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# Complete genome analyses of the first porcine rotavirus group H identified from a South African pig does not provide evidence for recent interspecies transmission events

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**Abstract**

Rotaviruses (RVs) are classified into eight species/groups (RVA-RVH) according to the migration patterns of their 11 genome segments, as well as by serological and molecular properties of Viral Protein 6 (VP6). In 1997 a new unclassified RV was reported infecting adults in Bangladesh and China. This virus was initially named novel adult diarrhoea rotavirus (ADRV-N), but later renamed as RVH. Since then, RVH has been detected in humans only very sporadically. However, RVH is increasingly being detected in pig populations in the USA, Brazil and Japan, but not yet in Africa. Unfortunately, whole genome sequence data of porcine RVH strains in GenBank is currently restricted to a single strain (SKA-1) from Japan.

Porcine diarrhoeic samples were collected in South Africa and analysed for rotavirus using an RVA ELISA and electropherotyping by PAGE. One sample displayed a 4:2:1:1:1:1:1 migration pattern, typical for RVH. In order to further investigate this strain, sequence-independent amplification followed by random sequencing using the 454/Roche GS FLX Sequencer was performed, resulting in the second complete porcine RVH strain (MRC-DPRU1575) available in databases.

Phylogenetically, all segments of MRC-DPRU1575 clustered closely with the SKA-1 strain and in some segments with known porcine RVH strains from Brazil and the USA. In contrast, the porcine RVH strains were only distantly related to human RVH strains from Asia and a partial RVH-like strain recently detected in bats from Cameroon.

Overall, strain MRC-DPRU1575 is the first complete genome of a porcine RVH from Africa and allows for the development of improved RVH screening methods. Our analyses indicate that RVH strains cluster according to their host species, not suggesting any evidence of recent interspecies transmission events. However, more RVH genomes from a wider host range are needed to better understand their evolutionary pathways and zoonotic potential.

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## 1. Introduction

Rotaviruses (RVs) are the major cause of severe dehydrating diarrhoea in both the young ones of humans and animals worldwide. RVs belong to the family *Reoviridae*, genus *Rotavirus* (Estes and Greenberg, 2013). RVs are further divided into eight distinct species or groups (RVA to RVH), based on their electropherotypes and VP6 based serology and sequence similarities (Estes and Kapikian, 2007; Matthijnssens et al., 2012). A recently identified canine RV species has been tentatively named *Rotavirus I (RVI)* and may represent the ninth RV group, although further ratification by the International Committee on Taxonomy of Viruses (ICTV) is warranted (Mihalov-Kovács et al., 2015). So far, RVH strains, which were initially referred to as adult diarrhoea rotavirus (ADRV-N) have been identified in human and pigs from Bangladesh, Brazil, Cameroon, China, Japan and United States (Yang et al., 2004; Alam et al., 2007; Jiang et al., 2008; Nagashima et al., 2008; Wakuda et al., 2011; Marthaler et al., 2014; Molinari et al., 2015). Recently, RVH-like rotaviruses have also been detected in bats from Cameroon (Yinda-CK, unpublished data).

Genomes of RV groups A-H can be easily distinguished from each other as their eleven dsRNA segments exhibit distinct electrophoresis migration patterns after polyacrylamide gel electrophoresis (PAGE) and silver nitrate staining, separating each gene segment according to its molecular weight (Herring et al., 1982). The most important PAGE patterns used to differentiate between RVA, RVB, RVC and RVH are 4:2:3:2; 4:2:2:3; 4:3:2:2 and 4:2:1:1:1:1:1, respectively. The use of PAGE as one of the molecular techniques to detect or screen for non-group A RVs is still applied at the South African Medical Research Council Diarrhoeal Pathogens Research Unit (MRC-DPRU) for RV research, during initial screening of samples because of the limited or inaccessibility of serological testing reagents against these non-classical RV groups, and its ability to identify mixed infections (Nyaga et al., 2015). This methodological approach resulted in the identification of the current strain which was further characterized.

This strain was identified in a sample collected from a farm in Kroonstad, Free State Province, South Africa, from a piglet during a study of domesticated animal rotaviruses at Sefako Makgatho Health Sciences University (previously MEDUNSA) and which exhibited a PAGE pattern very similar to that of human RVH strains J19 found in China and B219 from Bangladesh (Yang et al., 2004; Alam et al., 2007). The strain was characterized further by total genome sequencing and this manuscript is (to the best of our knowledge) the first to report the whole genome sequences of a RVH strain in Africa. The full genome sequences of strain RVH/Porcine-wt/ZAF/MRC-DPRU1575/2007/GXP[X] (MRC-DPRU1575) were compared with all the other available RVH sequences in GenBank.

