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# Genetic heterogeneity in the VP7 of group C rotaviruses

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**Abstract**

Evidence for a possible zoonotic role of group C rotaviruses (GCRVs) has been recently provided. To gain information on the genetic relationships between human and animal GCRVs, we sequenced the VP7 gene of 10 porcine strains detected during a large surveillance study from different outbreaks of gastroenteritis in piglets. Four GCRV strains were genetically related to the prototype GCRV porcine Cowden strain. A completely new VP7 genotype included 4 strains (344/04-7-like) that shared 92.5% to 97.0% aa identity to each other, but <83% to human GCRVs and <79% to other porcine and bovine GCRVs. A unique 4-aa insertion (SSSV or SSTI), within a variable region at the carboxy-terminus of VP7, represented a distinctive feature for these 4 unique strains. An additional strain, 134/04-18, was clearly different from all human and animal GCRVs (<85% aa identity) and likely accounts for a distinct VP7 genotype. The VP7 of a unique strain, 42/05-21, shared similar ranges of aa sequence identities with porcine and human strains (88.0–90.7% to porcine GCRVs and 85.2–88.2% to human GCRVs). Plotting the VP7 gene of strain 42/05-21 against the VP7 of human and porcine strains revealed discontinuous evolution rates throughout the VP7 molecule, suggesting different mutational pressure or a remote intragenic recombination event. These findings provide the need for future epidemiological surveys and warrant studies to investigate the pathogenic potential of these novel GCRVs in pigs.
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*Keywords:* Group C rotavirus; Enteritis; Pigs; Zoonosis

## Introduction

Rotaviruses (genus *Rotavirus*, family *Reoviridae*) are important enteric pathogens in humans and animals. Rotaviruses are classified into seven antigenically distinct groups (A to G) on the basis of a common group antigen, the inner capsid protein (VP6). Groups A, B, and C are associated with acute gastroenteritis in humans and animals while groups D, E, F, and G have been detected only in animals (Kapikian et al., 2001; Ciarlet and Estes, 2002). The recently described human rotavirus strains ADRV-N and B219 have not yet been taxonomically specified (Yang et al., 2004; Alam et al., 2007).

Group C rotaviruses (GCRVs) were first identified in swine in 1980s (Saif et al., 1980; Bohl et al., 1982). Subsequently, GCRVs have been detected in humans, cows, ferrets and dogs (Rodger et al., 1982; Torres-Median, 1987; Tsunemitsu et al., 1991; Chang et al., 1999; Otto et al., 1999; Mawatari et al., 2004). In humans, infection by GCRVs has been associated with sporadic episodes or large outbreaks of gastroenteritis in all age groups. This, along with their association with large outbreaks and deaths, raises public concerns of these viruses as important enteric pathogens (Bridger et al., 1986; Szucs et al., 1987; Arista et al., 1990; Caul et al., 1990; Saif and Jiang, 1994; Jiang et al., 1995; Kuzuya et al., 1998; Sánchez-Fauquier et al., 2003; Schnagl et al., 2004; Kuzuya et al., 2005; Rahman et al., 2005; Bányai et al., 2006).

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GCRVs resemble group A rotaviruses (GARVs) in their morphology and genomic organization. Like GARVs, GCRVs contain 11 segments of double-stranded RNA, but their RNA migration pattern (4–3–2–2) in polyacrylamide gel electrophoresis (PAGE) is different from that of GARVs (4–2–3–2) (Ciarlet and Estes, 2002). GARVs are classified on the basis of the two outer capsid proteins, VP7 and VP4, which are relevant for immune protection and for vaccine development, as they both elicit serotype-specific neutralizing antibodies (Ciarlet and Estes, 2002). Based on the two outer capsid proteins, a dual classification system by G serotypes (VP7) and P serotypes (VP4) has been adopted (Ciarlet and Estes, 2002). So far, based on genetic characterization, 15 VP7, and at least 27 VP4, gene alleles (genotypes) have been identified (Ciarlet and Estes, 2002; Martella et al., 2007; Steyer et al., 2007). GARVs belonging to the same G serotype usually share at least 90% amino acid (aa) sequence conservation, while rotavirus of different G serotypes usually share <85% aa identity (Green et al., 1988, 1989; Kapikian and Chanock, 1996). With a few exception (Ciarlet et al., 1997), strains sharing more than 89% of aa conservation are considered to belong to the same P genotype. Serotyping of GCRVs is hampered due to the difficulties in adapting GCRVs to cell culture (Fujii et al., 2000; Oseto et al., 1994). Sequence comparison suggests that genetic diversity exists among GCRVs and, like GARVs, a genotyping classification scheme based on VP4 (P type) and VP7 (G type) has been proposed (Tsunemitsu et al., 1992b, 1996; Fielding et al., 1994; Grice et al., 1994; Jiang et al., 1999a,b; Rahman et al., 2005). Porcine Cowden and bovine Shintoku GCRV strains were proposed as prototypes of different G types (G1 and G2, respectively) and the existence of a third G type, represented by the porcine HF strain has also been recognized by sequencing data (Tsunemitsu et al., 1992b, 1996). All human GCRVs share a high degree of conservation within the VP7 gene and belong to a distinct G genotype from the animal genotypes (Jiang et al., 1996; Adah et al., 2002; Castello et al., 2002; Rahman et al., 2005; Bányai et al., 2006), but one-way neutralization studies demonstrated antigenic relatedness of human GCRVs with Cowden-like porcine strains (Tsunemitsu et al., 1992b). At least 8 variable regions (VR-1 to VR-8) were recognized in the VP7 protein among antigenically distinct strains, in locations similar to those of the variable regions of GARVs (Tsunemitsu et al., 1996). Genetic analyses of the VP4 has revealed three distinct P genotypes, composed of human, porcine, and bovine GCRVs (Jiang et al., 1999a,b).

