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Identification of a G2-like porcine rotavirus bearing a novel VP4 type, P[32]

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Abstract – A porcine group A rotavirus (GARV) strain, 61/07/Ire, was isolated from a 4–5 week asymptomatic piglet, during an epidemiological survey of porcine herds in Southern Ireland, in 2007. The nucleotide (nt) and amino acid (aa) sequence of the full-length VP4 protein of the PoRV strain 61/07/Ire was determined. Based on the entire VP4 open reading frame (nt), strain 61/07/Ire displayed $\leq 76.5\%$ identity to representatives of the established 31 P-types, a value far lower than the percentage identity cutoff value (80%) established by the Rotavirus Classification Working Group (RCWG) to define a novel P genotype. Strain 61/07/Ire revealed low aa identity, ranging from 57.1% to 83.6%, to the cognate sequences of representatives of the various P genotypes. The aa identity was lower in the VP8* trypsin-cleavage fragment of the VP4, which encompasses the VP4 hypervariable region, ranging from 36.9% to 75.3%. Sequence analyses of the VP7, VP6, and NSP4 genes revealed that the GARV strain 61/07/Ire possessed a G2-like VP7, an E9 NSP4 genotype and an I5 VP6 genotype. Altogether, these results indicate that the GARV strain 61/07/Ire should be considered as a prototype of a new VP4 genotype, P[32], and provide further evidence for the vast heterogeneity of group A rotaviruses.

Rotavirus / pig / VP4 / P genotype

1. INTRODUCTION

Rotaviruses are classified into seven antigenically distinct groups (A to G). 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 [10, 25]. In humans, group A rotavirus (GARV) is estimated to cause 138 million cases of gastroenteritis annually, resulting in approximately 619 000 deaths, mostly in developing countries [21].

The rotavirus genome consists of 11 double stranded RNA segments, enclosed in a triple layered capsid. The outer capsid proteins, VP7

and VP4 (defining the G-types and P-types, respectively), both elicit serotype-specific neutralising antibodies and are important for immune protection and for vaccine development [10, 23, 65]. To date, 23 G-types and 31 P-types have been identified in humans and animals [1, 26, 34, 36, 41, 44, 58–61, 63]. Rotavirus strains within a G serotype share at least 90–91% VP7 amino acid (aa) sequence identity [10]. Rotavirus strains sharing $> 89\%$ VP4 aa sequence identities belong to the same P genotype, while those with $< 89\%$ VP4 aa sequence identities belong to different P genotypes [17]. Moreover, the greatest aa divergence in VP4 is seen between aa 71 and 204 of the VP4 trypsin-cleavage product VP8*, which tends to correlate with VP4 serotype specificity [29, 30].

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The middle capsid protein, VP6, bears the subgroup (SG) specificities which allow the classification of GARV into SG I, SG II, both SG I and II, or into neither SG, based on reactivity with SG specific monoclonal antibodies (MAbs) [10], or into genogroups I or II, based on sequence analysis of a fragment of the VP6 gene, known to correlate with SG specificity [24]. The non-structural glycoprotein NSP4 possesses enterotoxigenic activity, and has multiple functions in rotavirus morphogenesis and pathogenesis [10]. Sequence analyses of the NSP4 gene from human and animal rotavirus strains has revealed the presence of five distinct NSP4 genogroups, KUN- (A), Wa- (B), AU-1- (C), EW- (D), and avian-like (E) [9, 47]. Rotavirus strains isolated from rabbits, horses, cows, and pigs generally cluster according to species of origin, and all PoRV strains identified to date cluster into NSP4 genogroup B, Wa-like [9, 40].

More recently, based on sequence comparison of large data sets, including full-length rotaviral genome sequences, a novel classification system was proposed, based on nucleotide identity cut-off percentages, and different genotypes were defined for each genome segment. An alpha-numeric letter code, Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, is used to designate and classify the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 encoding genes, respectively [45]. Within this classification system, at least 13 VP6 genotypes (I1 to I13) and 12 NSP4 genotypes (E1 to E12), have been defined [45].

