occurrence of RVH was investigated in 103 Spanish pig herds. Nine farms were positive and the complete nucleotide sequences were achieved for four RVH isolates. Mean nucleotide identities with the RVH sequences available in GenBak ranged between 69.4 and 93.7 %. Phylogenetically, all genomic segments of Spanish RVH isolates clustered closely with other porcine RVH strains but were distantly related to human RVH as well as bat RVH strain. Moreover, based on the available tentative genotyping system for RVH, a new genotype for VP7 was proposed. To the best of our knowledge this is the first report of RVH on swine farms in Europe including its characterization by means of complete genome sequencing.

KEYWORDS

Rotavirus H, swine, whole genome, genotype classification, NGS.

INTRODUCTION

Rotaviruses (RVs) are members of the family *Reoviridae* and major causative agents of gastroenteritis in humans and animals worldwide. Their genome consists of 11 segments of double-stranded RNA that encode six structural proteins (VP1–4, VP6, and VP7) and five nonstructural proteins (NSP1–5) (Estes MK, 2007). According to the International Committee on Taxonomy of Viruses, the *Rotavirus* genus is divided into nine antigenically distinct groups or species (RVA, RVB, RVC, RVD, RVF, RVG, RVH, RVI and RVJ) based on the diversity of their inner capsid protein (VP6) sequence (Matthijnssens et al., 2012; Mihalov-Kovács et al., 2015).

RV infections are very prevalent on swine farms, frequently linked to suckling and post-weaning diarrhea, which ends in large economic losses to the pork industry. Main RV groups associated with diarrhea in swine include RVA, RVB and RVC. RVA affects piglets between three and five weeks of age while RVC is much more common in young piglets (<7 days of age) (Vlasova, Amimo, & Saif, 2017).

In 1997, a new human RV tentatively named as novel adult diarrhea rotavirus (ADRV-N) which did not belong to any previously established group was described in China causing an outbreak of gastroenteritis among adults (Alam et al., 2007; Yang et al., 2004). The ADRV-N was subsequently classified as RVH based on VP6 sequence analysis (Matthijnssens et al., 2012). In total, three human RVH strains from Asia (ADRV-N and J19 from Chine and B219 from Bangladesh) as well as a porcine RVH strain from Japan (SKA-1) were identified during 1997-2002 (Jiang et al., 2008; Nagashima et al., 2008). Since then, RVH has been reported in pigs from Japan (Suzuki & Inoue, 2018; Wakuda et al., 2011), USA (Marthaler et al., 2014), Brazil (Molinari, Lorenzetti, Otonel, Alfieri, & Alfieri, 2014), South Africa (Nyaga et al., 2016) and Vietnam (Phan et al., 2016). More recently RVH has been also reported in bats in Cameroon (Yinda et al., 2018).

To the best of our knowledge there are no reports of porcine RVH in Europe. Here we introduce the first detection and characterization of RVH in pigs with diarrhea from Spanish pig farms, the main European pig producer.

MATERIAL AND METHODS

The study was performed on 103 Spanish swine farms with diarrhea outbreaks in which a viral etiology was suspected between 2017-2019. The outbreaks affected nursing piglets (<21 days) (26 farms), postweaning-growing pigs (21-70 days) (11 farms) or fattening pigs (>70 days) (33 farms). The age of affected animals was unknown on 33 farms.

Total RNA was extracted from one pooled fecal sample (2-6 individual samples) per farm using QIAamp Viral RNA Mini Kit (Qiagen) following the manufacturer's instructions. Then, we performed a reverse transcription PCR (RT-PCR) using a newly designed primer pair, based on the VP6 gene from reference sequences of porcine RVH strains obtained from Genbank (Table 1), and amplifying a 1240 nt fragment. The RT-PCR reactions were carried out with the Verso 1-Step RT-PCR ReddyMix Kit (Thermo Scientific), following the manufacturer's recommendations, with the following cycling conditions: an initial step of 50^{o}C for 30 min and 95^{o}C for 2 min; followed by 45 cycles of 95^{o}C for 20 s, 50^{o}C for 30 s and 72^{o}C for 1 min; with a final extension step at 72^{o}C for 10 min.