A possible zoonotic role of animal GCRVs has been postulated based on increase sero-prevalence rates to GCRVs in human populations living in rural settings (Iturriza-Gómara et al., 2004). However, unlike GARVs, molecular analyses of human and animal strains does not seem to support this hypothesis, as all the human GCRVs sequenced so far appear to have a monophyletic origin (Rahman et al., 2005; Bányai et al., 2006). A possible explanation for these inconsistencies may be a result of the limited amount of sequence data currently available from strains of animal origin. At present, VP7 sequences are available for only 5 porcine strains, Cowden, WH, HF, 266, and 97D, and for 2 bovine strains, Yamagata and Shintoku (Qian et al., 1991; Kim et al., 1999; Mawatari et al., 2004; Tsunemitsu et al., 1996). Additional evidence for the zoonotic potential of porcine GCRVs have been revealed by analyses of archival faecal samples of Brazilian children, who were infected with porcine-like GCRVs (Gabbay et al., 2006). In addition, there is direct evidence that porcine GCRVs may infect cows, as the bovine strain WD534tc is actually a porcine strain (Chang et al., 1999). Accordingly, like GARVs, interspecies transmission by GCRVs may also occur, raising interesting questions about the ecology of human and animal GCRVs.

Gathering information on the genetic diversity of animal GCRVs is critical to trace the origin of unique, animal-like CGRV strains. To obtain further information on the genetic relationships between human and animal GCRVs, we determined the VP7 sequences of 10 porcine GCRV strains detected in different outbreaks of gastroenteritis in piglets during a large surveillance study in Italy from 2003 to 2005 (Martella et al., 2007).

## Results

The 10 porcine GCRV strains (118/05-1, 118/05-4, 118/05-18, 118/05-27, 134/04-18, 42/05-21, 344/04-7, 43/06-22, 134/04-2, and 43/06-1) were detected from distinct outbreaks of enteritis. However, strains 134/04-18 and 42/05-21 were identified in the same herd, in the province of Modena, in 2003 and 2004, respectively. All the samples but 2 (strains 134/04-2 and 344/04-7) were mixed viral infections, as they also contained either GARV or enteric calicivirus (ECV) RNA (Table 1).

The nearly-full length nt sequences (1043–1055 bp) of the VP7 of 10 porcine GCRV strains were determined along with their deduced aa sequences. The two in-phase initiations codon at nt 49 and 124 were conserved in all 10 VP7 genes of the GCRV strains analysed. The open reading frame (ORF) of strains 118/05-1, 118/05-4, 118/05-18, 118/05-27, 134/04-18, and 42/05-21 was 996 nt in length (nt 49 to 1044) and encoded

Table 1
List of reference animal and human strains with genotype/serotype designation

| | G type | Year | Origin | GARV | ECV | Accession |
|---|---|---|---|---|---|---|
| *Strain* | | | | | | |
| po/Cowden | 1 | – | US | | | M61101 |
| bo/Shintoku | 2 | – | Japan | | | U31750 |
| po/HF | 3 | – | US | | | U31748 |
| hu/Preston | 4 | – | UK | | | X77258 |
| *Italian strains* | | | | | | |
| po/118/05-1 | 1 | 2005 | Perugia | + | + | EF464651 |
| po/118/05-4 | 1 | 2005 | Perugia | + | + | EF464652 |
| po/118/05-18 | 1 | 2005 | Perugia | + | − | EF464648 |
| po/118/05-27 | 1 | 2005 | Perugia | + | − | EF464649 |
| po/134/04-18 [a] | 5 | 2003 | Modena | + | + | EF464653 |
| po/42/05-21[a] | 1/4 | 2004 | Modena | + | − | EF464650 |
| po/344/04-7 | 6 | 2004 | Perugia | − | − | EF464654 |
| po/43/06-22 | 6 | 2005 | Brescia | + | + | EF464657 |
| po/134/04-2 | 6 | 2003 | Brescia | − | − | EF464655 |
| po/43/06-16 | 6 | 2005 | Mantova | + | + | EF464656 |

[a] The strains were detected from the same herd.