GARV are one of the most frequently detected viral agents associated with diarrhoea affecting piglets between 1 and 8 weeks of age [53]. GARV may also be detected in non-diarrheic piglets [31]. Infection of pigs with GARV has been recognised in both enzootic and epizootic forms of diarrhoea, resulting in economic losses in commercial piggeries [54]. Antigenic and molecular characterization of GARV has revealed a broad heterogeneity in the VP7 and VP4 genes. The most common G-types identified in pigs are G3, G4, G5 and G11, associated with common P-types, P[6] and P[7] [19]. Porcine GARV strains with

additional P-types, P[13], P[19], P[23], P[26] and P[27] have also been described, but more sporadically. Furthermore, porcine strains displaying human-like G-types (G1, G2, G9 and G12) and P-types (M37-like P[6] and P[8]) and bovine-like G-types (G6, G8 and G10) and P-types (P[1], P[5] and P[11]) have also been described [6–8, 16, 19, 20, 35, 38, 40, 41, 50].

Several epidemiological investigations have revealed that infections by unusual, animal-like strains, or animal-human rotavirus reassortants may not be infrequent in humans [57]. Therefore, the study of animal rotaviruses is pivotal to fully understand the evolution and ecology of human rotaviruses [39]. Also, this is fundamental for the development of new instruments to improve the prophylaxis of rotaviral diarrhea in piglets. Between 2005–2007, a surveillance study for rotavirus was conducted on Irish porcine herds in Southern Ireland. Porcine faecal samples were collected from asymptomatic animals and screened by molecular assays to detect GARV RNA. In this report, we describe a novel VP4 genotype carried by a G2-like PoRV strain, 61/07/Ire.

2. MATERIALS AND METHODS

2.1. Sample collection

Porcine GARV strain 61/07/Ire was identified during an epidemiological survey in Irish pig herds, in 2007. A total of 292 faecal samples were obtained from 4–5 week old asymptomatic pigs from porcine herds in Ireland, over 3 years, from 2005–2007. The samples were collected from 3 different herds in County Cork. Eighty samples were collected in May 2005 (farm 1), 80 samples were collected in September 2006 (farm 2) and 102 samples in May 2007 (farm 3). A collection of 30 samples (farm 4) was also obtained in March 2007, in County Dublin, from 8–9 week old piglets.

2.2. RNA Preparation

Total nucleic acids were extracted from the samples by a standard phenol-chloroform method with

ethanol precipitation. The extracted nucleic acids were re-suspended in 100 µL of sterile DEPC H₂O and stored at -80 °C prior to use.

2.3. RT-PCR amplification of GARV and group C rotavirus (GCRV)

The extracted nucleic acids were tested for the presence GARV and GCRV by RT-PCR using oligonucleotide primers previously described [15, 55]. For amplification of the VP7, the primer pair Beg9/End 9 was used [18]. Prediction of the VP7 (G) type was carried out by multiplex PCR as described in previous studies [13, 18, 20, 66]. Partial amplification of the VP6 gene was determined with primer pair VP6F-VP6R [24]. For amplification of the NSP4 gene, the primers 151 and 152 were used [5].

2.4. RT-PCR amplification of the VP8* and VP4

For amplification of the VP8* subunit of the VP4 gene, the primers Con2 and Con3 were used [15]. Full-length amplification of the VP4 gene was achieved using the consensus primer 170 (ggt cac awc ctc tag mmr ytr ctt a) at the 3' end of the genome segment in combination with primer Con3 [40].

2.5. Sequence analysis

The RT-PCR-amplified products were sequenced by Genome Express (Meylan, France). The sequences were assembled, edited and analyzed using the Bioedit software package version 2.1 [22]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Arizona State University, USA) [28]. In addition, the sequences were analyzed using the web-based sequence analysis tool BLAST¹. The full-length sequence of the VP4 strain 61/07/Ire has been assigned the accession number FJ492835 in the Genbank database. The partial VP8* sequence of strains, 2F/05/Ire, 60/07/Ire, 1/07/Ire, 48/06/Ire, 2B/05/Ire and 2/07/Ire have been registered in the Genbank database as HM149304-309 respectively. The partial sequences of the VP7 strains 48/07/Ire and 61/07/Ire have been registered under the accession numbers FJ492832 and FJ492831, respectively. The partial sequences of the NSP4 strains 48/07/Ire and 61/07/Ire have been assigned the accession numbers FJ492833 and FJ492834, respectively. The partial sequence of

VP6 strains 61/07/Ire and 48/07/Ire have been registered as HM149310 and HM149311, respectively.

