



Short communication

First report of porcine respirovirus 1 in South America

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ABSTRACT

Porcine respirovirus 1 (PRV1) is an emerging virus in pigs that has been previously described in the USA and China. There are no reports of its presence in the rest of the world. The objective of this study was to determine the occurrence of PRV1 in Chile and to determine its phylogeny. Thus, we collected samples (oral fluids, nasal swabs, and lungs) from a swine influenza A virus (IAV) surveillance program, most of which belonged to pigs with respiratory disease. The samples were analyzed by RT-PCR, and the viral sequencing was obtained using RNA whole-genome sequencing approach. Maximum likelihood phylogeny was constructed with the available references. Thirty-one of 164 samples (18.9 %) were RT-PCR positive for PRV1: 62.5 % oral fluids, 19.0 % nasal swabs, and 8.6 % lungs. All 6 farms in this study had at least one positive sample, with 6–40 % of positive results per farm, which suggests that PRV1 is disseminated in Chilean swine farms. Twenty-one of 31 (677%) PRV1-positive samples were also positive for IAV, so the role of PRV1 as secondary pathogen in respiratory disease needs to be further evaluated. Near to complete genome of two PRV1s were obtained from two farms. The phylogenies, in general, showed low bootstrap support, except the concatenated genome and the L gene trees which showed clustering of the Chilean PRV1 with Asian sequences, suggesting a close genetic relationship. This is the first report of PRV1 in the Southern Hemisphere. Further studies are necessary to determine the genetic diversity of this virus in Chile.

1. Introduction

Genus *Respirovirus* belongs to the family *Paramyxoviridae*, order *Mononegavirales*, and including 6 viral species: *Bovine respirovirus 3*, *Caprine respirovirus 3*, *Human respirovirus 1*, *Human respirovirus 3*, *Murine respirovirus*, and *Porcine respirovirus 1* (PRV1) (Amarasinghe et al., 2018, 2019). Historically, these viruses have been related to respiratory infections in human and domestic animals (Li et al., 2016; Maclachlan et al., 2017; Park et al., 2019).

PRV1, also known as porcine parainfluenza virus 1 (PPV-1), was first described in 2013 in nasopharyngeal and rectal swab samples of deceased pigs collected from a slaughterhouse in Hong Kong (Lau et al., 2013). Although its pathogenicity is uncertain, this emerging virus has been associated with respiratory disease in pigs, evidenced by clinical signs that may include coughing, minor sneezing, and serous nasal discharge (Palinski et al., 2016). PRV1 has also been found in co-

infections with porcine reproductive and respiratory syndrome virus (PRRSV) and influenza A virus (IAV), suggesting that it may participate in the porcine respiratory disease complex (PRDC), increasing the severity of respiratory disease (Welch et al., 2017). Although PRV1 is genetically related to *Human respirovirus 1* (Lau et al., 2013), its zoonotic potential is still unknown.

PRV1 has a single-stranded, negative-sense linear RNA genome of ~15 kb in length, which encodes 6 major proteins: nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large (L) protein (Palinski et al., 2016; Park et al., 2017, 2019). The F and HN are the major envelope glycoproteins on the viral surface, which are crucial for receptor binding, entry, and fusion between the viral envelope and the host cell membrane. They are responsible for inducing the production of neutralizing antibodies (Henrickson, 2003), so they are subject to humoral immune pressure with the consequent genetic variability. Therefore, phylogenetic

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analysis of the F and HN genes may be useful to carry out molecular epidemiological studies of this virus (Park et al., 2019).

PRV1 has been detected in several states of the United States of America (USA), widespread in commercial swine farms (Palinski et al., 2016; Park et al., 2019). To date, PRV1 has only been described in the USA and China, and only 9 whole or near-complete genomes are available in public repositories (Park et al., 2019). Little is known about the epidemiology and distribution of PRV1 in the rest of the world. In this study, we report the first detection of PRV1 in South America and its genetic comparison with other previously described porcine respiroviruses.

