High genetic diversity of species A rotaviruses detected in swine farms in Chile

Hernán Cañon Jones,1,2,* Hernan Cortes,3 Aldo Gaggero,4 Jorge Levican,5 Mario Castillo-Ruiz,1 Trinidad Schlotterbeck1,8 and Ricardo San Martín6

Abstract

Rotavirus A is one of the main causative agents of diarrhoea in lactating and weaned pigs worldwide. Its impact in the swine industry is well documented. However, in Chile, the current epidemiological status of rotavirus on porcine farms is unknown. This study evaluated the current epidemiologic status of rotavirus A infection in Chile using on-farm detection techniques, electrophoretic confirmation, genotyping and phylogenetic clustering by analysis of partial sequences of VP4 and VP7 genes. Rotavirus A was detected in four out of five farms with an overall prevalence of 17.7% in diarrhoeic pigs. The average age of diarrhoea onset was at 32±6.2 days, corresponding to weaning pigs, and rotavirus was not detected in lactating piglets. Molecular characterization indicated that genotypes G5, G3, P[7] and P[13] are currently the most widely represented on these pigs farms. The phylogenetic analysis showed that farms shared similar G types (VP7), which might denote a common origin. Meanwhile, [P] types (VP4) showed considerable genetic diversity, and this might represent a high rate of reassortment of this genetic segment in rotavirus circulating in the researched area. These findings demonstrate the importance of considering both the geographical and production factors to accurately determine rotavirus prevalence status at the national level, and have relevant implications in determining effective strategies for rotavirus infection control on porcine farms.

INTRODUCTION

Species A rotavirus is one of the main causative agents of diarrhoea in lactating and weaned pigs [1]. The virus belongs to the genus Rotavirus within the family Reoviridae. It is a non-enveloped, icosahedral, triple-layered capsid containing the 11 segments of its dsRNA genome [2, 3]. Based on the antigenic properties and sequence diversity of VP6, nine species or groups (A–I) of rotavirus have been proposed [4, 5]. Rotavirus from species A is the most common in pigs and other mammalian species, including cattle and humans [4, 6–11].

The two structural outer capsid proteins, VP4 and VP7, define P (protease-sensitive) and G (glycoprotein) genotypes, respectively. The nucleotide sequence analysis of these genes is the most used technique for genotyping and has revealed valuable information in understanding the epidemiological aspects of rotaviral infections in swine [7, 10, 12–16].

Several different G and P genotypes have been found in cows [6] and children [7] in Ireland using current and novel reverse transcriptase PCR (RT-PCR) and genotyping techniques. Indeed, it has been possible to identify prevalent genotypes in specific geographical locations, which has contributed to producing more effective vaccines for piglets and sows [7, 10, 16, 17]. A molecular epidemiologic meta-analysis demonstrated G4, G5, P[6], P[7] and P[13] as the most prevalent genotypes in pigs worldwide, and extensive genetic diversity of rotaviruses exists depending on the geographical area [10]. Currently, an important aim of understanding the epidemiological molecular aspects of animal rotaviruses is the potential of cross-species infection based on the experimental and clinical evidence demonstrating the presence of animal rotavirus genotypes in human infections [18–23]. Recommendations have been established to obtain detailed molecular epidemiological data in order to elucidate the potential zoonotic implications of cross-species infections [8, 9], and also to establish adequate programmes for animal and human rotavirus infection surveillance [24]. The impact of rotavirus infections in the porcine industry is well documented, these causing high
morbidity and mortality and predisposing to colonization by highly pathogenic bacteria such as *Escherichia coli* O149, *Campylobacter* and *Salmonella* sp. [11, 25].

The worldwide prevalence of diarrhoea in pigs caused by rotavirus A is estimated to be about 15 % [1], but there are considerable differences depending on the geographical context and the detection methods used. For instance, using an ELISA, rotavirus A infections were detected independently in 9 % of diarrhoeic weaned piglets in New Zealand and USA [26, 27]. More recently, using molecular detection techniques, a prevalence of 63 % of rotavirus A infections was reported in Canada [12], while 34 % prevalence was reported in the UK [25]. In Latin America, rotavirus A has been detected in lactating and weaned pigs (up to 45 days old) with 3.3, 29 and 71.5 % prevalence in Argentina, Peru and Brazil, respectively [22, 28, 29].