3. RESULTS

3.1. RT-PCR screening for group A and C rotaviruses

A total of 19 out of 292 samples (6.5%) were found to contain GARV, while 13 samples (4.4%) contained GCRV [11]. None of the GARV-positive samples were found to contain GCRV. The GARV positive samples were detected in 3/4 herds surveyed (2005–2007). GARV were detected in 6/80 samples of the 2005 collection, 4/80 samples from 2006, 9/102 samples from 2007, all of which had been collected in County Cork. In addition, GARV infection was not diagnosed in 30 samples from the 2007 collection, made in County Dublin.

3.2. Sequence analysis of the VP8* fragment of the VP4 gene

The VP4 sequence of 15 out of 19 GARV samples was obtained while the sequence of four samples was not exploitable for further analyses. Sequence analysis allowed characterization of the majority of the strains as P[6], P[7], P[13], P[13]/P[22] and P[26]. However, two strains, 48/07/Ire and 61/07/Ire, were distantly related to representative rotavirus strains of established P genotypes (Fig. 1). Based on the VP8* fragment (about 880 bp), strain 48/07/Ire and 61/07/Ire displayed > 98% nucleotide identity to each other, therefore, the sequence of the full-length VP4 gene of one such strain, 61/07/Ire, was determined. The basic structure of the VP4 gene of the Irish strain was very similar to those of other rotavirus strains, in that it consisted of 2 362 bp with one open reading frame (ORF) beginning at nucleotide 10, and a single TAA stop codon at nucleotide 2 238. The predicted size of the porcine VP4 protein was 776 aa, similar to those of most rotavirus strains, which are 775 or 776 aa in length [10]. The deduced aa sequence of the VP4 of the PoRV strains 61/07/Ire was compared with those of representative rotavirus strains of the

¹ <http://www.ncbi.nlm.nih.gov>.