for a protein of 332 aa. The ORF of strains 344/04-7, 43/06-22, 134/04-2, and 43/06-16 was 1008 nt in length (nt 49 to 1056), due to a 12-nt-long insertion at nt 781 to 792, that did not alter the frame. As a result, the VP7 of these 4 strains was 336 aa in length, with a unique insertion (SSSV or SSTI) at residue 244, within the variable region VR8 at the carboxy-terminus of the protein (Tsunemitsu et al., 1996) (Fig. 1). The potential N-linked glycosylation sites (Asn-X-Ser/Thr) located at aa 67–69 and 225–227 were conserved in all 10 porcine GCRV strains. Similar to the human strains, an additional potential glycosylation site at residue 152–154 was present in the VP7 of strain 344/04-7, while another potential glycosylation site was present at position 318–320 (residues 314–316 of porcine strain Cowden) in the VP7 of strains 118/05-18 and 118/05-27. The putative signal cleavage site at residues 49–50 (AGQ) was highly conserved in the VP7 of almost all GCRVs. The cysteines at residue 3, 10, 78, 140, 170, 196, 212, 253 and 258 (residues 249 and 254 of strain Cowden) were highly conserved in the VP7 of all the GCRVs. The hydropathicity profile of the VP7 of the porcine GCRV strains was similar to the profile of the VP7 of the other GCRVs, with a highly hydrophobic region at the amino terminus of the protein, thus suggesting a structural conservation within the protein (data not shown).

A matrix of pairwise comparison between human and animal GCRV strains was elaborated (Table 2). The VP7 of the Italian strains 118/05-1 and 118/05-4, which were detected during the 2005 in different herds located in the province of Perugia, shared complete nt and aa identity (100%) to each other and were highly related (93.1–94.6% aa identity) to the VP7s of prototype porcine strains Cowden and WH. Similarly, the VP7 of porcine strains 118/05-18 and 118/05-27, also detected in 2005 in different herds of the province of Perugia, displayed 98.2% nt and 99.0% aa identity to each other and 92.0–92.4% aa identity to those of the prototype Cowden and WH strains. The VP7 of porcine strain 134/04-18 displayed <84.6% aa identity to those of all human and animal GCRVs. Interestingly,