2. Materials and methods

One hundred and sixty-four samples were obtained from 6 swine production companies, located in 5 administrative regions of Chile (Valparaíso, Metropolitan, O'Higgins, Maule, and Ñuble), where > 80 % of the Chilean intensive swine production is located. Samples were collected between 2015 and 2019 during a swine influenza surveillance program, including nasal swabs (121), oral fluids (8) and lungs (35). In general, the samples were collected in grower pigs between 3 to 11-weeks of age, specifically from pens where clinical symptoms of respiratory disease including sneezing and coughing was observed. The details of the sample collection are provided in Table 1 and supplemental information Table 1.

The PRV1 was detected by a conventional RT-PCR for specific amplification of PRV1, which was inspired by a RT-PCR with degenerated primers to amplify paramyxoviruses (van Boheemen et al., 2012; Neira et al., 2017). Thus, we designed the forward PRV1 F 5'-GAR GGT TAT TGT CAR AAA CTT TGG AC-3' and reverse PRV1 F 5'-TCA CRG CAA TTG CTT GGT TRT CR CC-3' primers, which amplified a 120 bp fragment (nt positions 10734–10854). Briefly, RNA was purified using Chomczynski-phenol solution (Winkler, BM-1755, Chile) and the RT-PCR was then carried out using the AgPath-ID™ One-Step RT-PCR Reagents kit (Life Technologies, 4387391, USA), following the manufacturer's instructions. The RT-PCR master mix contained 12.5 µL of 2X RT-PCR Buffer, 1 µL of RT-PCR Enzyme Mix, 0.4 µM of each primer, and nuclease-free water until complete 20 µL. Five µL of template RNA (or nuclease free-water for a negative control) were added to complete a final reaction volume of 25 µL. RT-PCR was initiated at 45 °C for 10 min (reverse transcription), followed by 95 °C for 10 min (initial denaturation) and 40 cycles of 2-step cycling comprising denaturation at 95 °C for 15 s and annealing-extension at 60 °C for 45 s. The PCR products were run in 2% agarose gel at 120 V for 30 min.

Eleven positive RT-PCR samples were selected to attempt viral isolation in African green monkey kidney (Vero) cells (ATCC® CCL-81™, USA). This is a previously used cell line to grow other parainfluenza viruses, including respiroviruses (Henrickson, 2003; Eberle et al., 2016). Briefly, cells were grown in minimum essential medium (MEM) with Earle's salts and L-glutamine (Corning, 10–010-CMR, USA)

supplemented with 10 % fetal bovine serum (GE Healthcare Life Sciences, SH30071.03, USA) and 1% antibiotic-antimycotic solution (Biological Industries, 03,033–1B, Israel). The inoculum was incubated for 1 h at 37 °C and 5% CO₂. Then, the inoculum was removed, and MEM supplemented with 1% antibiotic-antimycotic solution and 1 µg/mL of trypsin treated with N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) was added. Cells were incubated at 37 °C and observed for cytopathic effect (CPE) daily for 5 days. Complementarily, the supernatant was tested by the previously described RT-PCR to confirm the presence of PRV1, after 2 passages.

A selection of 8 samples with strong PCR amplification was submitted for RNA sequencing at Icahn School of Medicine at Mount Sinai, NY, USA, and Veterinary Diagnostic Lab, College of Veterinary Medicine, University of Minnesota. Library preparation was performed using the Illumina TruSeq kit, after depletion of ribosomal RNAs with the Ribo-Zero™ Magnetic Gold Kit Human/Mouse/Rat (Illumina). Following paired-end (2 × 100 nt) sequencing on the Illumina HiSeq platform, raw reads were trimmed for quality using Trimmomatic (Bolger et al., 2014). A fasta file containing all complete or near-complete genomes (> 14k bp long) of respiroviruses (n = 641) was obtained from GenBank and used as a reference to extract all reads mapped to these sequences using the Burrows-Wheeler Aligner (BWA-MEM) and Samtools (Li and Durbin, 2009). The mapped reads were then assembled into contigs using SPAdes (Nurk et al., 2013). The contigs were aligned to an annotated reference (JX857409) and visualized using Icarus (Mikheenko et al., 2016). The contigs were further queried in BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine their identity. The closest reference sequence (accession number JX857409) was used to create a final reference assembly with BWA-MEM and to create the final consensus sequences using Samtools mpileup. The consensus sequences were annotated using GATU (Tcherepanov et al., 2006). Phylogenetic analyzes were performed using the complete concatenated genome and all genes independently, using both nucleotide and amino acid sequences. All PRV1 reference sequences available in NCBI GenBank were used to construct the phylogeny (<https://www.ncbi.nlm.nih.gov/genbank/>). An outgroup for each protein-coding region phylogeny was obtained by querying the study sequence in BLASTX, and the closest *Human respirovirus 1* sequence was selected. The sequences were aligned using MUSCLE v3.8.3 (Edgar, 2004). The phylogenetic trees were constructed by the maximum likelihood method using IQ-TREE with substitution model selection (ModelFinder implemented in IQ-TREE) option and 1000 bootstrap (Nguyen et al., 2014). Also, phylogenetic analysis using Bayesian inference in mrBayes 3.2.7 was performed to support the ML analyzes (Ronquist et al., 2012).

