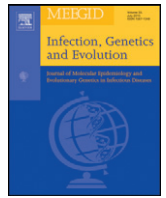




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## Research paper

# Characterization of a genetically heterogeneous porcine rotavirus C, and other viruses present in the fecal virome of a non-diarrheic Belgian piglet



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## ABSTRACT

Next-generation sequencing (NGS) technologies are becoming increasingly accessible, leading to an expanded interest in the composition of the porcine enteric virome. In the present study, the fecal virome of a non-diarrheic Belgian piglet was determined. Although the virome of only a single piglet was analyzed, some interesting data were obtained, including the second complete genome of a pig group C rotavirus (RVC). This Belgian strain was only distantly related to the only other completely characterized pig RVC strain, Cowden. Its relatedness to RVC strains from other host species was also analyzed and the porcine strain found in our study was only distantly related to RVCs detected in humans and cows. The gene encoding the outer capsid protein VP7 belonged to the rare porcine G3 genotype, which might be serologically distinct from most other pig RVC strains. A putative novel RVC VP6 genotype was identified as well. A group A rotavirus strain also present in this fecal sample contained the rare pig genotype combination G11P[27], but was only partially characterized. Typical pig RVA genotypes I5, A8, and T7 were found for the viral proteins VP6, NSP1, and NSP3, respectively. Interestingly, the fecal virome of the piglet also contained an astrovirus and an enterovirus, of which the complete genomes were characterized. Results of the current study indicate that many viruses may be present simultaneously in fecal samples of non-diarrheic piglets. In this study, these viruses could not be directly associated with any disease, but still they might have had a potential subclinical impact on pig growth performance. The fast evolution of NGS will be a powerful tool for future diagnostics in veterinary practice. Its application will certainly lead to better insights into the relevance of many (sub)clinical enteric viral infections, that may have remained unnoticed using traditional diagnostic techniques. This will stimulate the development of new and durable prophylactic measures to improve pig health and production.

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## 1. Introduction

In recent years, next-generation sequencing (NGS) has been applied to study the microbiota shed in the feces of different host species. The entirety of viruses present in the feces of a host is called the fecal virome and analysis of its composition in piglets is of great interest for several reasons. First, it allows investigators to distinguish viruses which can induce clinical disease (diarrhea), subclinical growth retardation, or even (apathogenic/beneficial) viruses which do not cause symptoms. Second, some of the viruses shed in the feces of piglets can also cause zoonotic

infections in humans and rotaviruses are a well described example (Martella et al., 2010; Zeller et al., 2012). In previous studies the fecal virome of healthy and diarrheic pigs has been investigated. Shan and colleagues reported the virome composition of healthy and diarrheic pigs on a high-density pig farm in North Carolina, United States. On average, 4.2 and 5.2 mammalian viruses were shed by non-diarrheic and diarrheic piglets, respectively. Most dominant viruses were kobuviruses, astroviruses, enteroviruses, sapoviruses, sapeloviruses, coronaviruses, bocaviruses and teschoviruses. However, boca- and coronaviruses were more abundant in diarrheic animals (Shan et al., 2011). A process-controlled method, based on the addition of a defined amount of bacteriophages to allow for comparison between different samples, was applied by Sachsenröder and colleagues to investigate the fecal virome retrieved from a pooled fecal sample of five 35 days old pigs. Two of five animals had diarrhea at the moment of sampling (Sachsenröder et al., 2012). Most viruses detected could be classified as bacteriophages (73.9%), mammalian viruses (23.9%) or plant viruses

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(0.8%). Kobuvirus, group C rotavirus, Chimpanzee stool associated circular ssDNA virus, astrovirus and enterovirus were the most abundant mammalian viruses found in this study (Sachsenroder et al., 2012). Zhang and colleagues reported the detection of 15 distinct mammalian viruses in a pooled fecal sample of 27 diarrheic piglets from China (Zhang et al., 2014). Porcine epidemic diarrhea virus, sapovirus, bocavirus, sapelovirus and torovirus were most abundant (Zhang et al., 2014). To study the virome composition, standardized procedures that are useful in laboratory settings are needed. Recently, a standardized protocol for sample preparation, called NetoVIR (Novel enrichment technique of VIRomes), was established in our laboratory to allow researchers to apply high throughput viral metagenomics to study fecal viromes (Conceicao-Neto et al., 2015).

Rotaviruses have been recognized as an important cause of diarrhea in young pigs and children. They belong to the *Reoviridae* family and possess a triple layered capsid, protecting the 11 segments of the dsRNA genome. The core (VP2) surrounds the genome and also encloses the viral enzymes VP1 and VP3. VP6 proteins form the inner capsid layer which is covered by the outer capsid proteins VP7 and VP4. The viral genome also encodes 6 different non-structural proteins (NSP1–NSP6). The genus *Rotavirus* is subdivided into 9 different species (A to H) (Matthijssens et al., 2012). Species I, recently detected in Hungarian sheltered dogs needs further ratification by the ICTV (Mihalov-Kovacs et al., 2015).

Group A rotaviruses (RVA) have been considered as clinically and epidemiologically most important RV species in humans and animals, including pigs. Due to its segmented genome, reassortant viruses can be created upon dual infection of a single cell with different virus strains. To detect such a reassortment event and to characterize rare RVA strains in human surveillance studies, a full genome-based classification system was established for RVAs (Matthijssens et al., 2008a; Matthijssens et al., 2008b). The outer capsid proteins VP7 and VP4 are important in this classification system, as they can induce the formation of neutralizing antibodies. Nevertheless, intracellular neutralizing antibodies targeting VP6 may also play an important role in clearing RVA infections (Aiyegbo et al., 2014; Aiyegbo et al., 2013). Many VP7 (n = 28) and VP4 (n = 39) genotypes have been detected in multiple species (Matthijssens et al., 2011; Matthijssens and Theuns, 2015; Trojnar et al., 2013). In pigs, a large diversity of VP7/VP4 genotypes can be encountered (Amimo et al., 2013; Chandler-Bostock et al., 2014; Collins et al., 2010; Martella et al., 2005; Papp et al., 2013; Pham et al., 2014). As an example, six different G-genotypes for VP7 (G2, G3, G4, G5, G9 and G11), and five different P-genotypes for VP4 (P[6], P[7], P[13], P[23] and P[27]) were recently encountered in 12 different G/P combinations in feces from Belgian diarrheic and non-diarrheic piglets (Theuns et al., 2014). Overall, these pig RVA strains possessed a relatively conserved pig genetic backbone for the other 9 gene segments, namely I5–R1–C1–M1–A8–N1–T7–E1–H1, which demonstrated a clear evolutionary relationship with genes of dominant human Wa-like RVA strains (Matthijssens et al., 2008a; Theuns et al., 2015).

Group C rotavirus (RVC) is being considered as an etiological cause of diarrhea in young pigs, but the virus has also been detected in fecal samples of non-diarrheic piglets at a lower prevalence rate (Bohl et al., 1982; Collins et al., 2008; Marthaler et al., 2014; Saif et al., 1980). A genotype classification system for RVC VP7 encoding genes has been established. Pig strains typically belong to genotypes G1, G3, G5, G6–G10, whereas human and cow RVCs belong to genotypes G4 and G2, respectively (Marthaler et al., 2013). Recently, two different VP4 nucleotide sequence identity cut-off values have been established to assign RVC strains into specific VP4 genotypes (Jeong et al., 2015; Suzuki et al., 2014a). Similar nucleotide sequence identity cut-off values have been identified to classify RVC VP6 genes into distinct genotypes (Jeong et al., 2015; Suzuki et al., 2014b). Still, only a limited number of RVC gene sequences have been analyzed to date and only one complete porcine RVC genome is available. This hampers not only our understanding of RVC evolution in different host species, but also the

development of a consensus full-genome characterization system for RVC (Bremont et al., 1992; Mawatari et al., 2014; Soma et al., 2013; Yamamoto et al., 2011).