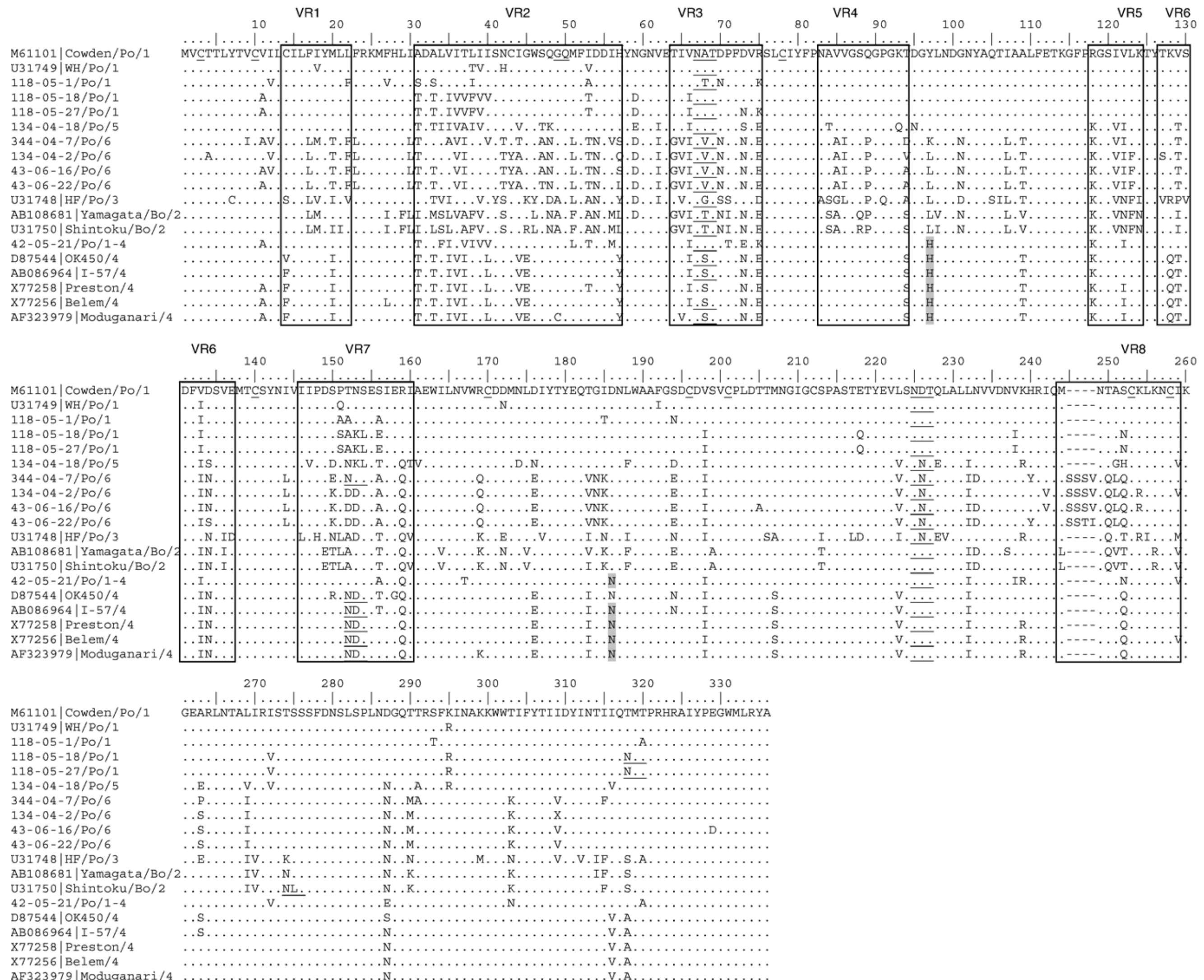


Fig. 1. Comparison of the deduced aa VP7 sequence from animal and human GCRVs. The tentative genotypes of GRCVs are also given. Identical aa are indicated by a dot and gaps are shown by dashes. The variable regions VR-1 to VR-8 (Tsunemitsu et al., 1996) are boxed. Potential N-linked glycosilation sites are underlined. Also, the conserved cysteine (C) residues and the putative signal cleavage site GQ are underlined in the consensus sequence (strain po/Cowden).

Table 2
VP7 comparison (amino acid) between human and animal group C rotaviruses

| | BCN6 | Preston | Belem | Moduganari | OK450 | 42-05-21 | Cowden | 118-05-18 | WH | 118-05-1 | 134-04-18 | 43-06-22 | 344-04-7 | 134-04-2 | 43-06-16 | Shintoku | Yamagata |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BCN6/G4 | – | | | | | | | | | | | | | | | | |
| Preston/G4 | 98.8 | – | | | | | | | | | | | | | | | |
| Belem/G4 | 97.9 | 99.1 | – | | | | | | | | | | | | | | |
| Moduganari/G4 | 97.3 | 98.5 | 98.2 | – | | | | | | | | | | | | | |
| OK450/G4 | 95.5 | 96.7 | 96.4 | 95.8 | – | | | | | | | | | | | | |
| 42-05-21/G1-G4 | 88.0 | 87.7 | 87.3 | 86.1 | 85.2 | – | | | | | | | | | | | |
| Cowden/G1 | 87.7 | 87.7 | 87.3 | 87.3 | 87.3 | 89.8 | – | | | | | | | | | | |
| 118-05-18/G1 | 86.9 | 87.2 | 86.2 | 85.6 | 85.9 | 90.7 | 92.4 | – | | | | | | | | | |
| WH/G1 | 85.5 | 85.5 | 84.9 | 84.9 | 84.9 | 88.6 | 97.0 | 92.0 | – | | | | | | | | |
| 118-05-1/G1 | 84.3 | 84.6 | 84.3 | 84.0 | 84.9 | 88.0 | 94.6 | 89.9 | 93.1 | – | | | | | | | |
| 134-04-18/G5 | 84.0 | 84.0 | 84.3 | 83.0 | 83.6 | 83.6 | 83.0 | 84.6 | 82.1 | 80.6 | – | | | | | | |
| 43-06-22/G6 | 81.9 | 82.5 | 81.6 | 81.9 | 81.6 | 77.8 | 78.9 | 78.0 | 77.1 | 78.3 | 77.8 | – | | | | | |
| 344-04-7/G6 | 80.4 | 81.3 | 80.4 | 80.7 | 80.1 | 77.8 | 77.7 | 76.8 | 76.2 | 77.4 | 76.2 | 93.2 | – | | | | |
| 134-04-2/G6 | 80.7 | 81.3 | 81.0 | 80.7 | 81.0 | 77.7 | 78.5 | 76.7 | 76.7 | 78.2 | 77.1 | 95.2 | 92.5 | – | | | |
| 43-06-16/G6 | 81.0 | 81.6 | 80.7 | 81.0 | 80.7 | 76.5 | 77.7 | 76.8 | 75.9 | 77.7 | 75.9 | 96.7 | 93.5 | 97.0 | – | | |
| Shintoku/G2 | 73.2 | 73.8 | 74.1 | 74.1 | 73.8 | 73.5 | 74.4 | 72.2 | 74.7 | 74.7 | 70.4 | 77.1 | 76.5 | 76.4 | 75.9 | – | |
| Yamagata/G2 | 73.5 | 73.5 | 73.8 | 73.8 | 73.5 | 73.8 | 74.7 | 73.7 | 75.0 | 75.0 | 71.3 | 78.6 | 78.0 | 77.9 | 77.4 | 96.1 | – |
| HF/G3 | 70.2 | 70.5 | 70.2 | 71.4 | 70.5 | 68.5 | 70.5 | 68.2 | 69.9 | 69.9 | 67.3 | 71.4 | 69.7 | 71.3 | 71.1 | 72.9 | 72.6 |

the VP7 gene of porcine strain 42/05-21 was equally genetically distant from those of human and animal strains, as it displayed 85.1% nt identity to the VP7 of human strain KA4 and 84.8% nt identity to porcine strain Cowden, while the deduced VP7 aa sequence displayed aa identities as high as 88.0–90.7% to those porcine GCRVs and 85.2–88.2% to those of human GCRVs. The VP7 of the other 4 Italian porcine strains, 344/04-7, 43/06-22, 134/04-2, and 43/06-16, exhibited 92.5–97.0% aa identity to each other, while the aa identity was 80.1–82.5% to those of human GCRVs, 75.9–78.5% to those of porcine GCRVs, 75.9–78.6% to those of bovine GCRVs, and <71.4% to that of the unique porcine strain HF.