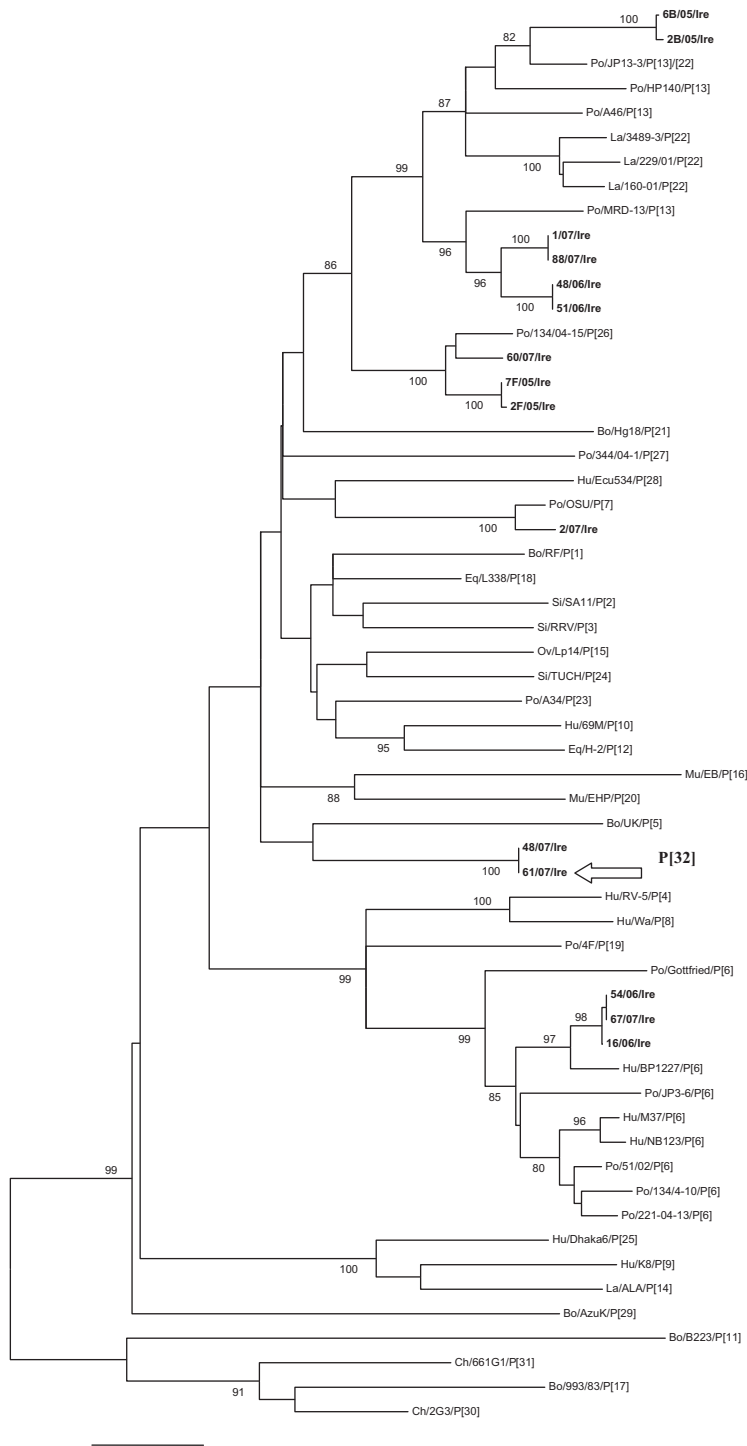


Figure 1. Neighbor-joining phylogenetic tree (2362 bp) of the VP4 nucleotide sequences displaying the Page 4 of 12 (*page number not for citation purpose*)

majority of the P genotypes. The conserved prolines and cysteines, and the potential trypsin-cleavage sites [2] were maintained in the VP4 of the Irish strain (data not shown). The additional cleavage sites identified in the VP5*, Lys-259, and putatively, Arg-467 [12], were also conserved (data not shown). The aa sequence of the full-length VP4 protein of PoRV strain 61/07/Ire showed aa identity values, ranging from 57.1% to 83.6% when compared to those of strains representatives of all P genotypes (Tab. I). Likewise, the aa sequence of the VP8* of 61/07/Ire shared low aa identity (36.9–75.3%), with the homologous sequences of representative strains of other P genotypes (Tab. I). Sequence comparison based on the nucleotide alignment of representative of the various P genotypes (Tab. I), and analysis by BLAST also revealed that in the 4th dsRNA segment strain 61/07/Ire shared < 76.5% nt identity with all the GARV strains identified thus far. This value is below the cutoff value proposed by Matthijnsens et al. [44] for the VP4 gene (80%).

3.3. Sequence analysis of the gene encoding the outer capsid protein VP7

By PCR genotyping using several pools of G-type specific primers [13, 18, 20, 66], strains 48/07/Ire and 61/07/Ire remained uncharacterized. Sequence analysis of the VP7 gene allowed the characterization of such untypeable GARV, 48/07/Ire and 61/07/Ire, as G2-like strains. A phylogenetic tree was constructed with a selection of human and porcine G2 strains. Two major lineages were observed, with one including the human G2 strains, and the other lineage consisting exclusively of porcine G2-like strains (Fig. 2).

The VP7 gene segments of strains 48/07/Ire and 61/07/Ire displayed the highest aa identity

(92.4–93%) to the porcine strain 34461/4, the reference G2-like porcine strain, identified in Spain [38]. The Irish G2-like strains displayed 12 and 7 nt mismatches in the binding sites of the G2-specific primers aCT2 and 9T1-2, respectively [13, 18], that likely prevented recognition by the genotyping probes (data not shown). In the VP7 antigenic regions A (aa 87 to 101), B (aa 141 to 152), C (aa 208 to 224) and F (235 to 242) [7, 14, 27, 48], strain 48/07/Ire differed in 9 residues and 61/07/Ire differed in 8 residues from the prototype G2-like porcine strain 34461/4 (data not shown).