This study was performed at Animal Virology Laboratory, Department of Animal Preventive Medicine, Faculty of Veterinary and Animal Sciences, University of Chile. The protocols for animal sampling were approved by the Ethical Scientific Committee for Animals and Environment Care from the University of Chile, certificate number

Table 1

Overall results of RT-PCR of Porcine Respirovirus 1 (PRV1) and Influenza A (IAV) in samples of 6 industrial swine farms of Chile.

Region	Farm	Sample	Year	Total Samples	RT-PCR PRV1 positive	RT-PCR IAV positive	PRV1 & IAV positive
Maule	Farm 1	Nasal Swab	2018	5	3 (60 %)	1 (20 %)	1 (20 %)
Maule	Farm 1	Oral Fluid	2018	4	3 (75 %)	4 (100 %)	3 (75 %)
Maule	Farm 1	Nasal Swab	2015	66	9 (14 %)	52 (79 %)	8 (12 %)
Maule	Farm 1	Oral Fluid	2015	4	2 (50 %)	2 (50 %)	2 (50 %)
Ñuble	Farm 2	Nasal Swab	2019	23	6 (26 %)	4 (17 %)	2 (9%)
O'Higgins	Farm 3	Nasal Swab	2019	8	2 (25 %)	6 (75 %)	2 (25 %)
O'Higgins	Farm 3	Lung	2019	4	1 (25 %)	3 (75 %)	1 (25 %)
Santiago	Farm 4	Nasal Swab	2019	4	0 (0%)	3 (75 %)	0 (0%)
Santiago	Farm 4	Lung	2019	31	2 (6%)	17 (55 %)	1 (3%)
Valparaíso	Farm 5	Nasal Swab	2019	5	2 (40 %)	2 (40 %)	1 (20 %)
O'Higgins	Farm 6	Nasal Swab	2019	10	1 (10 %)	4 (40 %)	0 (0%)
Total				164	31 (18.9%)	98 (59.8%)	21(12.8%)