## 2. Materials and Methods

### 2.1 Sample collection and Enzyme Immunoassay

This project was approved by the Medunsa Research Ethics Committee (MREC). Three samples were collected from 10-week old piglets with diarrhoea and diluted to a 10% suspension (w/v) using the kits sample diluent, prior to RV enzyme-immunoassay (EIA) testing for group A antigens (DAKO, Oxoid, Cambridge, UK) according to manufacturer's instructions. Two of the three samples were positive by EIA.

### 2.2 Extraction of viral dsRNA for Electropherotyping (PAGE)

Electropherotyping by PAGE was done on the EIA negative sample to identify non-RVA species (RVB-RVH). Briefly, 500 µl of the 10% stool suspension was treated with sodium acetate containing 1% sodium dodecyl sulphate. Total viral RNA was extracted using an equal volume phenol-chloroform treatment, followed by total RNA precipitation in absolute

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ethanol. For separation of the dsRNA segments, a 10% polyacrylamide gel was poured and run with a discontinuous glycine buffer system at room temperature for 18 h at 100 V. Analysis of the PAGE pattern was done after silver staining (Herring et al., 1982).

**2.3 Sequence-independent amplification method for dsRNA**

The sample was subjected to a one-tube whole genome dsRNA amplification method by sequence-independent amplification method developed by Potgieter and colleagues (Potgieter et al., 2009). Briefly, total RNA was extracted from a 10% stool suspension using TRI-Reagent-LS (Molecular Research Centre, USA) and the dsRNA was further cleaned-up using the QIAgen MinElute gel extraction kit (QIAGEN, Hilden, Germany). The eluate containing the dsRNA was treated with 2 M lithium chloride and the concentration and quality of the enriched extracted viral dsRNA was determined through electrophoresis in a 1% ethidium bromide stained agarose gel.

For the complete cDNA synthesis of all eleven dsRNA gene segments, a designed self-priming anchor-primer PC7-T7 loop was ligated in the presence of T4 RNA ligase (TaKaRa) to the dsRNA in a 37˚C incubator for 16 h. The ligated RNA was purified through a column from the NucleoSpin Extract II Column Kit (MACHEREY-NAGEL, Duren, Germany). The dsRNA was denatured by treating with 30 mM final concentration of methyl-mercury hydroxide (MMOH, Alfar Aesar) and reverse–transcription (RT) was carried out at 42˚C for 45 min and a further 15 min at 50˚C in the presence of 1 ul Transciptor Enzyme (Roche), 1 ul β-Mercaptoethanol (Sigma Aldrich), 0.5 ul RNAse inhibitor (Roche), 3 ul dNTPs (Roche), 6 ul Transcriptor Buffer (Roche), 8 ul dsRNA and sterile nuclease free water to a final volume of 30 ul per reaction. The following reagents 1 M NaOH (Sigma), 1 M Tris/HCl pH 7.5 (Sigma) and 1 M HCl (Sigma) at a final concentration of 0.1 M were added as a post RT clean-up step to remove all un-transcribed RNA.

After removal of excess RNA, the cDNA was hybridized for 60 min at 65˚C and 5ul of cDNA was amplified with a master mix consisting of 1 ul 25 pmol/ml primer PC1, which is complementary to primer PC7, 2.5 mM dNTPs (Roche), 0.5 ul ExTaq polymerase enzyme (TaKaRa), 5 ul 10 x ExTaq Buffer (TaKaRa) and water to a final volume of 50 ul. The cDNA was amplified as single cycle on 72˚C x 2 min and 94˚C x 2 min, then 19 cycles of 95˚C x 30 sec 65˚C x 30 sec, 72˚C x 3 min, with an extension of 72˚C x 5 min. The amplified products (cDNA) were run on a 1% TBE agarose gel together with initial extracted dsRNA in the presence of ethidium bromide (Potgieter et al., 2009).

**2.4 Sequencing**

The cDNA containing the complete genome (eleven segments) was sequenced using the method of ultra-deep DNA sequencing on the 454/Roche GS FLX Sequencer at Inqaba Biotechnical Industries (Pty) Ltd as described previously (Margulies et al., 2005). The assembly of the contigs for each segment was done by Lasergene 7 software (DNASTAR). To confirm and identify each segment, a BLAST analyses was performed on the assembled sequences using the NCBI GenBank online tool. The sequences were deposited in the NCBI GenBank under accession numbers KT962027-KT962037. The estimates of evolutionary divergence between all eleven segments of MRC-DPRU1575 and sequences available in GenBank were also determined (Suppl. Data 1). The analyses were conducted using the p-distance parameter model. Maximum likelihood phylogenetic trees and evolutionary analyses were conducted using the nucleotide substitution model inMEGA6 (Tamura et al., 2013).