In Chile, studies found rotavirus A prevalence of 14 and 23 % in lactating pigs [30, 31]. However, these reports have relevant geographical limitations since they are confined to the central zone of Chile and may not represent the actual epidemiological distribution in a region with a wide diversity of climate. Therefore, the current epidemiological status of rotavirus A on porcine farms in the climatologically diverse zones of Chile is unknown.

It is important to note that there are no approved porcine rotavirus vaccines currently available in Chile. Thus, any protection given by sows may be by their natural exposure to the virus.

Accordingly, the objective of the present study was to evaluate the current epidemiological status of rotavirus infection over a wide geographical area of Chile using on-farm detection, electrophoretic confirmation, genotyping and phylogenetic clustering by analysis of the partial sequences of VP4 and VP7 genes.

**RESULTS**

Using immunochromatographic on-farm rapid test kits initially, rotavirus was detected on four out of five porcine farms (80 %), with an overall prevalence of 17.7 % of diarrhoeic pigs (Table 1). Then, 29 positive samples were analysed using electrophoresis, PCR and genotyping techniques. Average age of diarrhoea onset was at 32±6.18 days, corresponding to 10 days from weaning. Clinically, diarrhoeic animals were mildly lethargic and anorexic, presenting liquid faeces that were predominantly khaki green (46 %), brown yellow (34 %), brown (15 %) or yellow (5 %) in colour.

The presence of rotavirus was confirmed by electrophoresis in 100 % of the samples positive according to immunochromatography.

Twenty-nine positive samples for rotavirus were selected and subjected to genotyping by nucleotide sequence analysis. Amplification with sequencing primers for VP4 and VP7 showed bands of the expected size in 24 cases for VP7 and 20 cases for VP4. The remainder were assigned to the category of non-typables (NTs) (Fig. 1a, b and Table 2).

**Table 1.** Prevalence of rotavirus A in diarrhoeic pigs from commercial farms in Chile

<table>
<thead>
<tr>
<th>Farm</th>
<th>Company</th>
<th>Positive (%), n</th>
<th>Negative (%), n</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>12 %, 12</td>
<td>88 %, 92</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>15 %, 40</td>
<td>85 %, 223</td>
</tr>
<tr>
<td>C</td>
<td>2</td>
<td>0 %, 0</td>
<td>100 %, 53</td>
</tr>
<tr>
<td>D</td>
<td>3</td>
<td>10 %, 5</td>
<td>90 %, 45</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
<td>93 %, 28</td>
<td>7 %, 2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>17.7 %, 85</td>
<td>82.3 %, 415</td>
</tr>
</tbody>
</table>

Identity matrix analysis of VP7 sequences gave a general divergence of 11 % (range 0–29.5 %). This high divergence was reflected in two clear genotypes, G5 and G3, as shown in Fig. 3 and Table S1 (available in the online Supplementary Material). The genetic divergence found within each genotype was low (0.5 %). Phylogenetic tree analysis by the maximum likelihood method (Fig. 2) also indicated that the strains belonging to genotype G5 segregated into two independent clusters. The first cluster (cluster 1) grouped strains that had almost the same sequence (strains 2, 3, 5, 8, 11, 12, 13, 14, 21, 22 and 23, bootstrap 85 %). The second cluster (cluster 2) consisted of strains A, AD and F (bootstrap 96 %), with a level of divergence 10 times higher than in cluster 1 (0.005 cluster 1 versus 0.05 cluster 2). Notably, clusters 1 and 2 were composed of strains obtained from farms 500 km apart; cluster 1 strains were from the southern region (E, purple squares in Fig. 2), while cluster 2 strains were sampled in the central-north region (farms A and D, green and blue squares in Fig. 2). The genetic divergence between these two clusters was 5 %.

On the other hand, two strains (E and G) were assigned
unambiguously to genotype G3 (bootstrap 97 %), and the divergence between these was only 0.2 %. These two G3 strains showed high identity with strain D86271 described in China and Japan in 1996 (bootstrap 97 %).