Porcine astrovirus (PoAstV), a member of the genus *Mamastrovirus* within the family *Astroviridae* is often detected during viral metagenomics studies (Sachsenroder et al., 2012; Shan et al., 2011). Cross-neutralization assays have been applied to classify astroviruses into different serotypes, but this has been hampered by the lack of viral growth in cell culture. Nowadays, classification is more often performed using genetic analysis of ORF2, which is the most variable astrovirus gene (De Benedictis et al., 2011; Lee and Kurtz, 1982). Still, the current classification is not able to cope with the wide genetic diversity among porcine astroviruses. It also remains difficult to associate these infections with disease, as these viruses have also been isolated from healthy pigs as well (De Benedictis et al., 2011; Mor et al., 2012; Xiao et al., 2013). This is in contrast to humans, where some species have been associated with diarrhea (De Benedictis et al., 2011; Jarchow-Macdonald et al., 2015; Medici et al., 2014; Xavier Mda et al., 2015). Other species of astroviruses have also been identified in cases of encephalitis in humans and cattle (Bouzalas et al., 2014; De Benedictis et al., 2011; Li et al., 2013; Naccache et al., 2015; Seuberlich et al., 2016). Interestingly, adenoviruses have also been recognized as an important cause of diarrhea in Irish children (Lennon et al., 2007). Also for enteroviruses in piglets, the role in the development of diarrhea is being questioned, while they are very often detected in the virome of piglets. Porcine enteroviruses belong to the species *enterovirus G* within the genus *Enterovirus* of the *Picornaviridae* family. Originally, porcine teschovirus and porcine sapelovirus were considered as distinct serotypes within the genus *enterovirus*, but they are now considered as distinct genera (Adams et al., 2013).

In a previous study, a low viral load (4.79 log<sub>10</sub> copies/g feces) of RVA was detected in a fecal sample from a non-diarrheic Belgian piglet (Theuns et al., 2014). This strain contained the rare pig G11P[27] genotype combination for VP7 and VP4. A much higher load of an RVC strain (9.56 log<sub>10</sub> copies/g feces) was later identified in this sample. Therefore, it was decided to unravel the entire virome content of this interesting fecal sample by means of NGS. It was aimed to reveal the second complete genome of a pig RVC strain. The evolutionary relationship between the RVC strain from the pig and those of other host species was investigated to better assess the risk for future interspecies transmission and reassortment events between pig and other RVC strains. Furthermore, the future application of NGS in pig veterinary diagnostics is being discussed.

## 2. Materials and methods

### 2.1. Processing of fecal sample

The fecal sample was collected at private diagnostic laboratory (DGZ Vlaanderen) from a non-diarrheic Belgian weaned piglet in 2012. A 20% fecal suspension of sample 12R021 was made in phosphate buffered saline containing 1000 U/ml penicillin (Continental Pharma, Puurs, Belgium), 1 mg/ml streptomycin (Certa, Braine l'Alleud, Belgium), 1 mg/ml gentamicin (Gibco BRL, Merelbeke, Belgium) and 0.01% v/v Fungizone (Bristol-Myers Squibb, Braine l'Alleud, Belgium) (Theuns et al., 2014). For Sanger sequencing, RNA was extracted from the fecal suspension using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and stored at –70 °C.

For Illumina sequencing, the fecal suspension was filtered twice. A first filtration step was done using 0.8 µm centrifugal membrane filters, followed by a second step using 0.45 µm filters (Merck Millipore, Darmstadt, Germany). Two microliters of Benzonase Nuclease (Merck Millipore, Darmstadt, Germany), 1 µl of Micrococcal Nuclease (New England Biolabs Inc., Ipswich, MA, USA), 1 µl of NEBNext® RNase III RNA Fragmentation Module (New England Biolabs Inc., Ipswich, MA, USA) and 7 µl of a buffer made in our laboratory (1 M Tris, 100 mM CaCl<sub>2</sub>

and 30 mM MgCl<sub>2</sub>) were added to 130 µl of fecal filtrate, and incubated for 2 h at 37 °C to destroy free-floating DNA/RNA. Next, 7 µl of EDTA was added to the sample for enzyme inactivation. Extraction of viral RNA was performed using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), but without addition of carrier RNA.

## 2.2. RT-PCR for RVA segments

Sanger sequencing was applied in an attempt to obtain the complete genome of RVA/Pig-wt/BEL/12R021/2012/G11P[27]. RNA was denatured at 95 °C for 2 min and immediately chilled on ice. For RT-PCR amplification of short gene segments (VP6, VP7, NSP2, NSP3, NSP4 and NSP5), reaction mixtures consisted of 5 µl RNA, 5 µl of 5× QIAGEN OneStep RT-PCR Buffer, 1 µl of dNTP Mix, 1.5 µl of forward and reverse primer (830 nM), 1 µl of QIAGEN OneStep RT-PCR enzyme mix, and nuclease free water in a total volume of 25 µl per reaction. Reaction volumes were upscaled to 50 µl for the longer gene segments (VP1, VP2, VP3, VP4, and NSP1). Primers used for RT-PCR amplification of the complete gene segments are available in the Supplemental Table 1.

Reverse transcriptase was performed at 50 °C for 30 min, followed by *Taq* polymerase activation at 94 °C for 15 min, and 35 cycles of amplification. For the longer fragments, denaturation was performed for 30 s at 94 °C, annealing for 30 s at 50 °C, and extension at 72 °C for 6 min. For the shorter fragments denaturation was performed for 30 s at 94 °C, annealing for 30 s at 47 °C, and extension at 72 °C for 2 min. A final extension step was performed for 10 min at 72 °C. Afterwards, 9 µl of PCR product were mixed with 1 µl of loading buffer and loaded on polyacrylamide gels. Electrophoresis was performed at 200 V for 36 min, followed by ethidium bromide staining of the gels for detection of positive samples. If RT-PCR was unsuccessful, two RNA extracts were combined and precipitated using 70% ethanol. Concentrated RNA and DNA was diluted in nuclease free water and used to repeat the RT-PCR.

## 2.3. Sanger sequencing and nucleotide sequence analysis

Samples were treated by adding 5 µl of PCR positive amplicons to 1 µl of USB ExoSAP-IT® PCR Product Clean-Up (Affymetrix, Santa Clara, California, USA) and sequenced with an ABI Prism BigDye terminator cycle sequencing reaction kit on an ABI Prism 3130xl apparatus (Applied Biosystems, Foster City, CA, USA) using forward and reverse primers. If possible, remaining parts of the coding regions were further covered by primer-walking sequencing. Analysis of nucleotide sequences obtained by Sanger sequencing was executed using the 4Peaks software (Mekentosj, Amsterdam, The Netherlands). RVA genotypes were determined using BLAST (Altschul et al., 1990) and Rotac<sup>2.0</sup> (Maes et al., 2009).