**3. Results**

A single RVH strain was recovered from a 10-week old piglet with diarrhoea, during a study of rotavirus disease in domesticated animals in South Africa.

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### 3.1 EIA and PAGE

The sample containing RVH strain MRC-DPRU1575 was negative by the RVA specific EIA and thus was subjected to PAGE. The PAGE pattern observed from MRC-DPRU1575 was different from the ordinary known migration pattern of RVA (4:2:3:2) and RVB (4:2:2:3). The pattern was also compared in an agarose gel electrophoresis with known RVA, RVB and RVC controls available in the MRC-DPRU laboratory (Fig. 1). MRC-DPRU1575 was considered unique because it represented a profile distinct from that of RVA, RVB and RVC, but almost identical to that of RVH strains J19 and B219 (Yang et al., 2004; Alam et al., 2007). This pattern possessed a more equal segment separation between the smaller five gene segments and grouped as 4:2:1:1:1:1:1 (Fig. 1).

### 3.2 Phylogenetic analyses

High pairwise identities on the nucleotide (81-94%) and amino acid (81-100%) level were observed for all segments of strain MRC-DPRU1575 when compared with strain SKA-1 and other porcine RVH strains across the genome (Table 1). On the other hand, strain MRC-DPRU1575 was distantly related to human RVH strains at the nucleotide (58-73%) and amino acid (38-80%) level, as well as to the partial nucleotide (48-74%) and amino acid (29-80%) sequences available for a bat RVH-like strain tentatively named RVH/Bat-wt/CMR/CMBAT/2014/GXP[X] (Table 1). The gene segment encoding the bat NSP5 gene segment was not available for this comparison and none of the open reading frames (ORFs) for the 10 available segments were complete.

The genome of strain MRC-DPRU1575 was also phylogenetically analysed. The comparison was based on reference sequences obtained from GenBank for human RVH strains ADRV-N, J19 and B219 from Asia (Yang et al., 2004; Alam et al., 2007; Jiang et al., 2008; Nagashima et al., 2008) and porcine RVH strain SKA-1 from Japan (Wakuda et al., 2011), 29 porcine RVH strains from the USA (Marthaler et al., 2014) and also BR59, BR60, BR61, BR62 and BR64 from Brazil (Molinari et al., 2015).

Phylogenetically, all gene segments of strain MRC-DPRU1575 were closely related to strain SKA-1 and distantly related to human strains J19 and B219 from Asia and the Cameroonian bat strain. The characterized genes encoding VP4, VP6, VP7 and/or NSP4 of porcine strains from the USA and Brazil also clustered closely according to their host species with MRC-DPRU1575 (Fig. 2).

## 4. Discussion

Since the first report of RVH (named novel-adult diarrhoea rotavirus (ADRV-N) at that time), as a cause of outbreaks of gastroenteritis in humans in China in 1997 (Yang et al., 2004), a limited number of studies have shown that RVH in pigs are increasingly being detected across the globe, probably due to improved molecular diagnostic tools that are being employed, rather than a global expansion of the RVH group.

The role of RVH in pathogenesis of diarrhoea in pigs remains unstudied raising the need for more *in vivo* and extensive molecular epidemiologic studies (Marthaler et al., 2014). Furthermore a recent study of pigs samples in the USA identified that RVH frequently occurred in co-infections with other RV groups mostly RVA, RVB and RVC (Marthaler et al., 2014). It is interesting that in other South African porcine rotavirus studies, RVB and RVC strains were also recovered (Geyer et al., 1996), but their prevalence and co-infection rate remains to be determined. RVH were also reported from a Brazilian pig farm (Molinari et al., 2015), where PAGE patterns of the genome segments of the Brazilian RVH samples were similar to those of RVB, but VP6 analyses showed that they were indeed RVH. However, since the new VP6 based RV classification approach identified RVH as an

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independent RV group and by design of RVH specific primers, identification of more RVH porcine strains has been made possible (Matthijnssens et al., 2012).

In this study, PAGE and sequence independent amplification assays were used to obtain the RNA migration patterns and cDNA for sequencing the whole genome of the porcine RVH strain MRC-DPRU1575, respectively. The migration pattern of MRC-DPRU1575 was distinctive from the pattern of other RV groups but was similar to the RVH (ADRV-like) PAGE pattern. Phylogenetic analysis of the complete ORFs obtained for all gene segments of strain MRC-DPRU1575 showed that it most closely related to the pig RVH strains from Japan, Brazil and USA and evolutionarily distinct from human RVH strains from Bangladesh and China (Yang et al., 2004; Alam et al., 2007; Jiang et al., 2008; Nagashima et al., 2008; Wakuda et al., 2011; Marthaler et al., 2014; Molinari et al., 2015) and also distinct from a bat RVH-like strain from Cameroon (Yinda et al., unpublished data). In addition to the close identities across the genome between our study strain and Japanese RVH strain SKA-1, gene segments coding for VP4, VP6, VP7 and NSP4 were also closely related to porcine RVH strains from Brazil, and for VP6 also with porcine RVH strains from the USA (Fig. 2). MRC-DPRU1575 was only distantly related to human RVH strains (J19 and B219), and the bat RVH-like strain included in this analysis. Since the bat RVH-like strain was rather distantly related to established RVH strains (example: 62% identity on the amino acid level of the partial VP6 sequence, (table 1), further complete genome analyses of this strain needs to be performed before it can be definitely classified as RVH.