Analysis of VP4 sequences revealed an overall divergence of 28.2 % (range 0.4–53.7 %). This high divergence confirmed the presence of three genotypes: P[7], P[13] and P[19] (Fig 3. and Table S2). The genetic divergence found within each genotype was not uniform and was much higher in genotype P[13] than in genotype P[7] (i.e. 24 versus 3.7 %, respectively). This last feature can be observed on the phylogenetic tree comparing the strains obtained in this study to those available in GenBank using the neighbour-joining method (Fig 3.). This analysis indicates that the samples grouped in genotype P[7] are closely related, forming a single cluster (91 % bootstrap). Only one sample escaped this group (sample G), and the evolutionary divergence analysis showed a 9 % divergence regarding other P[7] strains.

Finally, the combined analysis of VP4 and VP7 regarding farm of origin showed the following: while all the VP7 sequences from farm E shared a high identity, possibly reflecting a common origin (G5 cluster 1), purple squares in Fig 2.), the VP4 sequences showed considerable diversity reflected in three P types, P[7], P[13] and P[19]. In this context, strain 22, the VP7 sequence of which grouped with the dominant G5 type (cluster 1), carried a VP4 sequence that clustered with P[19], suggesting acquisition of a different VP4 genomic segment in a stable VP7 (G5) background (compare KT906396 in the VP7 tree to KT906387 in the VP4 tree).

### DISCUSSION

The prevalence of rotavirus A in diarrhoeic pigs from commercial porcine farms detected in this study is in agreement with that reported by other studies conducted in Chile decades ago: Rivas [30], Reinhardt et al. [33] and Berrios et al. [31] showed prevalences of 14, 11 and 23 %, respectively. However,
Fig. 2. Phylogenetic tree based on the VP7 nucleotide sequence of porcine rotavirus A strains. Note: A 864 nt fragment corresponding to a partial VP7 coding region was analysed. The evolutionary history was inferred using the maximum likelihood method based on the Kimura two-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The analysis involved 46 nt sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5. Chilean porcine strains from the present study are denoted by coloured squares.
Fig. 3. Phylogenetic tree based on the VP4 nucleotide sequence of porcine rotavirus A strains. Note: A 834 nt fragment corresponding to a partial VP4 coding region was analysed. The evolutionary history was inferred using the neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Kimura two-parameter method. The analysis involved 45 nt sequences. Evolutionary analyses were conducted in MEGA5. Chilean porcine rotavirus A strains from the present study are denoted by coloured squares.
our result reflects notable differences regarding the type of population where rotavirus was detected. In the present study, 0–40-day-old pigs with acute diarrhoea were sampled, and rotavirus was found only in post-weaning pigs, while the three studies above detected rotavirus only in lactating pig populations. Rotavirus infection has been associated with highly stressful events such as weaning or feeding changes, both of which occurred a few days prior to the onset of acute diarrhoea [26, 34]. Age of presentation was later in this study than in previous reports, and rotavirus was not detected in lactating piglets. This divergence can be partially explained by the changing nature of the rotaviral infection over time, and may be associated with changes in production procedures such as biosecurity protocols.

Interestingly, our results showed highly divergent prevalence levels among farms, which may be explained by climatic variation within the geographic zones where the samples were taken. For example, there was no rotavirus present in acute diarrhoeic pigs in farm D located in the central, and warmer, zone of Chile (18.3°C annual mean), while farm E, located further south and in a colder zone of Chile (14.0°C annual mean), had 93% rotavirus prevalence [35].

These findings demonstrated the importance of considering geographical and production factors to accurately determine rotavirus prevalence status at the national level, as has been suggested by several studies in veterinary epidemiology [36, 37]. It is known that rotavirus infections are more prevalent in colder and wet weather [2] and that stressful production procedures such as weaning time and food changes during weaning can trigger rotaviral infections [11]. Clinical signs of diarrhea and disease were in accordance with previous studies in post-weaning rotaviral infections in Japan [38, 39].

Rotavirus is highly variable at the genetic level. To date, 32 G and 47 P genotypes with different combinations have been described for rotavirus A found in many host species [40, 41]. In addition, various studies have shown that porcine rotavirus A strains exhibit G genotypes G2–G5, G9 and G11 and P genotypes P[6], P[7], P[19] and P[23] [10, 40, 42, 43]. In concordance, the genotypes most frequently detected in this study were G5 and P[7] (75.86 and 51.72%, respectively), which in combination totalled 46.43% of all samples analysed.

Comparative phylogenetic analysis of VP7 sequences regarding representative GenBank reference sequences indicated that the most prevalent strain, G5, clustered with KP057833, EF672588, L79916 and DQ515961 strains from Kenya (2012), Brazil (2007), Brazil (1986) and Thailand (2006) [44–47], respectively, confirming a wide temporal and geographical distribution of this genotype (bootstrap 73%).