## 2.4. Preparation of nucleic acids for Illumina sequencing

Total RNA was amplified using the Whole Transcriptome Amplification Kit 2 (WTA2, Sigma Aldrich, Diegem, Belgium). Library Synthesis Solution (0.5 µl) was added to 2.82 µl of RNA, followed by denaturation for 2 min at 95 °C. RNA was cooled to 18 °C and 0.5 µl Library Synthesis Buffer, 0.4 µl Library Synthesis Enzyme and 0.78 µl of water were immediately added to the reaction. The mixture was subjected to the following temperature profile: 18 °C, 25 °C, 37 °C, 42 °C and 70 °C for 10, 10, 30, 10 and 20 min respectively. Samples were cooled down to 4 °C, followed by a brief centrifugation step. A mastermix containing 60.2 µl of nuclease free water, 7.5 µl of Amplification Mix, 1.5 µl of WTA2 dNTP mix and 0.75 µl Amplification Enzyme was added to the sample and incubated as follows: 94 °C for 2 min and 30 cycles at 94 °C for 30 s and 70 °C for 5 min. WTA2 products were purified with the MSB® Spin PCRapace kit (STRATEC Molecular, Birkenfeld, Germany) and prepared for Illumina sequencing using the KAPA DNA Library Preparation Kit (Kapa Biosystems, Wilmington, NC, USA).

## 2.5. Illumina sequencing and nucleotide sequence analysis

Fragments ranging from 350 to 600 bp were selected using BluePippin (Sage Science, MA, USA). Sequencing of the samples was performed on a HiSeq™ 2500 platform (Illumina, Inc., San Diego, CA, USA) for 300 cycles (150 bp paired-ends). Raw reads were trimmed for quality and adapters using Trimmomatic (Bolger et al., 2014), and were *de novo* assembled into contigs using SPAdes (Bankevich et al., 2012). Scaffolds were classified using a tBLASTx search against all complete viral genomes in GenBank using an e-value cut-off of 10<sup>-10</sup>. Scaffolds with a significant tBLASTx hit were retained and used for a second tBLASTx search against the GenBank nucleotide database using an e-value of 10<sup>-4</sup> (Altschul et al., 1990).

## 2.6. Phylogenetic analysis

GenBank accession numbers of gene segments of the porcine RVA and RVC strains, and astro- and enterovirus are provided in Table 1. RVC genotypes for VP7 were assigned using the nucleotide identity cut-off values proposed by Marthaler and colleagues (Marthaler et al., 2013). Multiple sequence alignments were performed using the ClustalW plug-in in MEGA 6.06, followed by manual editing. For each RVC gene segment, maximum-likelihood phylogenetic trees were constructed and bootstrap-analysis was set at 500 replicates. Substitution models were determined for each gene segment separately. Pairwise distances were calculated using the p-distance model with 500 bootstrap replicates. Genetic analyses of the astro- and enteroviruses were executed on the amino acid level, because of a high genetic diversity present at the nucleotide level.

## 3. Results

### 3.1. Partial characterization of the Belgian pig RVA strain

Only the partial genome of pig strain RVA/Pig-wt/BEL/12R021/G11P[27] was determined using Sanger sequencing, and a further attempt to obtain the remaining parts of the genome using NGS proved unsuccessful. The partial characterization can be explained by the very low viral load of the RVA strain in the fecal sample. Its genotype constellation in relationship to other porcine RVA strains is shown in Table 2. The VP7 and VP4 genotypes were already previously described as G11

**Table 1**  
GenBank accession numbers.

Virus	Gene	Accession number	Number of NGS reads	
RotavirusC/Pig-wt/BEL/12R021/2012/G3P5	VP1	KP982879	30,568	
	VP2	KP982880	18,820	
	VP3	KP982881	14,619	
	VP4	KP982882	26,910	
	VP6	KP982883	18,925	
	VP7	KP982884	6782	
	NSP1	KP982874	22,087	
	NSP2	KP982875	19,830	
	NSP3	KP982876	22,134	
	NSP4	KP982877	2530	
	NSP5	KP982878	7151	
	Rotavirus A/Pig-wt/BEL/12R021/2012/G11P[7]	VP6	KU510404	Sanger sequencing
		NSP1	KU510402	Sanger sequencing
		NSP2	KU510401	Sanger sequencing
	Porcine Astrovirus/BEL/12R021	Complete genome	KP982872	104,775
Complete genome		KP982873	11,833	



**Table 2**  
Genotype constellation of RVA strain 12R021 and other porcine RVA strains<sup>a</sup>.

Strain	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5	Ref.		
RVA/Pig-wt/BEL/12R021/2012/G11P[27]	<b>G11</b>	<b>P[27]</b>	<b>I5</b>	Rx	Cx	Mx	<b>A8</b>	<b>N1</b>	<b>T7</b>	Ex	Hx	TS		
<i>Concensus pig RVA genotype constellation</i>	<b>G5</b>	<b>P[7]</b>	<b>I5</b>	<b>R1</b>	<b>C1</b>	<b>M1</b>	<b>A8</b>	<b>N1</b>	<b>T1</b>	<b>T7</b>	<b>E1</b>	<b>E9</b>	<b>H1</b>	(Theuns et al., 2015)
<i>Human Wa-like RVA constellation</i>	<b>G1</b>	<b>P[8]</b>	<b>I1</b>	<b>R1</b>	<b>C1</b>	<b>M1</b>	<b>A1</b>	<b>N1</b>	<b>T1</b>	<b>E1</b>	<b>H1</b>	(Matthijnsens et al., 2008a)		
RVA/Pig-wt/BEL/12R022/2012/G2P[27]	<b>G2</b>	<b>P[27]</b>	<b>I5</b>	<b>R1</b>	<b>C1</b>	<b>M1</b>	<b>A8</b>	<b>N1</b>	<b>T7</b>	<b>E9</b>	<b>H1</b>	(Theuns et al., 2015)		
RVA/Pig-tc/MEX/YM/1983/G11P[7]	<b>G11</b>	<b>P[7]</b>	<b>I5</b>	<b>R1</b>	<b>C1</b>	<b>M1</b>	<b>A8</b>	<b>N1</b>	<b>T1</b>	<b>E1</b>	<b>H1</b>	(Matthijnsens et al., 2010)		
RVA/Pig-tc/VEN/A253/1988/G11P[7]	<b>G11</b>	<b>P[7]</b>	<b>I5</b>	<b>R1</b>	<b>C2</b>	<b>M1</b>	<b>A1</b>	<b>N1</b>	<b>T1</b>	<b>E1</b>	<b>H1</b>	(Matthijnsens et al., 2008a)		
RVA/Pig-wt/CAN/F6-4/2006/G11P[13]	<b>G11</b>	<b>P[13]</b>	<b>Ix</b>	Rx	Cx	M1	<b>A8</b>	<b>N1</b>	<b>T7</b>	Ex	Hx	(Martel-Paradis et al., 2013)		
RVA/Pig-tc/USA/Gottfried/1983/G4P[6]	<b>G4</b>	<b>P[6]</b>	<b>I1</b>	<b>R1</b>	<b>C1</b>	<b>M1</b>	<b>A8</b>	<b>N1</b>	<b>T1</b>	<b>E1</b>	<b>H1</b>	(Matthijnsens et al., 2010)		
RVA/Pig-tc/USA/OSU/1977/G5P[7]	<b>G5</b>	<b>P[7]</b>	<b>I5</b>	<b>R1</b>	<b>C1</b>	<b>M1</b>	<b>A1</b>	<b>N1</b>	<b>T1</b>	<b>E1</b>	<b>H1</b>	(Matthijnsens et al., 2010)		

<sup>a</sup> Belgian strain from this study is shown in bold. Color code: blue, typical pig genotype; green, Wa-like genotype; TS is this study.

and P[27], respectively (Theuns et al., 2014). Here, the genes encoding VP6, NSP1, NSP2 and NSP3 were characterized, which possessed the typical pig I5, A8, N1 and T7 genotypes, respectively.