The phylogenetic tree (Fig. 2) revealed at least six genetic lineages (G genotypes). All the human GCRV strains clustered in a tightly related genetic group, designated genotype G4. The porcine GCRV strains Cowden, WH, 118/05-1, 118/05-4, 118/05-18 and 118/05-27 formed a second lineage, designated genotype G1, while strains HF and 134/04-18 represented each a distinct genotype, G3 and G5, respectively. The porcine strains 344/04-7, 43/06-22, 134/04-2, and 43/06-1 formed a monophyletic cluster (genotype G6), as did the bovine GCRV strains Shintoku and Yamagata (genotype G2).

To show the genetic relatedness within the VP7 gene of the various human and animal GCRV strains, identity plots were elaborated (Fig. 3). Interestingly, this analysis provided evidence for different rates of diversification across the VP7 gene of GCRVs. The 5′ portion of the VP7, spanning nt 150 to 350, appeared to undergo the highest extent of variation. This highly variable region corresponds to the aa region VR-2, VR-3 and VR-4 (aa, 31–57, 64–75 and 83–94, respectively) (Fig. 3A). A region of lower variation spanned between nt 380 and 700 (aa 110 to 215) and included the variable regions VR5, VR6 and VR7 (aa 118–124, 127–137 and 146–160, respectively). Downstream of this region, an area of moderate variation spanned nt 700 to 820 (aa 216 to 257), thus including VR8 (aa 244–259). Interestingly, the plot of the VP7 of the unique porcine GCRV strain 42/05-21 against those of genotype G4 (human) and G1 (porcine) strains, revealed discontinuous rates of variation throughout the VP7 gene (Fig. 3B). Porcine strain 42/05-21 was equally related to genotype G4 and G1 strains in the first tract (hypervariable), while it was more similar to genotype G1 strains in the second tract (85.7% vs. 83.7% nt) and more similar to genotype G4 strains in the moderately variable tract (84.1% vs. 79.8% nt). Direct inspection of the aa VP7 alignment revealed synapomorphies (shared-derived substitutions), such as 92-H and 186-N, between strain 42/05-21 and genotype G4 strains, and a number of additional changes were retained, even if not exclusive to, between strain 42/05-21 and genotype G4 strains, such as 159-Q, 232-I and 239-R. However, computer-aided analysis did not provided firm evidence for a recombinant origin of the strain.

## Discussion

GCRVs are an important cause of acute gastroenteritis in swine, cattle, and man (Ciarlet and Estes, 2002). Limited seroepidemiologic surveys suggest that group GCRVs are