VP4 analysis showed that strains grouped in the P[7] genotype are closely related, with the only exception being G strain, which formed a unique cluster (bootstrap 91%) separated from other reference P[7] strains.

Strains grouping in the cluster P[13], E, M, 24 and 25, had wider variability and are closely related to different reference strains. Strain E is highly related to the cluster formed by strains JN974810 (Canada, 2006), KJ135198 (UK, 2011) and HM149308 (Ireland, 2005) [25, 42, 48]. Strain M grouped with strain HM149306 (Ireland, 2007) [42]. Strains 24 and 25 clustered with the strain AB573650 (Japan, 2006) [49].

Genotype P[19] has formerly been described as porcine rotavirus, and few reports of detection among animals and humans exist since the first isolation and characterization in 1994 [50–53]. Consistent with RotaC analysis, in which strain 22 was classified as P[19] with a low grade of confidence, in the phylogenetic analysis, this strain is grouped within cluster P[19] but is located in a separate branch from the GenBank reference sequences and might represent a new variant. Regarding the combination of G and P genotypes of this strain, while the former (G5) was closely related to that of other strains on the same farm (E), its P type was highly divergent from the dominant genotype and might represent independent segregation of the genetic segments, suggesting reassortment regarding locally co-circulating rotavirus strains [54–56]. To elucidate this phenomenon further, it will be necessary to conduct long-term surveillance.

Results showed that farms shared many genetically similar rotaviruses, specially regarding G genotypes, which might denote a common origin independent of the location as has been demonstrated in other studies on rotavirus [39]. On the other hand, the results showed a high heterogeneity among [P] types, suggesting reassortment of genetic segments. True recombination of segment fragments of the rotavirus genome has also been described as a mechanism for generating the diversity of genotypes in nature, but was not observed in this study [57, 58].

Future efforts are necessary towards conducting full genetic profiling to elucidate the presence of a new strain circulating among farms at the national level, and to determine the biological impact as an emerging rotavirus strain. The results presented here demonstrate that rotavirus is currently present in Chile, mainly in post-weaned pigs, and that circulating genotypes are both common and potentially novel. From an animal production point of view, the information obtained from this study reinforces our knowledge regarding the prevention and control of rotavirus infections, which can translate into better strategies of prevention and control of infection in young pigs. Currently, most preventive methods are based on exposure of viral antigens in the form of specific vaccines of the circulating strains [59]. However, in Chile, there are no rotavirus vaccines available or registered for pigs, suggesting that protection against rotavirus in sows is limited to passive immunization during lactation. The genetic diversity found in this study reinforces the need for new preventive approaches focusing, for example, on blocking viral attachment at the host cell membrane by thiazolides [60] or natural compounds such as saponins [61–63]. In addition, this study showed the importance of constant monitoring of known and emerging rotavirus strains in order to achieve effective immunological responses to currently used vaccines.
In conclusion, the correct identification of circulating genotypes across time and geographically diverse zones could translate into a greater impact in decreasing rotavirus presence at the farm and national levels, thereby improving the overall health and welfare of animals and economic prospects for producers.

**METHODS**

**Study design and sample collection**

Five hundred stool samples for rotavirus detection were collected between July 2013 and July 2014 from five different commercial porcine farms in Chile, between Santiago (33° 27′ 24′′ S 70° 38′ 53′′ W) and Concepción (36° 36′ 23′′ S 72° 06′ 12′′ W). This area represents more than 90 % of all intensive swine production in Chile [64]. Large swine farms (ranging from 2000 to 6000 reproductive sows) located at least 50 km apart from each other and representing the major Chilean pig-producing companies were selected. Anal or rectal diarrhoea samples were obtained using cotton swabs from 0- to 40-day-old pigs with acute diarrhoea (no greater than 1 day since onset). Diarrhoeic pigs were initially detected visually as those having a distended abdomen with a hyperaemic, eroded or swollen anal region. The selected pigs were lifted, and gentle abdominal pressure was applied to stimulate defeation and confirm the presence of diarrhoea. If the latter occurred, a sterile cotton swab was introduced anally to collect a sample consisting of either loose epithelium or diarrhoea. Samples were then transferred to a sterile sample plastic pouch for further analysis. The age of the animal(s), colour and consistency of the diarrhoea and any other clinical symptoms were recorded.