From each positive pooled sample by RT-PCR to RVH, total RNA was extracted using a TRIzol LS reagent (Thermo Scientific) protocol. The total RNA extraction was directly sequenced at the Genomics Bioinformatics Service (SGB) of the Autonomous University of Barcelona (UAB), without using any primer or amplification step. Next Generation Sequencing (NGS) was carried out using an Illumina Miseq Platform. RVH sequences were obtained from NGS outputs applying a tailor-made, virus-specific script developed by us (Cortey et al., 2019). We confirmed each segment identified by BLAST analyses on the assembled sequences using the NCBI GenBank online tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were aligned using CLUSTALW. The evolutionary relationships among sequences were analyzed with a phylogenetic analysis, using the Maximum-Likelihood method (ML) and the Tamura-Nei substitution model with MEGAX software (Kumar, Stecher, Li, Knyaz, & Tamura, 2018). Sequences were deposited in the NCBI GenBank with the accession numbers MT644949-MT644992.

RESULTS AND DISCUSSION

We detected RVH in nine out of the 103 pooled samples (8.7%, Figure 1), most of them from fattening pigs (6 positive samples out of 33) or postweaning-growing pigs (2 positive samples out of 11) while only one positive sample was detected in nursing piglets. A similar result was reported in commercially raised pigs in the USA (15% of positive fecal samples) being the odds of RVH positive 5.9 in the >55-day group as compared with odds for the 4-20-day piglets (Marthaler et al., 2014). However, in our research, the number of RVH outbreaks did not differ significantly between age groups when compared using Fisher exact test (p=0.139). Although no significant differences were demonstrated in the number of RVH outbreaks between age groups, clearance of maternal antibodies together with the mix of piglets after weaning may explain a higher percentage of positive samples in postweaning pigs (>21 days-old) as compared with suckling piglets (18% versus 4%).

Whole genome sequencing was attempted in all the strains and we achieved the complete genome (11 segments) in three of them (SP-VC18, SP-VC29 and SP-VC36). In another strain (SP-VC19) complete segment sequence was achieved for VP4, VP6, VP1, NSP1, NSP2, NSP4 and NSP5 while partial for VP7, VP2, VP3 and NSP3. The nucleotide identity among sequences varied between 82.0%–100% for VP7, 83.2%–100% for VP4, 86.1%–100% for VP6, 87.0%–100% for VP1, 84.4%–100% for VP2, 81.6%–100% for VP3, 84.0%–100% for NSP1, 91.0-%–100% for NSP2, 83.5%–100% for NSP3, 81.5%–100% for NSP4 and 92.3%–100% for NSP5.

The four sequences of porcine RVH strains recovered from Spanish farms were compared with those available in Genbank, including partial and complete genome sequences of porcine RVH isolates from Japan (n=11), USA (n=2), Brazil (n=3), South Africa (n=1) and Vietnam (n=5), as well as human (n=3) and bat (n=1) RVH strains.

High pairwise identities at the nucleotide levels (69.4-93.7%) were observed for all genomic segments of the Spanish porcine RVH strains when compared with the proposed porcine RVH genotypes (Appendix). In contrast, Spanish porcine RVH strains were distantly related to human RVH strains at the nucleotide levels (31.3–71.3%), as well as to the bat RVH strain (15.9-68.0%). Accordingly, the ML phylogenetic trees shown that Spanish porcine RVH strains were more closely related to the porcine RVH strains from Japan, USA, Brazil, South Africa and Vietnam, and more distantly related to human RVH strains from Bangladesh and China and were also distinct from a bat RVH strain from Cameroon (Figure 2). Phylogenetic analyses indicate that genomic sequences of RVH isolates recovered from different host species cluster in clearly distinct sub-groups (porcine, human and bat), suggesting the lack of recent interspecies transmission events. However, this observation is based on a very limited number of fully sequenced RVH strains recovered until now in these three different host species, particularly in humans and bats. More global whole genome RVH sequences are needed to conclusively determine the RVH evolutionary pathways and their zoonotic potential.