### 3.2. Characterization of a genetically divergent Belgian pig RVC strain

#### 3.2.1. Pairwise distances between the Belgian pig strain and other RVCs

In contrast to the RVA strain, the entire genome of the Belgian RVC strain could be obtained from the NGS analyses. The number of RVC reads used to reconstruct the RVC genome is shown Table 1. Overall, the Belgian pig RVC was genetically distantly related to the only other completely characterized pig RVC strain, Cowden, and even more divergent from RVC strains isolated from cows and humans (Table 3). Interestingly, the VP1, VP2, VP3 and VP6 encoding genes of Belgian strain 12R021 were slightly more related to those of human RVCs than those of cow RVCs. For genes encoding nonstructural proteins, there was a much higher genetic distance between genes from pig RVCs and those of human and cow RVCs. Overall, the genetic diversity was most pronounced for the gene encoding NSP1.

#### 3.2.2. Genes encoding outer capsid proteins VP7 and VP4

A maximum-likelihood phylogenetic tree was constructed to analyze the VP7 and VP4 coding genes, using the general time reversible

model with gamma distribution and invariant sites for both genes. As shown in Fig. 1, the genes encoding the RVC outer capsid protein VP7 were subdivided into 9 official genotypes (G1 to G9) and one tentative genotype (G10) (Marthaler et al., 2013; Moutelikova et al., 2015). Pig RVC strains were found in genotypes G1, G3, G5, G6, G7, G8, G9 and G10. Genotype G4 is formed by a cluster of highly related human RVC strains in between genetically diverse pig RVC genotypes G1, G5, G7, G9 and G10. Furthermore, the G2 genotype consisted of only cow RVCs, genetically distinct from pig and human RVCs. The Belgian pig RVC strain 12R021 belonged to genotype G3, and only showed between 85.1 and 88.9% nucleotide similarity with the other pig G3 strains.

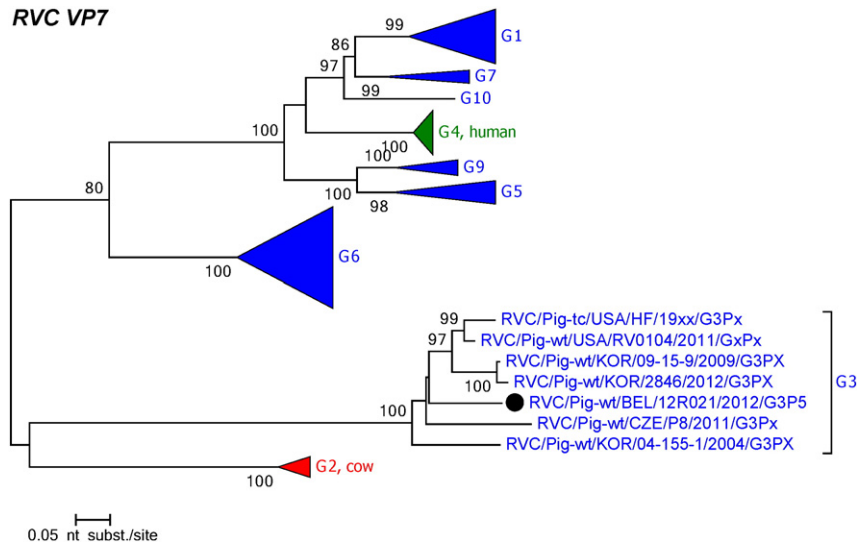
The genes of the outer capsid protein VP4 could be phylogenetically divided into distinct clusters (Fig. 2). One cluster contained genetically related human RVCs, whereas the other cluster consisted of genetically heterogeneous pig and cow RVCs. Belgian strain 12R021 clustered together with pig strains from the US, South Korea, Japan and Czech Republic in the P5 genotype, but at a relatively high genetic distance (81.0 to 86.9% nt similarity).

#### 3.2.3. Genes encoding structural proteins VP6, VP1, VP2 and VP3

A Tamura 3 model with gamma distribution and invariant sites was used to construct a phylogenetic tree for VP6 (Fig. 3). A major cluster within the tree consisted of genetically heterogeneous pig RVCs,

**Table 3**  
Nucleotide similarities between pig RVC strain 12R021 and other RVC strains.

Strain	Nucleotide similarities (%) between genes of strain 12R021 and other strains										
	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVC/Pig-tc/USA/Cowden/1980/G1P1	72.6	74.5	85.4	88.5	86.8	87.4	73.2	81.3	76.8	82.6	85.4
RVC/Cow-wt/JPN/Toyama/2010/G2P3	71.2	73.6	81.4	79.7	78.8	78.1	68.8	80.8	77.2	66.4	75.7
RVC/Cow-wt/JPN/Y/08/2008/G2P3	71.7	73.6	81.8	79.6	79.4	78.2	68.7	80.4	77.0	67.8	75.4
RVC/Human-wt/GBR/Bristol/1988/G4P2	72.8	71.0	83.0	84.6	83.9	83.3	65.5	80.8	78.2	68.3	74.8
RVC/Human-wt/BGD/BS347/2005/G4P2	72.5	71.0	82.9	84.7	83.6	82.7	64.1	80.3	77.5	68.5	74.9
RVC/Human-wt/CHN/Wu82/2001/G4P2	72.8	70.8	83.1	84.8	83.3	86.4	65.7	80.9	77.4	68.5	74.4
RVC/Human-wt/KOR/CAU 10-312/2010/G4P2	72.6	70.9	82.5	84.7	83.7	82.4	63.9	79.8	77.3	68.3	74.3

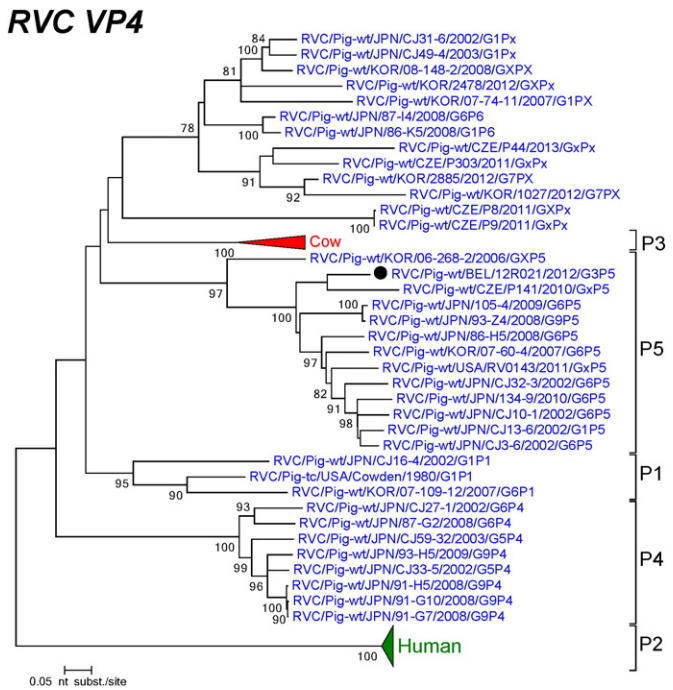


**Fig. 1.** Maximum-likelihood phylogenetic tree based on the coding sequences of RVC VP7 genes. Bootstrap values (n = 500 replicates) lower than 70% are not shown. Pig, cow and human RVC strains are colored blue, red and green, respectively. Belgian strain RVC/Pig-wt/BEL/12R021/2012/G3P5 is marked with a circle.