**Virus detection and identification**

**Immunochromatographic detection**

Initial detection of rotavirus was carried out on-farm using an immunochromatographic kit (Porcine Rotavirus Ag Rapid Test, Cat. N° DTS 464, Creative Diagnostics), following the manufacturer’s instructions. The kit has reported as having >95 % accuracy and precision for the detection of pig rotavirus in faeces. In order to validate the on-farm immunochromatographic kit, 30 random positive and negative samples were subjected to electrophoretic analysis. False positives and false negatives were within acceptable levels (<5 % for both), confirming a satisfactory performance of the on-site kits to be used for the detection of rotavirus.

**Electrophore confirmation**

All samples detected as positive by immunochromatography were analysed using classical PAGE to detect the rotavirus genome as previously described [65].

**Molecular characterization**

Twenty-nine stool samples positive for rotavirus were subjected to molecular characterization by RT-PCR according to previously described [66] and nucleotide sequence analysis of the partial coding region of VP4 and VP7 (P and G types, respectively). The samples were selected from the most geographically distant farms.

**Viral RNA extraction**

A 10 % (w/v) stool suspension was prepared in 500 µl sterile PBS and centrifuged for 5 min at 5000 g for 5 min for clarification. An aliquot of 250 µl of the supernatant was mixed with digestion buffer (100 mM Tris/HCl, pH 7.5, 12 mM EDTA, 150 mM NaCl, 1 % SDS) and 10 µl K proteinase and incubated for 1 h at 65 °C. Digestion products were extracted using TRIzol (Life Technologies) following the manufacturer’s instructions. The extracted RNA was resuspended in 20 µl of PCR-grade water and stored at −80 °C prior to use.

**cDNA synthesis**

Reverse transcription was carried out using 2 µg extracted RNA, 200 U M-MLV reverse transcriptase (Promega), 25 U recombinant RNasin (Promega) and random hexamers (Promega) following the manufacturer’s instructions. cDNA was obtained in a final volume of 25 µl and kept at −20 °C until analysis.

**Amplification of partial coding region of VP4 and VP7 by PCR**

The primers Con2/Con3 [66] were used to amplify the VP4 gene, and the VP7 gene was amplified with consensus primers 9con1 and 9con2 [67], as previously described. The amplification reaction was carried out in a final volume of 100 µl. The reaction mixture included 1 x Paq5000 reaction buffer (2 mM MgCl₂ final), 0.4 mM of each dNTP, 0.6 µM of each primer, 5 µ of DNA polymerase Paq5000 (Agilent Technologies) and 5 µl of cDNA template. The reaction was carried out using a 2729 thermal cycler (Applied Biosystems) with the following cycling program: 95 °C for 2 min, 35 cycles at 94 °C for 30 s, 42 °C for 30 s and 72 °C for 1 min followed by an extension phase at 72 °C for 7 min. The amplified product was resolved in 1.5 % agarose gel in TAE buffer (40 mM Tris/acetate, 1 mM EDTA) containing GelRed stain (Biotrin) and visualized using an UV transilluminator.

**Purification of PCR products**

The corresponding rotavirus electrophoretic bands were excised from the gel and purified using an E.Z.N.A gel extraction kit (Omega, BioTek) following the manufacturer’s instructions. Quality control and alignment of the sequences were performed using BioEdit software.

**Sequence analysis**

Initially, identity was crosschecked using BLAST (NCBI), and rotavirus identified sequences were transformed to FASTA and analysed using RotaC 2.0 for automatic genotyping of group A rotavirus (http://rotac.regatools.be/) [32]. The cut-off value of identity for assignment to a genotype was 80 % for both VP4 and VP7.

**Phylogenetic analysis**

Phylogenetic analysis was carried out by comparison to previously reported strains in GenBank using MEGA software [68].
Evolutionary distance was calculated using the Kimura two-parameter method. The construction of the phylogenetic tree was performed using neighbour-joining and maximum-likelihood algorithms. The robustness of phylogenetic associations was confirmed using the bootstrap of 1000 replicates.

**Epidemiological and statistical analyses**

Prevalence of rotavirus was obtained at both the local (farm) and global (overall) level. Descriptive statistical analyses was confirmed using the bootstrap of 1000 replicates.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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