3.4. NSP4 and VP6 analysis

In the NSP4-based tree (Fig. 3), strains 48/07/Ire and 61/07/Ire were clustered with the human strain A_G4_120 and the porcine strains P21-5 and CMP034, within the E9 genotype, while the vast majority of porcine viruses are E1. In the NSP4, strain 48/07/Ire and 61/07/Ire displayed 99% nucleotide identity to each other. The Irish GARV strains displayed the highest nt identity (93.6–93.9%) to E9 strains (P21-5, CMP034 and A_G4_120), while nt identity to other E types was lower than the established cutoff value (85%).

The VP6 genotype was determined by amplification of a 379-bp fragment, spanning from amino acids 241 to 367 of the VP6, with primer pair VP6F-VP6R [24]. In the VP6, strain 48/07/Ire and 61/07/Ire displayed only 83.1% nucleotide identity to each other. Based on this short fragment, strain 48/07/Ire closely resembled human SGII ($\leq 99.2\%$ nt identity), within the I1 genotype, while strain 61/07/Ire resembled porcine SGI strains (92.6% nt identity to porcine strain CM), within the I5 genotype (Fig. 4). Accordingly, the two Irish strains possessed a diverse VP6 gene, likely due to a reassortment event.

relationship between strains 61/07/Ire (designated with an arrow and referred to as P[32]) with all the known P genotypes. Genbank accession of the VP4 sequences are in Table I. Abbreviations: Si, simian; Eq, equine; Po, porcine; Ca, canine; Bo, bovine; Mu, murine; Hu, human; Ov, ovine; La, lapine. All phylogenetic trees in this study were statistically supported by bootstrapping of 500 replicates. Bootstrap values below 80% were not shown in the phylogenetic trees in this study. The scale bar represents 0.1 substitutions per site.

Table 1. Comparison of the nucleotide and amino acid sequences of VP8*, VP5* and VP4 of porcine strain 61/07/Ire with those of representatives of established P genotypes. Abbreviations: Si, simian; Eq, equine; Po, porcine; Bo, bovine; Hu, human; Ov, ovine; Ca, caprine; Mu, murine. In boldface the highest identity matches.

Species/Strain/Accession No/P type	VP4 (nt)	VP8* (aa)	VP5 (aa)	VP4 (aa)
Bo/RF/U65924/P[1]	73.1	69.8	83.9	78.9
Si/SA11/M23188/P[2]	73.7	71.5	84.7	80
Si/RRV/M18736/P[3]	72.3	71.5	83.7	79.3
Ca/K9/D14725/P[3]	73.1	69.4	85.4	79.6
Hu/RV-5/M32559/P[4]	69.9	59.4	75.9	70.6
Bo/UK/M22306/P[5]	68.8	61.9	77.6	72.1
Hu/M37/L20877/P[6]	69.3	61	79	73.1
Po/Gottfried/M33516/P[6]	68.5	60.6	77.2	72
Po/OSU/X13190/P[7]	75.1	71.9	86.6	81.5
Hu/Wa/M96825/P[8]	69.8	59.4	75.3	70.2
Hu/K8/D90260/P[9]	65.5	55.6	73.6	67.7
Hu/69M/M60600/P[10]	72.5	69.4	84.3	79.2
Bo/B223/D13394/P[11]	59.3	36.9	67.1	57.1
Eq/H-2/L04638/P[12]	71	64.8	83	76.9
Po/A46/AY050274/P[13]	75.9	66.3	87.7	80.4
Hu/Pa169/D14724/P[14]	66.3	58.1	74.4	69
Ov/Lp14/L11599/P[15]	72.3	69.8	83.3	78.8
Mu/EB/L18992/P[16]	66.5	55.2	78.6	71
Bo/993/83/D16352/P[17]	62.5	41.4	70.2	60.8
Eq/L338/D13399/P[18]	72.9	67.7	82.4	77.7
Po/4F/L10359/P[19]	69.8	59.8	79.9	73.3
Mu/EHP/U08424/P[20]	69.1	67.7	81.2	76.6
Bo/Hg18/AF237665/P[21]	72	62.3	83.3	76.4
La/160/01/AF526374/P[22]	nd	65.1	nd	nd
Po/A34/AY174094/P[23]	nd	64	nd	nd
Si/TUCH/AY596189/P[24]	71.5	70.2	84.7	79.7
Hu/Dhaka6/AY773004/P[25]	66.4	56	72.7	67.1
Po/134/04-15/DQ061053/P[26]	76.5	75.3	87.7	83.6
Po/344/04-1/DQ242615/P[27]	69.9	61	78.6	72.8
Hu/Ecu534/EU805773/P[28]	69.8	64	81.1	75.3
Bo/AzuK/AB454420/P[29]	64.5	47.3	70	62.3
Ch/Ch-2G3/EU486956/P[30]	60.9	42.6	68.9	59.9
Ch/Ch-661G1/EU486962/P[31]	61.9	41	69.7	60.2