Recently a full genome-base genotyping system for RVH has been proposed (Suzuki & Inoue, 2018). Based on the recommended cut-off values, the Spanish porcine RVH strains were classified into one or two different genotypes for each genomic segment (Table 2 and Figure 2). We proposed a new genotype for VP7 (G11), since the pairwise sequence identities of SP-VC29 and SP-VC36 exceeded cut-off values proposed for the genomic segments (86%) when compared with the rest of the available strains (Appendix). However, this result should be taken with caution due to limited number of RVH sequences available. This is particularly obvious in the VP6 segment (Figure 2), where the ML tree clustering seems to point to a single – very diverse – group, but according to the proposed thresholds the isolate SP-VC36 might be considered a new genotype (Appendix). More RVH sequences should be added to the proposed genotyping system and cut-offs for each genome segment should be reviewed based on them.

Herein we report the first complete genome sequence of four porcine RVH strains from Spain being the first RVH strains identified in Europe. Our data indicate that RVH is relatively widespread in Spanish swine population being identified in almost 9% of the investigated diarrhea outbreaks. In addition, full genome sequencing showed its usefulness in the characterization the isolates, will facilitate further RVH surveillance in pigs globally as well as the development of improved diagnostic methods for RVH detection on swine farms.

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CONFLICT OF INTEREST

None of the authors of this study has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to, and the appropriate ethical review committee approval has been received. The regional guidelines for the care and use of animals were followed.

DATA AVAILABILITY STATEMENT

Data are available in the GenBank database and by direct contact with the correspondence author.

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Table 1. Oligonucleotide primers based on the VP6 gene designed for the detection of porcine RVH in fecal samples using RT-PCR. The nucleotide position was based on VP6 complete gene of RVH strains SKA-1 (AB576626), MRC-DPRU1575 (KT962031), MN.9.65 (KU254587) and OK.5.68 (MH230121).

Primer	Sequence (5' -3')	Nucleotide position
RVH-VP6-F	GTGACCCACAAGGATGGATCTCAT	19-42
RVH-VP6-R	GAACACTGGATCCCAGTGCGTGAC	1234-1257

Table 2. Genotypes for individual genes of the four porcine Spanish RVH strains identified in this study according to the full genome-based genotyping system proposed by Suzuki and Inoue, 2018.

Strains	Gene Segment	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSI
	Cut-off in percentage	86	86	87	85	87	86	84	67	87	83
Porcine RVH SP-VC18	Porcine RVH SP-VC18	G3	P1	I3	R1	C3	M1	A6	N1	T1	E4
Porcine RVH SP-VC19	Porcine RVH SP-VC19	G3	P1	I3	R1	C1	M1	A6	N1	T1	E4
Porcine RVH SP-VC29	Porcine RVH SP-VC29	G11*	P3	I3	R3	C3	M3	A6	N1	T1	E3
Porcine RVH SP-VC36	Porcine RVH SP-VC36	G11*	P3	I3	R3	C1	M3	A5	N1	T1	E4

*New genotype proposed

Figure 1**.** Identification of the porcine RVH positive samples by the visualization under UV light of the RedSafe staining 2% agarose gel electrophoresis of the amplification of a 1240 bp fragment of the RVH VP6 gene. The pooled positive samples are identified as VC9, VC18, VC19, VC29, VC32, VC36, VC39 VC41 and VC52 and the negative control as NEG.

Fig 2. Maximum Likelihood trees constructed with the Tamura-Nei model for the VP7, VP4 and VP6 RVH segments. Numbers along the tree represents the confidence value for a given internal branch based on 500 Bootstrap replicates, only values larger than 70 are shown. The symbols (filled circles) above the strains indicate the porcine Spanish RVH strains identified in this study. GenBank accession number, country and

year of collection of fecal sample are also shown below the strains. Genotypes are indicated on the right of the bracket. Scale bars indicate nucleotide substitutions per site.