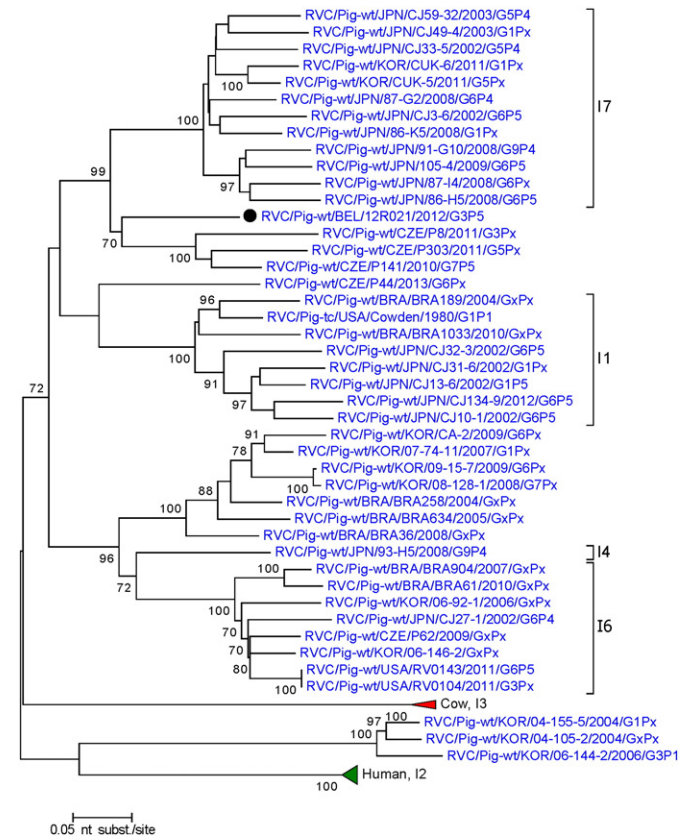
whereas cow and human RVCs were genetically distantly related to pig RVCs. Using the nucleotide sequence identity cut-off value of 90%, which was proposed by Jeong and colleagues, it was not possible to assign strain 12R021 to any known VP6 genotype (Jeong et al., 2015). The strain formed a separate subcluster at a high genetic distance from pig strains isolated in the Czech Republic (86.5 to 87.8% nt similarity).

For the other gene segments encoding structural proteins (VP1 to VP3) of RVCs, only a limited number of nucleotide sequences were available in GenBank. The following substitution models were used to construct phylogenetic trees, namely Hasegawa-Kishino-Yano model with gamma distribution for VP1, Hasegawa-Kishino-Yano model with

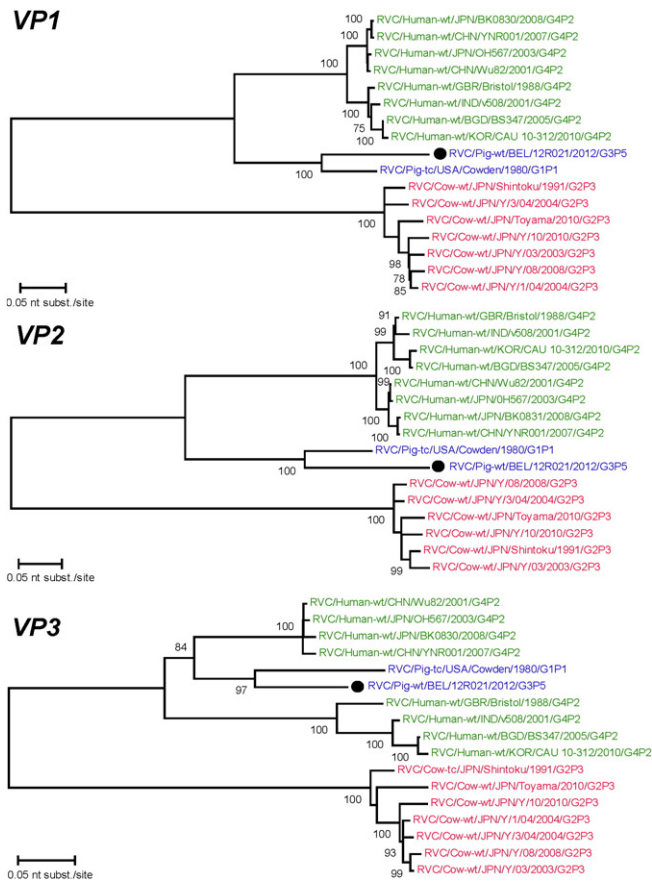
gamma distribution for VP2 and general time reversible model with gamma distribution and invariant sites for VP3. The phylogenetic trees of genes encoding structural proteins are shown in Fig. 4. For VP1 and VP2, genes clustered together according to the host species of origin. For VP3, the pig strains were located in between two distinct human subclusters.



**Fig. 2.** Maximum-likelihood phylogenetic tree based on the coding sequences of RVC VP4 genes. Bootstrap values (n = 500 replicates) lower than 70% are not shown. Pig, cow and human RVC strains are colored blue, red and green, respectively. Belgian strain RVC/Pig-wt/BEL/12R021/2012/G3P5 is marked with a circle.



**Fig. 3.** Maximum-likelihood phylogenetic tree based on the coding sequences of RVC VP6 genes. Bootstrap values (n = 500 replicates) lower than 70% are not shown. Pig, cow and human RVC strains are colored blue, red and green, respectively. Belgian strain RVC/Pig-wt/BEL/12R021/2012/G3P5 is marked with a circle.



**Fig. 4.** Maximum-likelihood phylogenetic trees based on the coding sequences of RVC VP1, VP2 and VP3 genes. Bootstrap values ( $n = 500$  replicates) lower than 70% are not shown. Pig, cow and human RVC strains are colored blue, red and green, respectively. Belgian strain RVC/Pig-wt/BEL/12R021/2012/G3P5 is marked with a circle.

### 3.2.4. Genes encoding non-structural proteins NSP1 to NSP5

The phylogenetic trees of genes encoding non-structural RVC proteins are shown in Fig. 5. The Tamura 3 model with gamma distribution was used for NSP1, NSP2 and NSP4, whereas the Tamura-Nei model with invariant sites and Hasegawa-Kishino-Yano model with gamma distribution were used for NSP3 and NSP5, respectively. Also for the genes encoding non-structural proteins, a limited number of sequences were available. Those encoding NSP1, NSP4 and NSP5 of the Belgian RVC strains clustered together with other known porcine RVC strains. However, within the NSP1 tree the genetic distance between the Belgian strain and Cowden was high (73.2% nt similarity). Nonetheless, the Belgian strain fell within typical porcine subclusters in the phylogenetic tree of NSP4 (81.7 to 88.1% nt similarity) and NSP5 (85.4 to 87.5% nt similarity), albeit at a significant genetic distances. In contrast, the NSP2 (80.8 to 81.6% nt similarity) and NSP3 (76.8% nt similarity) genes of the Belgian strain were highly divergent from all other known RVC strains, including that of the other characterized porcine RVC strain Cowden.

### 3.3. Astrovirus genome organization and phylogenetic analysis

A porcine astrovirus (PoAstV) with a genome length of 6350 nt (ssRNA) was recovered from the fecal virome as well. The genome was reconstructed from a total of 104,775 NGS reads. The genome organization of PoAstV-BEL-12R021 is presented in Fig. 6. Three major open reading frames (ORFs) were identified: ORF1a (2493 nt), ORF1b (855 nt) and ORF2 (2346 nt). A slippery sequence (5'-AAAAAC-3')

was present between nucleotides 2471 and 2477, which is responsible for a ribosomal frameshift. As such, the entire ORF1 had a total size of 3954 nucleotides. Furthermore, a poly(A) tail was present at the 3' end of the viral genome.