The RVH sequences from the different host species (porcine, human and bat) available in this study cluster in clearly distinct sub-clusters, not suggesting any evidence of recent interspecies transmission events. Overall our analyses suggest that porcine and human RVH strains seem to be slightly more closely related to each other suggesting a more recent common ancestor in comparison to the partially sequenced RVH bat strain. However, these observations are based on only a very small number of RVH strains found in three different host species, and many more global whole genome RVH sequences are needed to conclusively determine the RVH evolutionary pathways and zoonotic potential.

To our knowledge, this manuscript is the first to perform a complete genome analyses of porcine RVH strains and MRC-DPRU1575 is the first RVH strain reported from Africa and the second porcine RVH strain with all 11 gene segments (at least all 11 ORFs) being sequenced.

## 5. Acknowledgments

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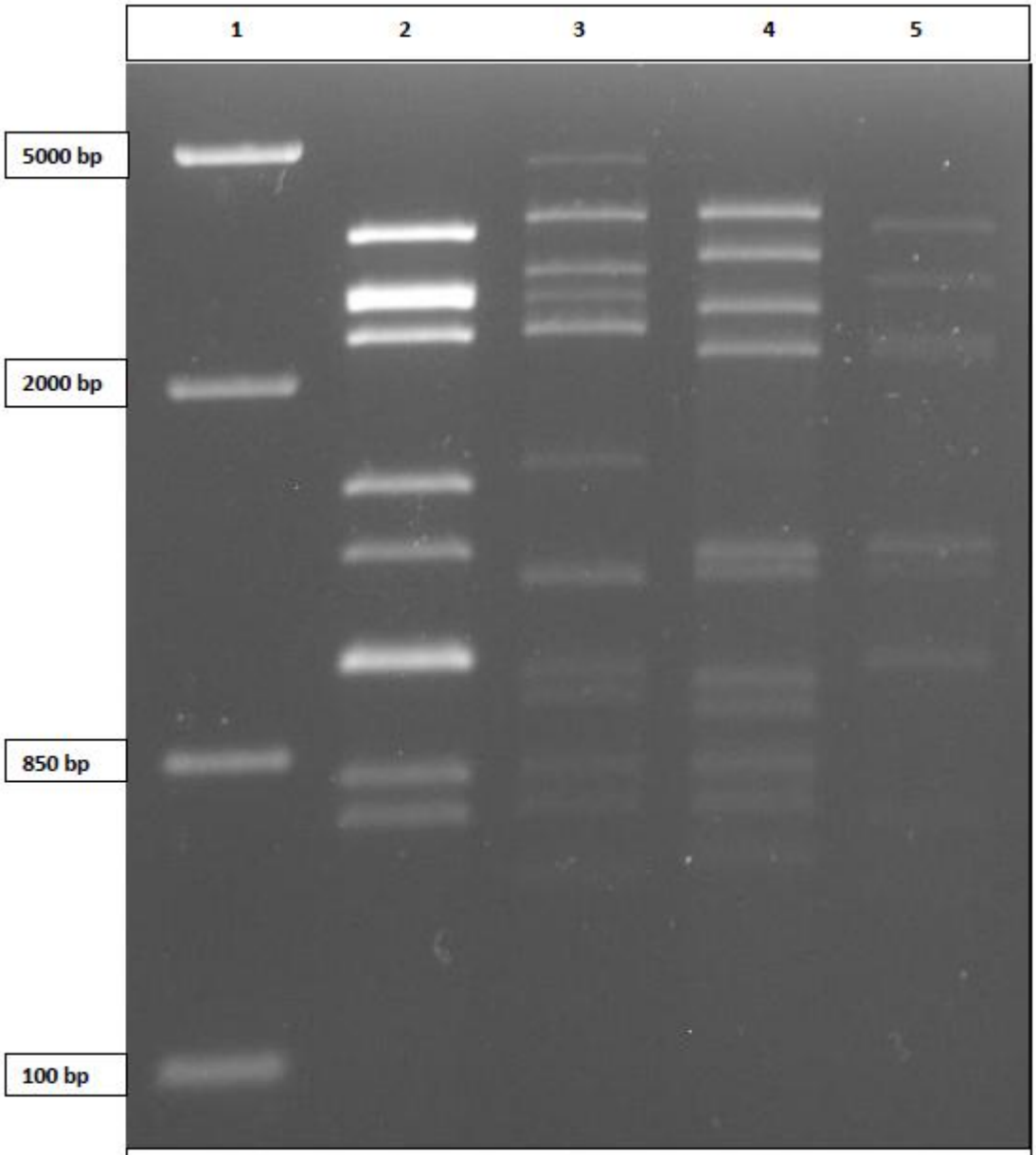