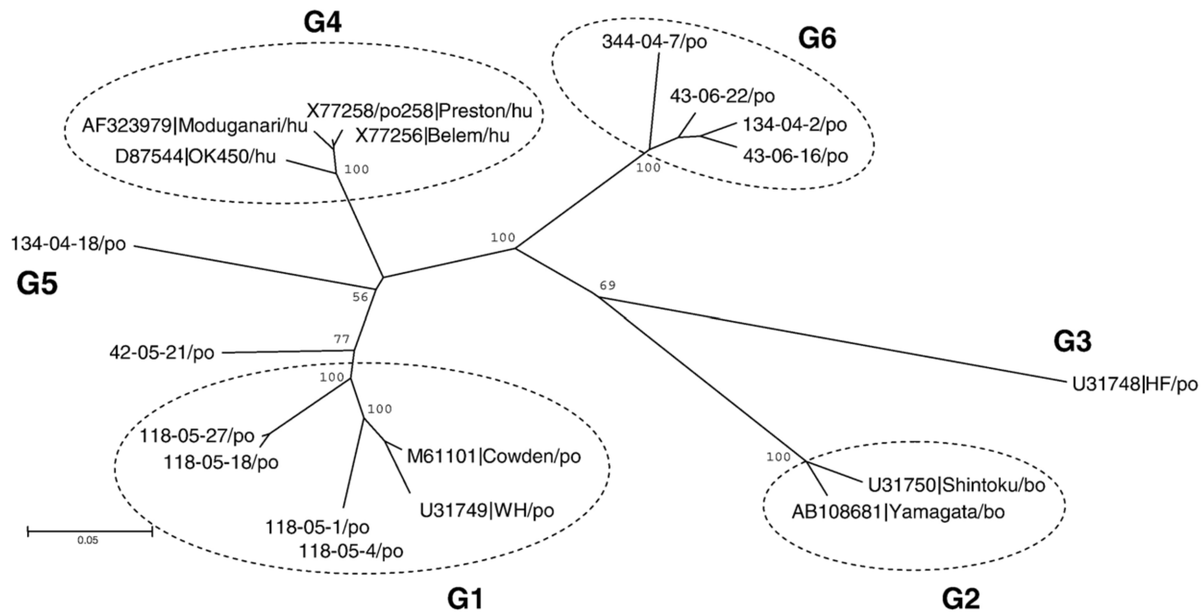


Fig. 2. Neighbor-joining unrooted tree based on the complete VP7 aa sequence of porcine GCRVs described in this study and reference strains: porcine (Po), Cowden, HF; WH; bovine (Bo), Shintoku, Yamagata; human (Hu), Belem, Bristol, Moduganari, OK450 and Preston. The scale bar is proportional with the phylogenetic distance.

widespread and presumably enzootic in pig herds (Terrett et al., 1987; Tsunemitsu et al., 1992a; Saif and Jiang, 1994). Direct detection of the virus from clinical specimens has been, however, rarely performed resulting in significant gaps in our knowledge about the virological and epidemiological features, as well as about the ecology, pathogenic and zoonotic potential of these enteric pathogens.

During a passive surveillance study for virus enteric pathogens in piglets with gastroenteritis, GCRVs were detected in 32/102 fecal specimens (31.3%) from 12/12 swine herds. GRCVs were also identified from a large collection of samples collected between 2003 and 2005 at the Istituto Zooprofilattico of Brescia, Italy. Out of 3453 samples examined in this time span, rotavirus-like particles were detected by electron microscopy in 411 samples (11.9%), with marked yearly variations (6.4% to 18.8%) (Lavazza et al., unpublished). A subset (86) of this collection was screened for GCRV, and GCRVs were detected in 22 (25.5%) samples (Martella et al., 2007). The possibility to evaluate the presence of GCRVs in a large number of gastroenteritis outbreaks allowed to gather relevant information on the genetic heterogeneity of GCRVs. In the present study, the VP7 genes of 10 porcine strains were sequenced, revealing the presence of several G genotypes, some of which were not previously recognized until now.

Four porcine strains, 118/05-1, 118/05-4, 118/05-18 and 118/05-27, resembled the prototype porcine GCRV strains Cowden and WH. However based on sequence analysis, five porcine strains could not be assigned to any previously established VP7 genotypes, as they shared <85% (134/04-18) and <81% aa identity (344/04-7-like strains) to all previously recognized GCRVs, and they were therefore assigned to new VP7 genotypes, proposed as G5 (134/04-18) and G6 (344/04-7-like). To classify the various strains into distinct genotypes, we applied an 89% aa cutoff, which was adopted considering previous GCRV studies (Tsunemitsu et al., 1992b, 1996; Rahman et al., 2005) and also taking into account the values accepted for distinction of GARV G types (Green et al., 1988, 1989; Kapikian and Chanock, 1996). When combining the results of antigenic and sequence analysis, no antigenic relationships was observed among the porcine strain Cowden (genotype G1, serotype G1), porcine Shintoku (genotype G2, serotype G2) and porcine HF (genotype G3, serotype G3), that share 69.9–74.7% aa identity (Tsunemitsu et al., 1992b, 1996). In contrast, one-way antigenic relationship was observed between representatives of the G1 strains (Cowden and WH) and a single genotype G4 human strain, Ehime, which share 85.2–87.7% aa identity (Tsunemitsu et al., 1996). Accordingly, although classified as distinct genotypes (Rahman et al., 2005), the G4 human GCRVs and G1 porcine GCRVs retain conserved epitopes. The human GCRVs G4 appear to have evolved relatively recently from a unique bottle-neck event and are highly conserved to each other, while a surprisingly high genetic heterogeneity was observed among the VP7s of porcine GCRV, even within a particular genotype.

Of interest, a porcine GCRV strain, 42/05-21, displayed intermediate genetic features between human G4 and porcine G1 strains. Strain 42/05-21 shares 90.7% aa identity with its closest relative within genotype G1 strains, and only 88.0% with the most distantly related G1 strains, suggesting that it may be an ancestral strain for this genotype. By plotting the VP7 gene of strain 42/05-21 against the VP7 of G4 human and G1 porcine strains, a bewildering pattern was observed, with a fragment more related to human G4 viruses and a fragment more related to porcine G1 viruses. This pattern may be interpreted, either as a remote recombination phenomenon (Suzuki et al., 1998; Parra et al., 2004), or as independent