Phylogenetic analysis of the amino acid sequence coded by the ORF2 gene was performed using the LG amino acid model with frequencies and gamma distribution. The phylogenetic tree showed large genetic variation between strains from different host species (Fig. 6). The Belgian astrovirus 12R021 clustered together with astroviruses isolated from pigs, cows, wild boar and deer, in different countries around the world. Nevertheless, the distance between these strains and the Belgian strain was high, with amino acid sequence similarities between 56.0 and 67.5%. Within this cluster, the Belgian astrovirus was most closely related to porcine astrovirus PoAstV14-4 from Canada (67.5% aa similarity).

### 3.4. Enterovirus genome organization and phylogenetic analysis

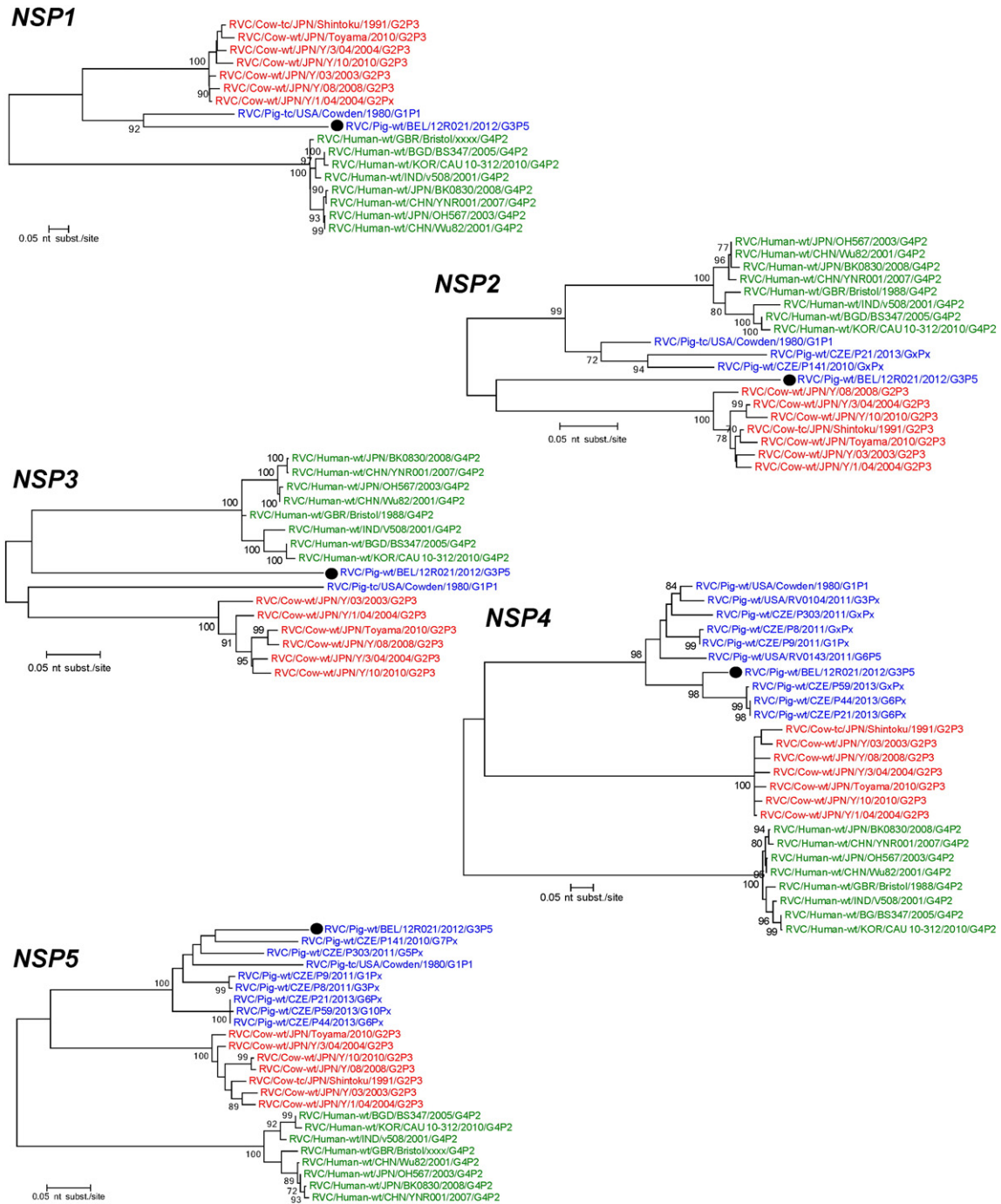
The fecal virome also contained an enterovirus, which had a ssRNA genome containing a single ORF of 6507 nt, preceded by a large untranslated 5' region of 798 nucleotides (Fig. 7). The genome was reconstructed from 11,833 NGS reads. The single ORF encodes a polyprotein that is split into 4 different structural (VP1 to VP4) and 7 non-structural proteins (2apro, 2b, 2c, 3a to 3d). The enterovirus strain belongs to species *Enterovirus G* within the *enterovirus* genus. Phylogenetic analysis of the amino acid sequence of the complete polyprotein was done using the LG amino acid model with gamma distribution and showed that the Belgian enterovirus clustered most closely to pig enterovirus K23 from Hungary, with an amino acid identity of 90.4%.

## 4. Discussion

The fecal virome of piglets can harbour a wide diversity of viral species, including bacteriophages, mammalian and plant viruses. In this study, the eukaryotic fecal virome content of a single Belgian non-diarrheic piglet was reported. This fecal sample was of interest because it contained a porcine group C rotavirus, a porcine group A rotavirus, an astrovirus and an enterovirus. The complete genome characterization of the RVC strain revealed some interesting features. Currently, our knowledge on the evolution of group C rotaviruses from different host species is relatively scarce. Only one archival pig RVC strain (RVC/Pig-tc/USA/Cowden/1980/G1P1) has been completely characterized (Bremont et al., 1992). Using NGS it was possible to obtain the complete genome sequence of a Belgian pig RVC, and this will help us to better understand their evolution. This strain was genetically distinct from pig strain Cowden and RVCs from humans and cows. Generally, most RVC gene segments clearly clustered according to the host species of origin. Unfortunately, only 2 pig RVC strains have been fully characterized to date, and both are genetically very divergent from each other, a characteristic which is less pronounced in human and bovine RVC strains (Bremont et al., 1992; Mawatari et al., 2014; Soma et al., 2013; Yamamoto et al., 2011). To date, it is not possible to conclude for any ancestral roles between pig, cow and human RVCs. Analysis of more complete genomes of RVC strains from different host species should give answers to these questions. However, at least for the RVC VP3 gene, a closer relationship between genes from pig and human RVCs can be suggested, as pig RVCs clustered in between two human VP3 subclusters (Yamamoto et al., 2011).

An accurate serotype classification system does not exist for RVCs, due to difficulty in growing the virus in cells. As alternative, genetic classification methods have been developed, but only for a limited number of RVC gene segments and being the most elaborate for those encoding outer capsid proteins VP7 and VP4, and inner capsid protein VP6 (Marthaler et al., 2013; Suzuki et al., 2014a, 2014b). The VP7 encoding gene of RVC strain 12R021 belonged to genotype G3, which was only discovered in a relatively small number of pig RVC strains from South





**Fig. 5.** Maximum-likelihood phylogenetic tree based on the coding sequences of available RVC NSP1–NSP5 genes. Bootstrap values ( $n = 500$  replicates) lower than 70% are not shown. Pig, cow and human RVC strains are colored blue, red and green, respectively. Belgian strain RVC/Pig-wt/BEL/12R021/2012/G3P5 is marked with a circle.