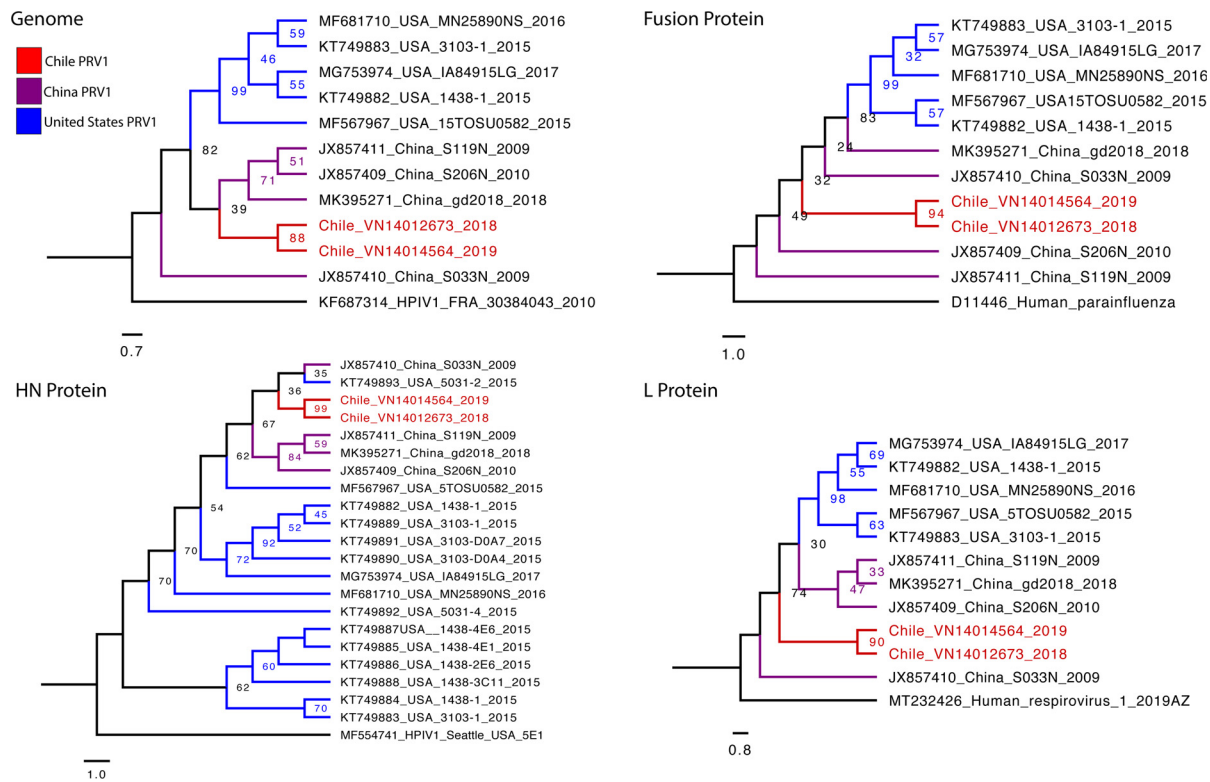


Fig. 1. Phylogenetic analysis of amino acid sequences of PRV1. Phylogenetic trees were constructed using the sequences obtained in this study and all PRV1 sequences available in GenBank. *Human respirovirus 1* was used as outgroup. The phylogenetic trees were inferred using maximum likelihood method in IQ-TREE based on the model FLU + G4 for the genome, fusion and HN protein and JTT + G4 for L protein with 1000 bootstrap replicates. Bootstrap values are indicated at each node. The Chilean strain is represented in red, purple for Chinese and blue for the USA strains. GenBank accession numbers for each sequence are given next to strain name (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

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3. Results and discussion

Thirty-one of 164 total samples (18.9 %) were RT-PCR positive for PRV1. The overall diagnostic results are included in Table 1 and supplemental information Table 1. From all the six evaluated farms at least one positive sample was obtained, and the within-farm percentage of positive samples ranged between 6 and 40 %. These results suggest that PRV1 is disseminated in Chilean swine farms, similar to the results found in the USA (Palinski et al., 2016). However, more studies with a higher number of samples and farms are needed to confirm the widespread of PRV1 in Chile. The virus was detected in all types of samples analyzed in this study: oral fluids (5/8, 62.5 %), nasal swabs (23/121, 19.0 %), and lungs (3/35, 8.6 %). This is consistent with a previous study that also detected a higher percentage of positive samples in oral fluids and nasal swabs than in the lungs (Park et al., 2019). Palinski et al. (2016) showed that PRV1 replicates in epithelial cells of the upper respiratory tract, resulting in nasal shedding (Palinski et al., 2016). Therefore, oral fluids and nasal swabs are likely to be the best samples for detecting the virus.

Twenty-one of 31 (677%) PRV1-positive samples were also positive for IAV, by RT-PCR. No clinical symptoms have been associated directly with PRV1 during pathogenesis studies, raising the possibility that PRV1 infection is asymptomatic in the absence of additional factors, such as coinfections (Palinski et al., 2016). PRV1 has been previously detected in coinfections with IAV and PRRSV (Welch et al., 2017), although its relevance in the PRDC has not yet been evaluated. Due to the high percentage of samples positive for both PRV1 and IAV in this study, the role of PRV1 as a secondary pathogen in respiratory disease warrants further evaluation.