4. DISCUSSION

A surveillance study was conducted in Southern Ireland, to investigate the diversity of GARV strains circulating in asymptomatic animals in porcine herds in Ireland from 2005-2007. Sequence analysis of the VP7 and the VP4 genes of the strains detected in this study identified a spectrum of G and P genotypes, including

G2-like, G4, G5, G9, G11, P[7], P[13], and P[26]. In addition, sequence analysis of the VP8* subunit of the VP4 gene (Fig. 1) identified two GARV strains, 48/07/Ire and 61/07/Ire, which could not be placed into any of the previously established VP4 genotypes. Epidemiological studies from diverse geographical settings worldwide have demonstrated the existence of several novel P genotypes in pigs [26, 40, 41, 60].

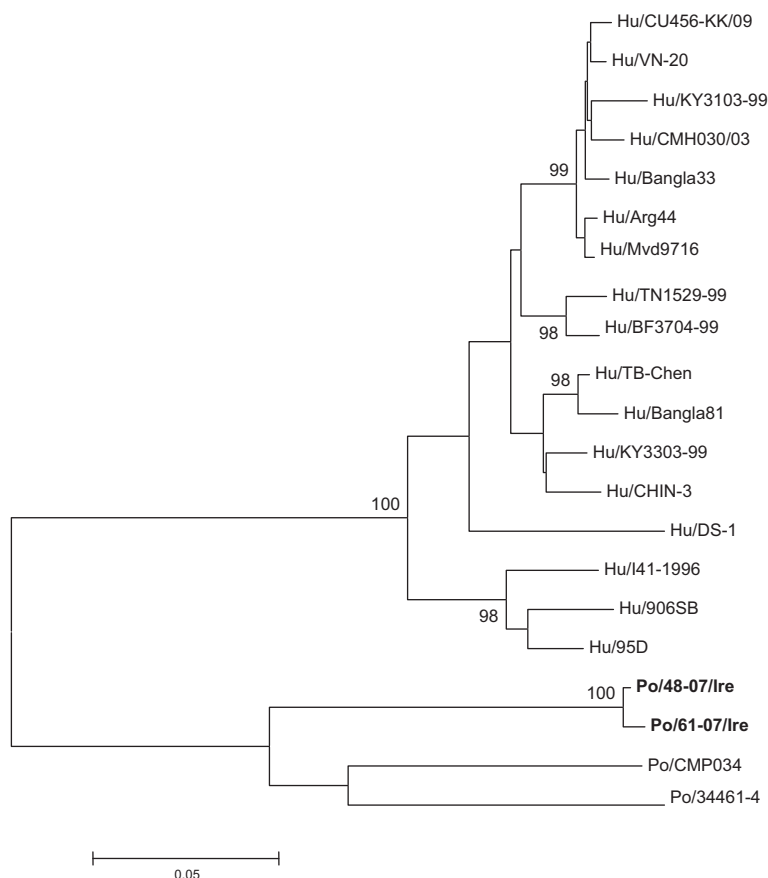


Figure 2. Neighbor-joining phylogenetic tree (920 bp) based on the VP7 nucleotide sequences of various porcine G2-like and human G2 porcine strains with porcine strains 48/07/Ire and 61/07/Ire. The Irish porcine strains are highlighted in bold. The scale bar represents 0.05 substitutions per site.