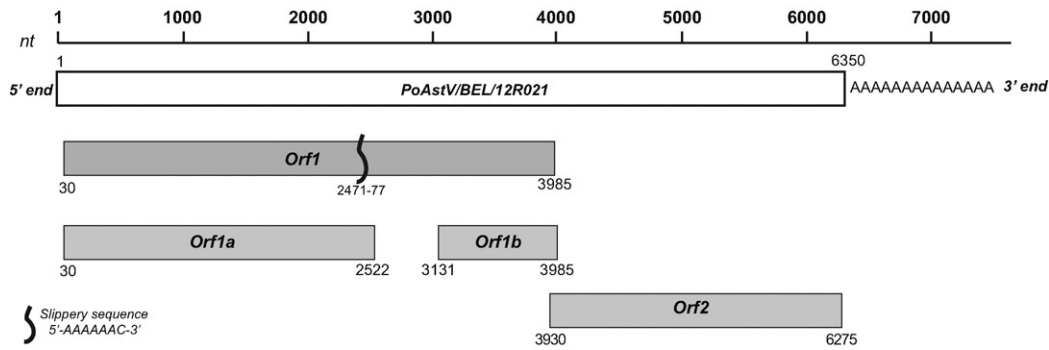
Korea and Ohio, United States (Amimo et al., 2013; Jeong et al., 2015). In a large study from the United States and Canada, the G3 genotype was not found in diarrheic pigs (Marthaler et al., 2013). Furthermore, in a recent study conducted in Belgian diarrheic suckling piglets, the G3 genotype was not found (Theuns et al., 2016). In general, genotypes G6 and G1 are most dominant in the pig population (Jeong et al., 2015; Marthaler et al., 2013; Theuns et al., 2016). Tsunemitsu et al. (1992) demonstrated that one of these G3 strains (HF), likely belonged to a distinct serotype than strain Cowden (genotype G1) and Shintoku (genotype G2). This may indicate that strain 12R021 is not only genetically, but also serologically distinct from most other pig, human and cow RVC strains isolated to date (Tsunemitsu et al., 1992). However, this

hypothesis needs further investigation using relevant enterocyte cell culture models.

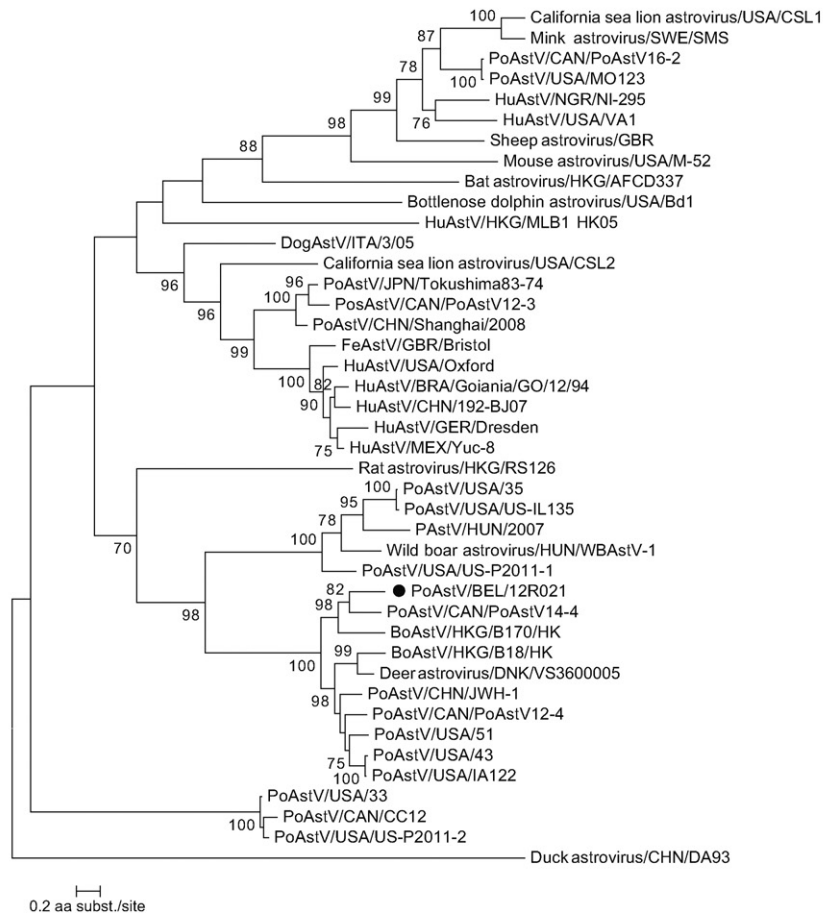
Here, a large genetic diversity was also observed among VP4 encoding genes of pig and cow RVC strains, which was in large contrast to the high relatedness among human RVC VP4 genes. Recently, two research groups proposed distinct nucleotide sequence identity cut-off values to divide VP4 genes into distinct genotypes. Jeong and colleagues used an 83% cut-off value to classify the genes into 7 genotypes (P1 to P7), whereas Suzuki and colleagues used a cut-off value of 80% to distinguish 6 genotypes (P1 to P6). Still, it is difficult to find a consensus for all genotypes between both classification systems, which hampers the correct classification of new RVC strains, especially those of pigs. The



## Astrovirus genome organization



## Phylogenetic analysis of astrovirus Orf2

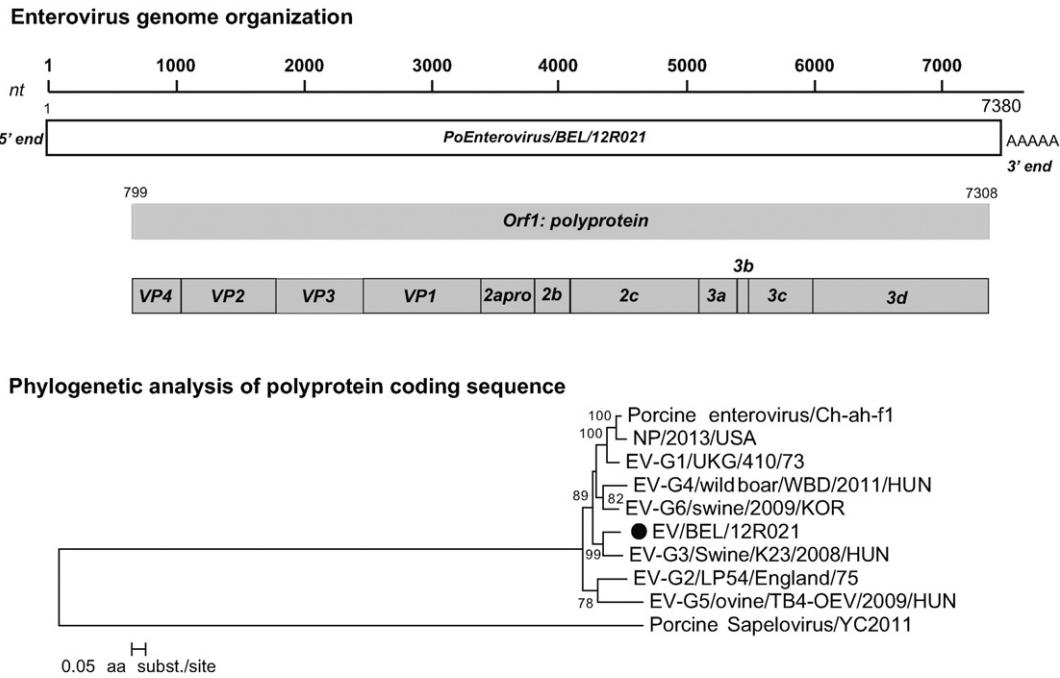


**Fig. 6.** Genome organization of pig astrovirus isolated from fecal sample 12R021 (upper panel). Maximum-likelihood phylogenetic tree based on the amino acid coding sequences of Orf2. Bootstrap values ( $n = 500$  replicates) lower than 70% are not shown (lower panel). Belgian pig astrovirus 12R021 is marked with a circle.