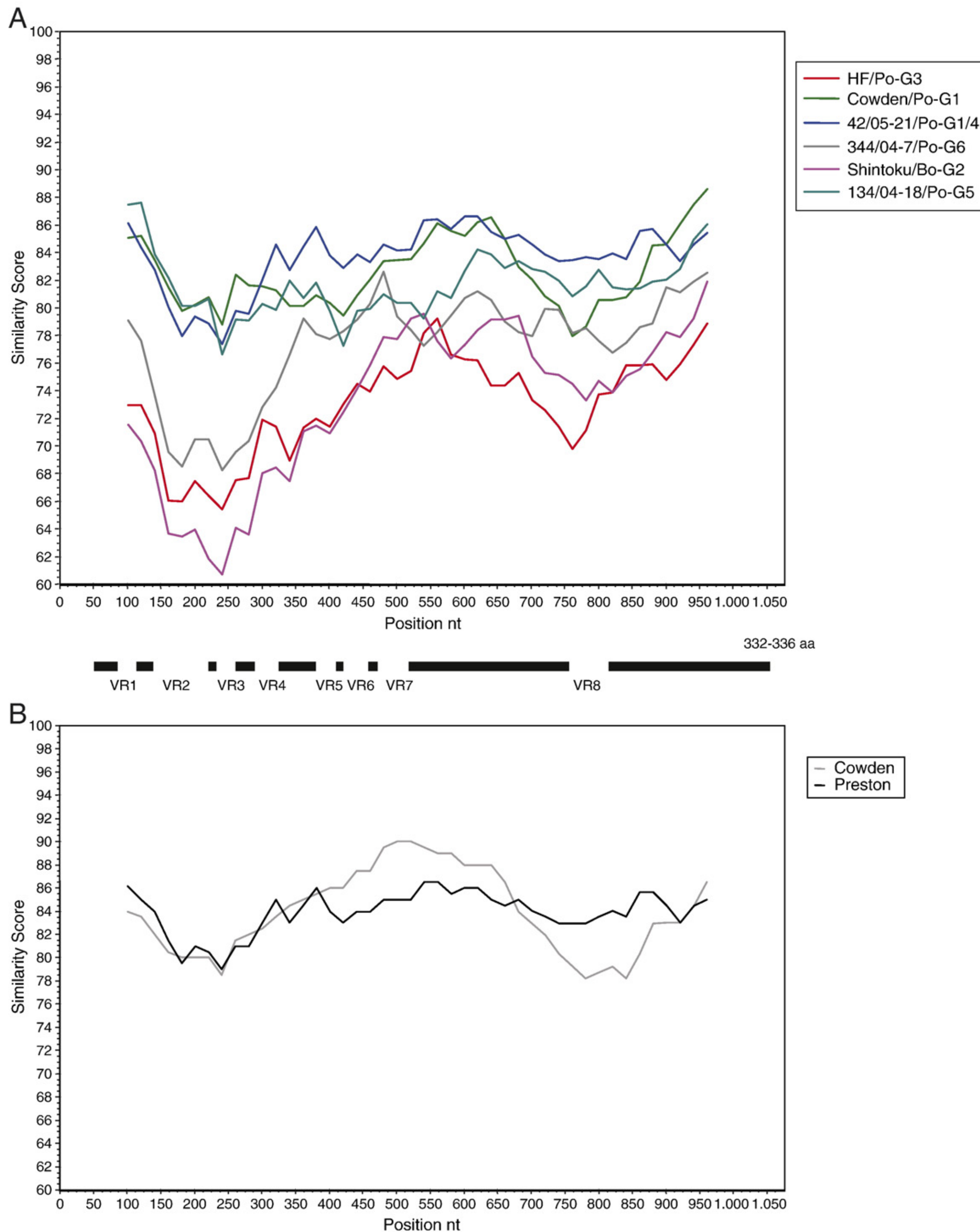


Fig. 3. Similarity plots generated by Simplot. Plots were generated to show the genetic relatedness of the various animal strains to the human G4 GCRVs (A). At the bottom, the VP7 aa sequence with the variable regions VR1 to VR8 are shown. Similarity plots were also generated to show the relationships between the unusual strain po/42/05-21 and G4 (human) and G1 (po/Cowden-like) strains (B).

evolution of the strains from a common ancestor, with different selective pressure acting over the various regions of the VP7 protein. Interestingly, the G1/G4 porcine strain 42/05-21 was detected in the same herd as the G5 strain 134/04-18, in province of Modena. Strain 42/05-21 was associated with a gastroenteritis outbreak in December 2004, while strain 134/04-18 was identified from an outbreak occurred in June 2003. For GARVs, repeated introductions of various strains have been observed over the various seasons and years, a pattern that is consistent with mechanisms of selective pressure due to the immunity raised in the human population (Arista et al., 2006). It is possible that similar mechanisms may drive the evolution of GCRVs, with temporal fluctuation of the various strains under the influence of the immune pressure against to the dominant GCRV strains in the animal host. Large prospective epidemiological studies are needed to understand better these

mechanisms driving the evolution of GCRVs in animals and humans.