**Fig. 1:** A 1% ethidium bromide agarose gel showing the quality and migration patterns of rotavirus dsRNA from VP1 (3302 bp) to NSP5 (667 bp) after column purification step for dsRNA used for Sequence Independent Amplification. Lane 1; Molecular Weight marker (Fermentas SM1113MM, ThermoFisher Scientific, Inc), lane 2; RVA pattern, lane 3 RVB (with a non-RV unidentified band at 4000 bp), lane 4; MRC-DPRU1575/2007 showing (4:2:1:1:1:1:1), and lane 5; RVC profile.

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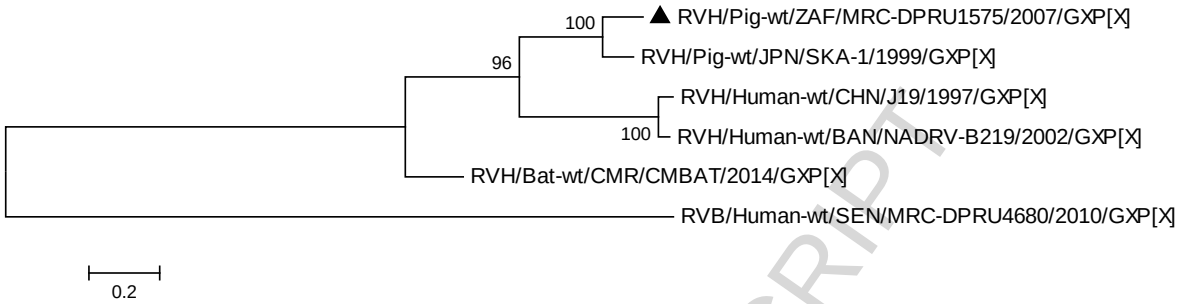
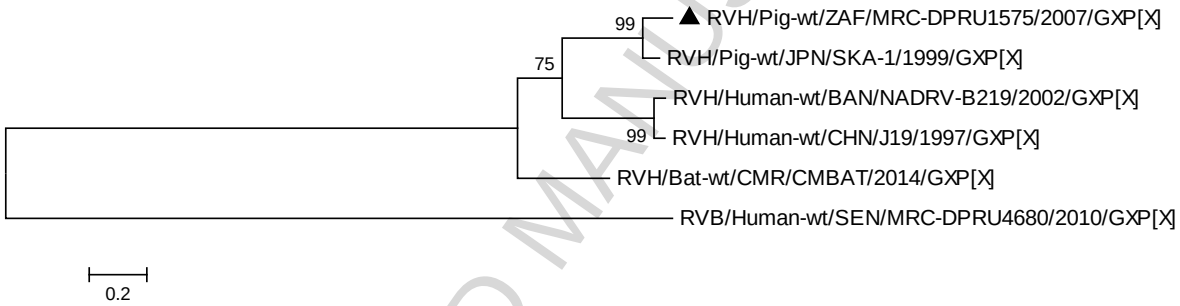
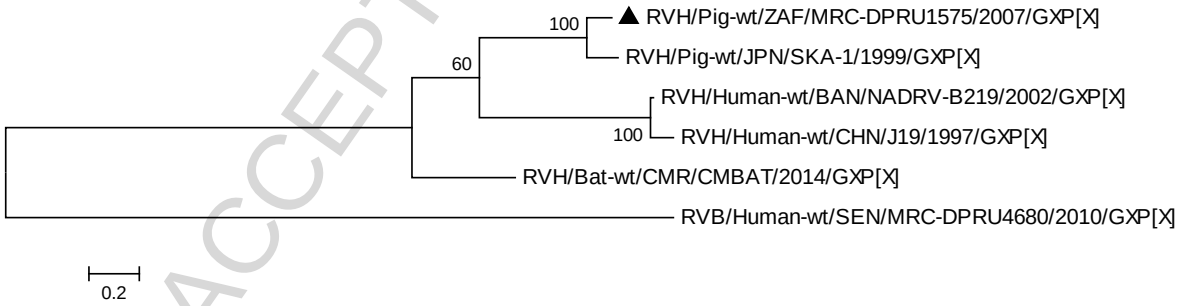
**a. VP1**