genotype P5 is shared between both classification systems, and contained the Belgian pig strain. It has been demonstrated for the RVA VP4 proteins that the highly variable VP8\* domain within VP4 possesses the capacity to recognize different carbohydrate moieties dependent on the genotype (Coulson, 2015). As an example, strains bearing the typical pig genotype P[7] recognize sialic acids, whereas differences in carbohydrate recognition have been observed between important human RVA genotypes P[6] and P[8] (Huang et al., 2012; Liu et al., 2012; Nordgren et al., 2014). RVCs also possess a highly variable VP8\* domain. The dependence on sialic acids for binding and infection of cells was shown earlier for pig strain Cowden. This strain was also capable of hemagglutinating human type O, guinea pig and mouse red blood

cells (Svensson, 1992). Still, it might be that the diversity observed among genes encoding VP4 proteins of contemporary pig RVC strains results in the capacity to recognize and bind to a wider range of carbohydrate moieties present on mucus and enterocytes. In contrast to the high diversity of VP4 genes of pig RVCs, it is more likely that human RVCs only use a single conserved type of carbohydrate for attachment, as they are genetically much more related to each other.

Interestingly, the VP6 inner capsid protein encoding gene of strain 12R021 could not be classified into one of the existing genotypes, using either the cut-off values of Jeong and colleagues or Suzuki and colleagues (Jeong et al., 2015; Suzuki et al., 2014b). Also here, no consensus has yet been reached. As such, 12R021 can be seen as a tentative new



**Fig. 7.** Genome organization of pig enterovirus isolated from fecal sample 12R021 (upper panel). Maximum-likelihood phylogenetic tree (lower panel) based on polyprotein coding amino acid sequences. Bootstrap values ( $n = 500$  replicates) lower than 70% are not shown. Belgian pig enterovirus 12R021 is marked with a circle.

genotype, together with strains from the Czech Republic. For pig RVAs, and for human RVAs from different genogroups, it was described that genotype-specific mutations were present at the VP7 interface of the VP6 proteins (Heiman et al., 2008; Theuns et al., 2015). The diversity observed among pig RVCs might have similar purposes. First, it may allow for a better recognition of different VP7/VP4 proteins encoded by different genotypes, resulting in a better structural fit in mature particles. Second, the high diversity observed in VP6 might be the cause of immunological selection pressure as well. Indeed, VP6 proteins have an important immunological function which has long been underestimated. For RVAs it was demonstrated that the majority of antibodies are elicited against VP6, and unexpectedly less against the outer capsid proteins VP7 and VP4 (Svensson et al., 1987). Anti-rotavirus immunoglobulin A (IgA) antibodies are internalized at the basal side of enterocytes, undergo transcytosis, followed by excretion in the lumen as secretory IgA (SIgA). During transcytosis, antibodies against the RVA VP6 proteins are able to intracellularly neutralize viral replication by sterical blocking of type I channels in viral double layered particles, thereby hampering RNA egress during transcription (Aiyegbo et al., 2013). A similar mechanism might be in place for RVC, whereas a wide diversity among VP6 proteins allow the viruses to evade immune pressure. The availability of RVC susceptible cells will allow us to investigate these hypotheses.

An RVA strain (RVA/Pig-wt/BEL/12R021/2012/G11P[27]) was also present in the feces of this non-diarrheic Belgian piglet. A rare pig G11P[27] genotype combination was found for VP7 and VP4, but this strain was only present at a low viral load ( $4.79 \log_{10}$  copies/g feces). Here it was attempted to reveal the entire genome of this strain, which was only partially successful. This was most likely due to the low viral load of the RVA strain in the fecal sample. Another hypothesis might be that most of the excreted RVA RNA was unprotected by virions, and thus digested by the RNase digestion step during the viral purification step for NGS preparation. For successful RT-PCR amplification of some gene segments, concentration of the RNA extracts using ethanol precipitation was necessary, and typical pig genotypes I5, A8 and T7 could be assigned to some of the genes, including those encoding the inner capsid protein (VP6), the interferon antagonist (NSP1) and the translation enhancer (NSP3), respectively. Similar pig genotypes have

been disclosed from other pig RVA strains around the world, and have recently also been detected in Belgian pig strains (Ghosh et al., 2010; Kim et al., 2012; Martel-Paradis et al., 2013; Matthijnsens et al., 2008a; Matthijnsens et al., 2010; Monini et al., 2014; Okitsu et al., 2013; Shi et al., 2012; Theuns et al., 2015). It was suggested that proteins encoded by these pig genotypes may allow for a better match with the cellular machinery of the pig intestinal epithelial cells during viral replication (Theuns et al., 2015). Furthermore, our RVA strain also possessed an N1 NSP2 genotype, further confirming the evolutionary relationship between pig RVAs and human Wa-like RVAs (Matthijnsens et al., 2008a; Theuns et al., 2015).

Finally, results from this study also highlight that many viral species may reside in the intestine of piglets without even being noticed using traditional diagnostic approaches (Sachsenroder et al., 2012; Sachsenroder et al., 2014; Shan et al., 2011; Zhang et al., 2014). Indeed, most viral species are not routinely investigated in cases of diarrhea on pig farms. Here, a porcine astrovirus was detected in the fecal virome, which showed most similarity to a porcine astrovirus strain from Hungary. The clinical relevance of these astroviruses remains enigmatic and should be further investigated. A porcine enterovirus was also present in the fecal virome, but also for enteroviruses in piglets their role in the development of enteric diseases is being questioned in piglets, and have been more frequently associated with neurological problems and reproductive problems in pigs (Knowles, 2006). Indeed, the roles of PoAstV and the porcine enterovirus in the pathogenesis of piglet diarrhea can also be questioned here, as this piglet did not demonstrate any symptoms of diarrhea at the moment of sampling. Still, these subclinical infections may have had an impact on pig growth, which has been ascribed for instance to porcine circovirus infections (Maes, 2012). Nonetheless, the detection of all these viruses provided only a picture at one time-point, and the history and outcome of these infections is unknown. In view of future studies in the field, analyses of the fecal viromes of diarrheic and non-diarrheic piglets will likely enable the discovery of new viruses that are part of yet unspecified intestinal disease complexes. These might contribute to clinical enteric diseases or result in growth retardation in subclinically infected young piglets. Standardized protocols, which allow studying viral metagenomics in a high throughput manner without introducing major bias (such as the NetoVIR protocol), are definitely needed to execute such studies (Conceicao-Neto

et al., 2015). This will eventually lead to innovative diagnostic protocols and control measures of (sub)clinical enteric infections in young pigs.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.meegid.2016.05.018>.

### Author contributions

ST and NCN carried out the wet-lab experiments. ST, NCN, MZ and EH analyzed the nucleotide sequence data. ST, LMB, NCN, MZ, EH, IDR, HJN and JM assisted in data-analysis and writing of the manuscript. JM, HJN and MVR coordinated the study. All authors read and approved the final manuscript.

### Conflicts of interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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