We were not able to isolate PRV1 in Vero cells. The supernatant was

positive for PRV1 by RT-PCR in the first passage but became negative in the second passage. Previous studies described the difficulty of isolating PRV1 (Palinski et al., 2016). Recently, PRV1 was successfully isolated in LLC-MK2 cells (ATCC® CCL-7™) (Park et al., 2019). This cell line should be used in further studies to isolate Chilean PRV1 but is not yet available in the country.

We obtained next-generation sequencing reads of PRV1 in 7 of 8 samples. However, near-complete genomes were obtained only from 2 samples (Chile/VN14012673/2018 and Chile/VN14014564/2019) (Accession numbers MT497920 - MT497921), which were used for phylogeny. The final consensus sequences for both samples obtained by reference assembly represents ~97 % of the PRV1 genome. The Chile/VN14012673/2018 sample was obtained from 1066 reads and assembled in 2 contigs with coverage of $7 \times$. On the other hand, the Chile/VN14014564/2019 sample was obtained from 843 reads and assembled in 5 contigs with coverage of $8 \times$. Even though some gaps were observed due to partial sequencing, the genomic organization is typical of viruses belonging to the family *Paramyxoviridae* containing all representative genes (N, P, M, F, HN, and L). For the Chile/VN14012673/2018 sample, the M, F, and HN genes were completely sequenced, and N (91.2%), P (97.2%), and L (99.8%) genes were partially sequenced. Nucleotide positions for the partial genes cover are detailed in supplemental information Table 2. For the Chile/VN14014564/2019 sample, the N, P, and M genes were completely sequenced, and F (86.8%), HN (94.7%), and L (95 %) were partially sequenced. The two near-complete genomes were obtained from samples belonged to 2 different farms with no geographical or epidemiological relationship. On the other hand, no other paramyxoviruses were obtained from the samples but reads related to Porcine Astrovirus, Porcine circovirus, and Suid Herpesvirus 2 were commonly found in all samples analyzed.

The Chilean PRV1s showed > 95 % nucleotide identity with PRV1

strains detected in China (GenBank accession numbers: JX857409.1 - JX857411.1). Blastn results are included in supplemental information. We used all PRV1 available sequences for the phylogeny. For the genome, N, P, M, F and L genes the trees were constructed with 9 PRV1 reference sequences, while the phylogeny of the HN gene was constructed with 19 PRV1 reference sequences. An outgroup with a *Human respirovirus 1* sequence was always included. The details of the substitution models used are described in Fig. 1 and supplemental information Figure 2. In general, the Chilean PRV1s were grouped into a sub-clade among Chinese PRV1 sequences, and the North American PRV1 sequences were grouped into another monophyletic cluster. The trees did not have high bootstrap values to determine a clear relationship of Chilean viruses to Asian or North American viruses (Fig. 1, Supplemental information Figure 2). However, the complete genome and the L gene trees had the highest bootstrap support, and the topology of the amino acid and nucleotide trees were more consistent and similar to the Bayesian analysis. The topology of these trees supports the close relationship between Chilean and Chinese PRV1 sequences and the presence of a North American monophyletic cluster. Overall, these results suggest that Chilean PRV1s are genetically related to strains from the USA and China, but, due to the limited information available, their origin cannot be determined.

4. Conclusion

This is the first report of PRV1 in the Southern Hemisphere. To date, PRV1 has only been reported in China and the USA. The results suggest that PRV1 is a ubiquitous virus in Chilean swine farms and that it is highly geographically distributed. It should be noted that the whole-genome sequencing was attempted in 8 samples and two PRV1 near to complete genome were obtained. Therefore, using only two sequenced strains, it was not possible to determine the genetic diversity of PRV1 in Chile. However, the results confirm the genetic relationship between Chilean and other PRV1s identified in China and the USA. Further studies are necessary to obtain the complete genome of Chilean PRV1 strains, determine the genetic diversity of this virus in the country, and its implication and importance in swine respiratory disease.

Data availability statement

All the data that support this study are available upon request.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.vetmic.2020.108726>.

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