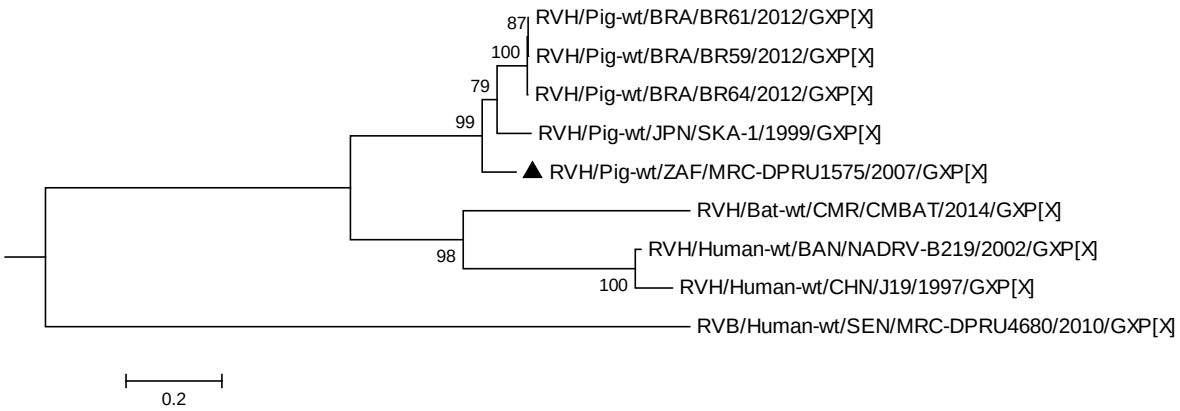

**b. VP2**




**c. VP3**




**d. VP4**



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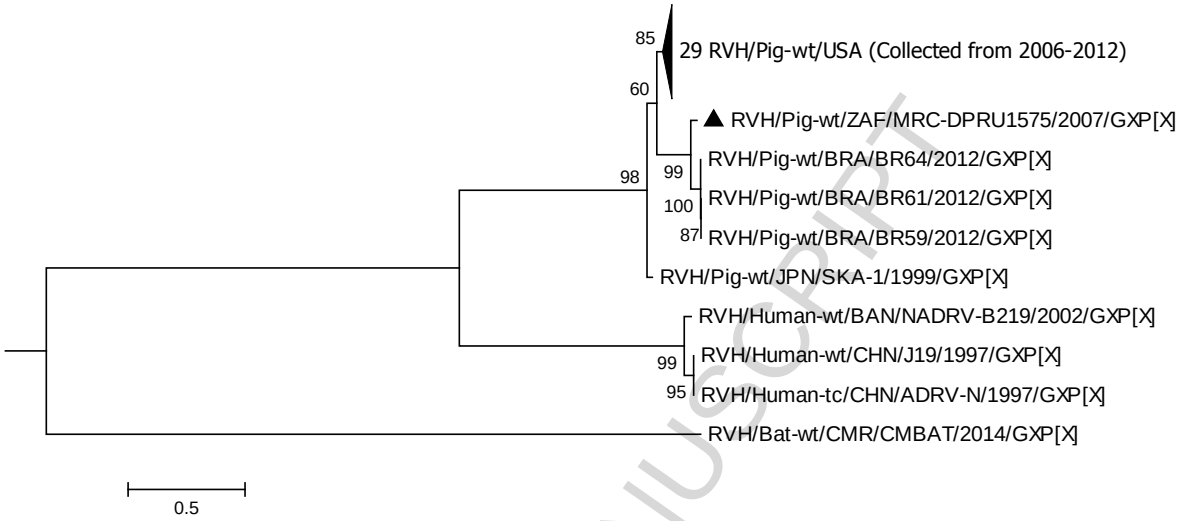
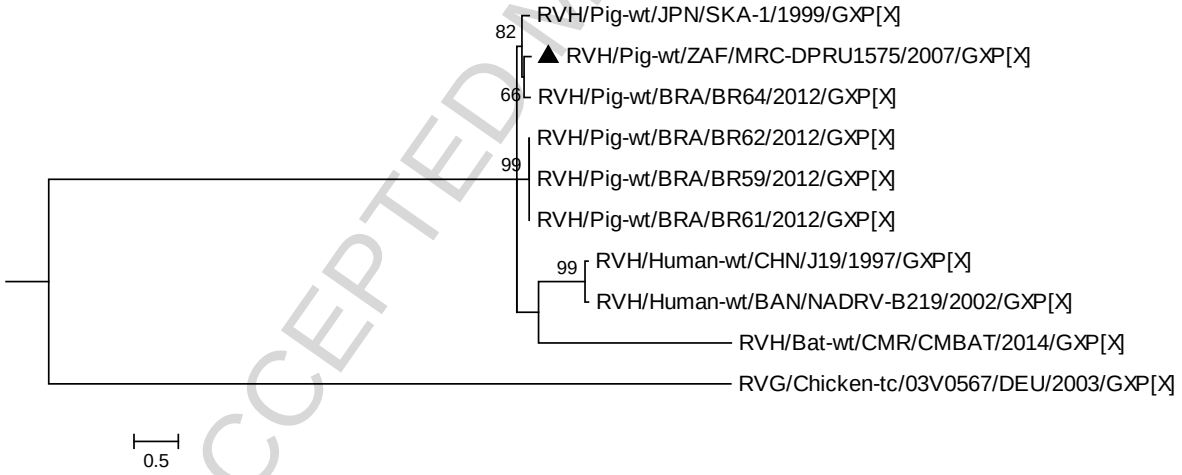
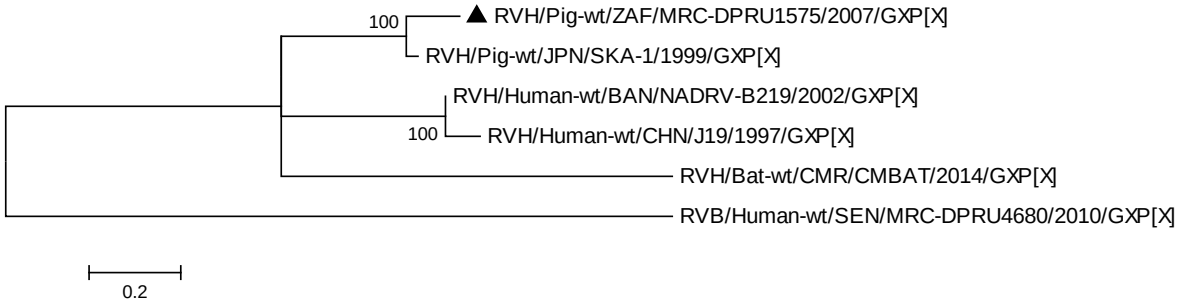
**e. VP6**