The GARV strains 48/07/Ire and 61/07/Ire were collected from 4–5 week old piglets in the same farm in County Cork, in 2007. Based on analysis of the VP4, strain 61/07/Ire displayed the highest identity (83.6% aa) to strain Po/134/04-15 [40]. By comparing the nt sequence of the entire VP4 ORF, the strain 61/07/Ire displayed $\leq 76.5\%$ identity to all other VP4 full-length sequences available in the databases, a value which was far lower than the percentage identity cutoff value (80%) established by the Rotavirus Classification Working Group (RCWG) to define a novel P genotype [44]. The sequence was submitted to

the RCWG and was assigned a new P genotype, P[32].

Analysis of the VP7 gene identified strains 48/07/Ire and 61/07/Ire as G2-like, since they displayed the highest aa identity (92.4–93%) to the G2-like porcine strain 34461-4. G2-like strains have been identified in pigs in Europe [38] and in Asia [26]. These porcine VP7 genes are similar to human G2 viruses, although they cluster clearly in a separate mono-phyletic branch.

When the NSP4 gene sequence was analysed, the Irish strains 48/07/Ire and 61/07/Ire were placed separately from rotaviruses of the

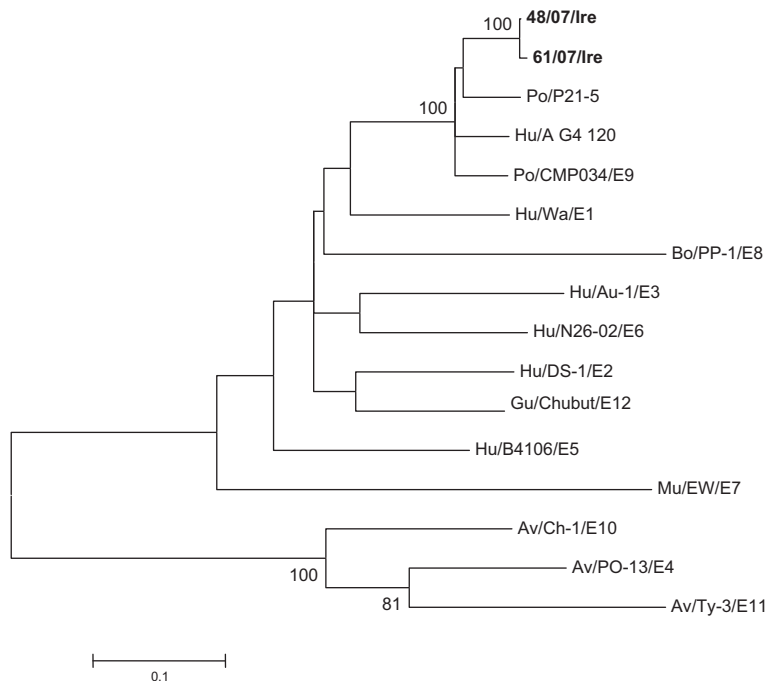


Figure 3. Neighbor-joining phylogenetic tree (729 bp) based on the NSP4 reference strains, E1–E12. The scale bar represents 0.1 substitutions per site.

E1 genotype, which encompasses the majority of porcine viruses. Interestingly, the Irish strains 48/07/Ire and 61/07/Ire clustered with a small group of viruses, including the unusual human strain A_G4_120 and the porcine strains P21-5 and CMP034, within the E9 genotype. Phylogenetic analyses of the VP6 gene revealed that strain 61/07/Ire resembled porcine strains of genotype I5. Conversely, strain 48/07/Ire clustered with genotype I1 viruses. Therefore, 61/07/Ire possessed a different VP6 gene, suggesting a genetic reassortment. Since different strains were found to co-circulate at the same time in each of the examined herd (three strains in farm 1, two in farm 2 and seven in farm 3). Reassortment is frequent among rotaviruses and, along with positive accumulation of single point mutations, inter-segmental recombination, rearrangement, and inter-species transmission, contributes to their evolution [10, 42].