Testing for GARVs and ECVs, revealed that out of 54 samples positive for GCRVs only 5 samples contained exclusively GCRVs. Due to inappropriate management and to overcrowded conditions, weaned pigs may be exposed to multiple enteric pathogens. Mixed viral infections are frequent and may result in synergetic mechanisms of enhancement of pathogenicity. Indeed, synergetic mechanisms have been reproduced experimentally in calves co-infected with GCRV and GARV or with GARV and *Escherichia coli* (Chang et al., 1999; Gouet et al., 1978), and in rabbits co-infected with GARV and *E. coli* (Thouless et al., 1996). Of the 10 porcine GCRV strains sequenced in this study, 8 were mixed viral infections (GARV and/or ECVs), while 2 contained exclusively GCRVs. Of interest, both of the strains, 134/04-2 and 344/04-7, were characterized as genotype G6. In experimental conditions, the virulent GCRV strain Cowden alone was able to induce disease in pig, while the attenuated virus Cowden was not (Chang et al., 1999). This finding may raise the question whether GCRV strains, like GARVs (Bridger, 1994; Dunn et al., 1993), may vary markedly in their pathogenic nature.

In conclusion, in this study we identified novel genotypes of the GCRV VP7 gene and we detected a GCRVs strain with intermediate genetic features between human and porcine strains, suggesting a wide diversity of porcine GCRVs. These findings provide the basis for future epidemiological studies and warrant investigations on the pathogenic potential of these novel GCRVs in pigs.

## Materials and methods

### *Samples*

The 10 porcine GCRV strains 118/05-1, 118/05-4, 118/05-18, 118/05-27, 134/04-18, 42/05-21, 344/04-7, 43/06-22, 134/04-2, and 43/06-1 are listed in Table 1. All 10 strains were detected in diarrheic piglets between 1 and 3 months of age during a passive surveillance study for viral gastroenteritis in weaning and post-weaning piglets. The samples were collected from distinct gastroenteritis outbreaks that occurred in Italian piggeries in Northern and Central Italy in the period 2003–2005 (Martella et al., 2007).

### *Detection of group C rotaviruses (GCRVs) by RT-PCR*

The viral RNA was extracted from dilutions of rectal swabs or stool samples. The RNA was extracted from 200 μl of 10% (wt/vol) fecal suspensions in phosphate buffered saline (PBS), using guanidinium isothiocyanate/silicae in according with the procedure of Boom et al. (1990). RNA was eluted in 50 μl $H_2O$ DEPC with RNasin (0.2 μg/μl, Promega) and used in RT-PCR. A one-step RT-PCR procedure (Superscript III One step, Invitrogen, UK) was utilized to amplify the 3′ end of the viral VP6 gene using oligonucleotide primers described elsewhere (Sánchez-Fauquier et al., 2003). The amplicons were analyzed in 2% agarose gels following ethidium–bromide staining and UV-light transillumination.

### *Detection of group A rotaviruses (GARVs) and of porcine enteric caliciviruses (ECVs) by RT-PCR*

All the samples were screened for the presence of GARVs and ECVs by reverse transcription-polymerase chain reaction (RT-PCR), using oligonucleotide primers previously described (Gentsch et al., 1992; Jiang et al., 1999a,b).

### *RT-PCR amplification of the VP7 gene*

A one-step RT-PCR procedure (Superscript III One step, Invitrogen, UK) was utilized to amplify the 8th genome segment, that encodes the VP7, from nucleotide (nt) 20 to the 3′ end (nt 1062), using oligonucleotide primers GrC-VP7-20 and GrC-VP7-1062 described elsewhere (Rahman et al., 2005). The amplicons were analyzed in 2% agarose gels following ethidium–bromide staining and UV-light transillumination. Fifteen out of 20 GCRV-positive samples yielded specific amplicons of about 1040 bp, but only 10 were suitable for sequence analysis, based on the intensity of the bands by agarose gel visualization.

### *Sequence determination and phylogenetic analyses of the VP7 of porcine group C rotavirus (GCRV) strains*

The PCR products were extracted from gel slices using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany). Cycle sequencing was carried out using the BigDye kit (v1.1; Applied Biosystems, Foster City, CA) with primers GrC-VP7-20 and GrC-VP7-1062, specific for the VP7 ends of GCRVs, and with internal primers designed on the actual sequences with an overlapping strategy. Dyelabeled products were run on an automated sequence analyzer (ABI Prism type 3100; Applied Biosystems, Foster City, CA).

The sequences were assembled using Bioedit software package version 2.1 (Hall, 1999) and compared to cognate sequences in the genetic databases using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and FASTA (http://www.ebi.ac.uk/fasta33) web-based programs. Phylogenetic reconstruction was carried out with various algorithms of distance correction and the neighbor joining method, supported with bootstrapping over 1000 replications. Distance analysis and phylogenetic inference were carried out using the Mega 3.0 software package (Kumar et al., 2004). The nucleotide sequences are available in GenBank under accession numbers EF464648 to EF464657.

The Simplot computer program (Lole et al., 1999) was used to generate identity plots of the VP7 gene of the porcine GCRVs. The VP7 sequences of porcine GCRV Cowden, WH, and HF strains, and bovine GCRV Yamagata and Shintoku strains, and 35 VP7 sequences of a selection of human GCRV strains, were retrieved from the databases and included in our study. The sequences were analyzed using a window size of 200 and step size of 20 with gap strip off and Jukes–Cantor correction on. The RDP software v2.0 (Martin et al., 2005) was used to identify

potential recombination events in the newly identified sequences, by using the default settings as search options.

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