**f. VP7**




**g. NSP1**



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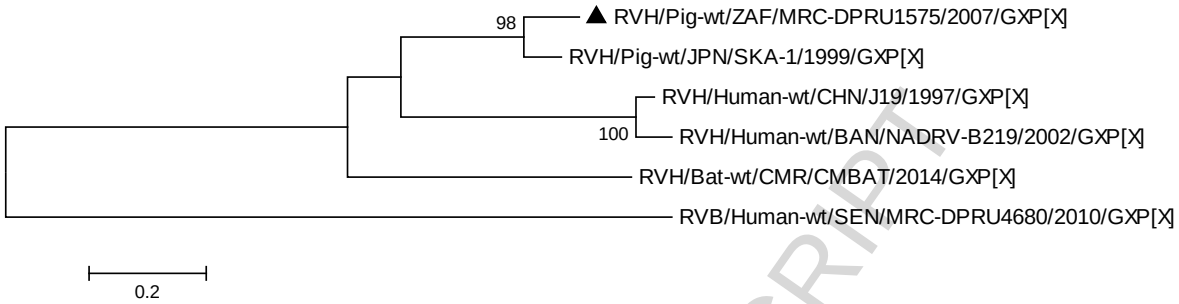
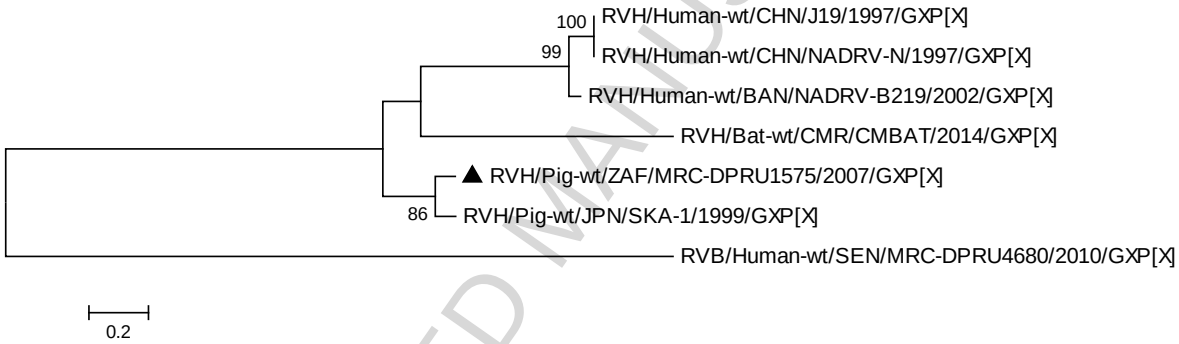
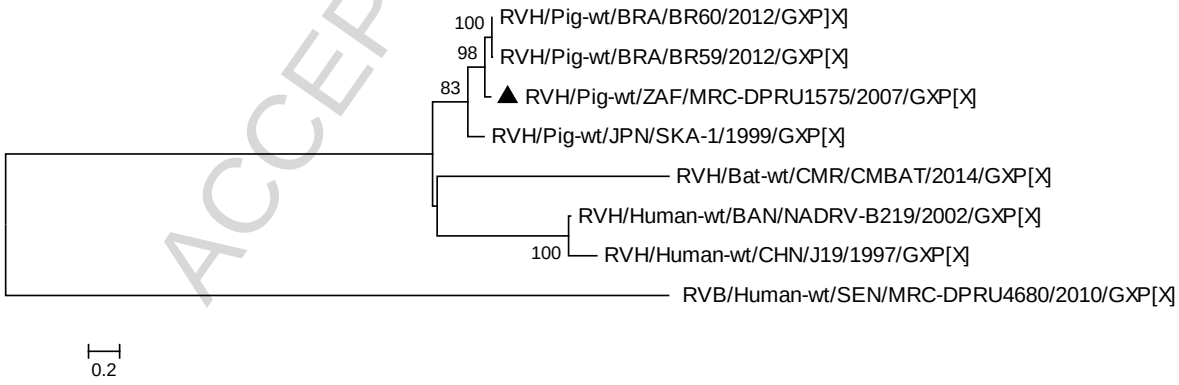
**h. NSP2**