In recent years, rotavirus surveillance has intensified throughout the world, monitoring

the appearance of novel antigenic types and providing evidence for a significant genetic/antigenic diversity of GARV. Together with data presented in this study, at least 32 rotavirus P genotypes and several novel lineages within the epidemiologically relevant human VP4 genotypes (e.g. P[8] and P[6]) have been recognized, some of which presumably account for novel antigenic P types or subtypes [3, 4, 32, 33, 37, 46, 49, 51, 52].

Along with cattle, pigs are regarded as important reservoirs for human rotavirus diversification, and there is evidence that heterologous rotaviruses of porcine origin or porcine-human reassortants may have occurred and spread successfully throughout human populations in a few occasions in Latin America [43, 56], South East Asia [62, 64] and Europe [4, 39]. Human GARV with unusual G-types such as G5, G6, G8, G9, G10, G11 and G12 associated with P-types P[3], P[6], P[9], P[11], P[14] and P[25] have been detected

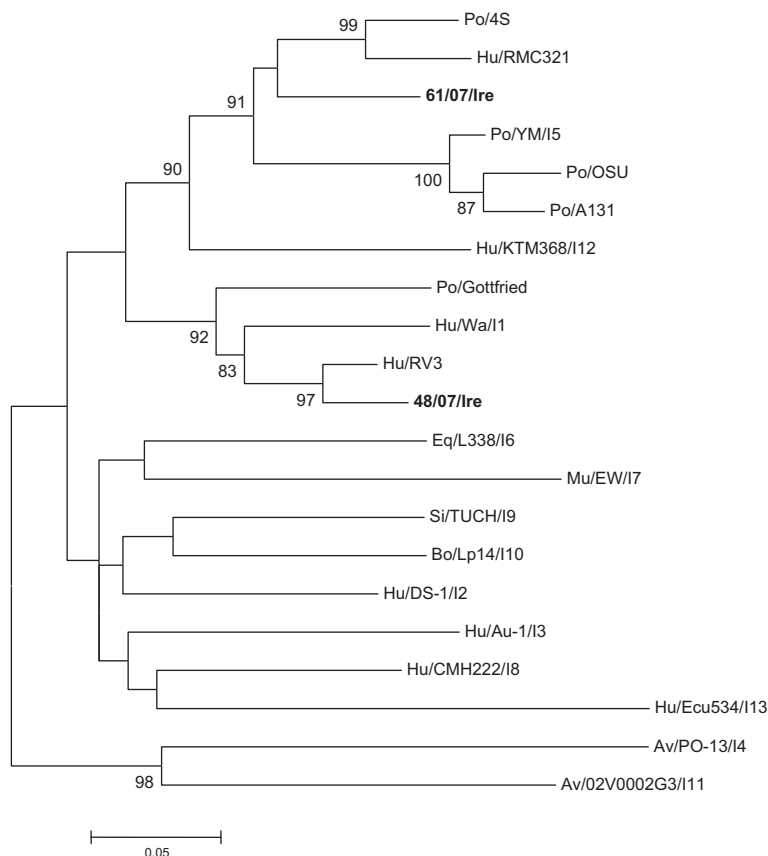


Figure 4. Neighbor-joining phylogenetic tree (379 bp) based on the VP6 reference strains, I1–I13. The scale bar represents 0.05 substitutions per site.

[51, 57]. Therefore, mapping the precise distribution of rotavirus G and P-types in the various animal species is important to provide a baseline to readily identify the source of atypical GARV infections in humans. Also, it is important to develop more effective tools for the prophylaxis of rotaviral diarrhea in animals. A better understanding of rotavirus epidemiology will help understanding the global ecology and the evolution of rotaviruses.

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