**i. NSP3**




**j. NSP4**




**k. NSP5**

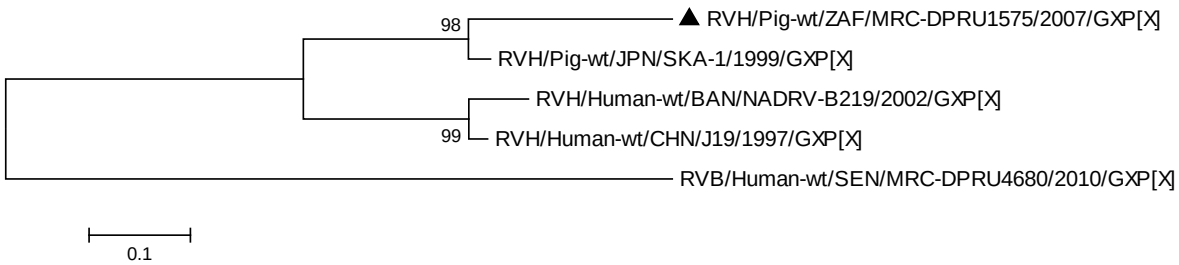


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**Fig. 2 a-k:** Phylogenetic trees constructed from nt sequences of strain MRC-DPRU1575 aligned against all other RVH genome segments in GenBank. Bootstrap values (500 replicates) above 60 are shown. The black triangle represents the RVH study strain in all trees. A RVB or RVG strain was used as an out-group for each of the 11 gene segments, except for VP6, as the RVB and RVG sequences were highly divergent. Scale bars of 0.1, 0.2 and 0.5 substitution per nucleotide were used depending on the divergence for each tree.

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**Table 1:** Highest nucleotide (amino acid) identity for MRC-DPRU1575 when compared to RVH pig, human and bat species.

| | Gene segments | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | VP1 | VP2 | VP3 | VP4 | VP6 | VP7 | NSP1 | NSP2 | NSP3 | NSP4 | NSP5 |
| Pig | 87 (96%) | 87 (97%) | 85 (91%) | 86 (91%) | 94 (100%) | 88 (92%) | 85 (89%) | 89 (97%) | 89 (94%) | 92 (92%) | 81 (81%) |
| Human | 72 (78%) | 73 (80%) | 65 (60%) | 59 (53%) | 72 (76%) | 58 (58%) | 58 (48%) | 72 (73%) | 65 (63%) | 59 (38%) | 62 (53%) |
| Bat * | 74 (80%) | 70 (74%) | 64 (58%) | 61 (58%) | 62 (62%) | 53 (39%) | 48 (33%) | 72 (74%) | 57 (51%) | 51 (29%) | |

* Pairwise identities were calculated based on partial VP1 (nt 1893 -3510), VP2 (nt 2525-2949), VP3 (nt 1680-2168), VP4 (nt 1899-2463), VP6 (nt 752-1191), VP7 (nt 618-728), NSP1 (nt327-1205), NSP2 (nt 830-946), and NSP4 (nt 418-674) except for NSP3 which was complete and NSP5 which was not included in this analyses

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Highlights

- Study reports the first rotavirus group H (RVH) from Africa
- The strain is the first compete porcine RVH genome globally
- Data will allow for improved RVH screening possibilities

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