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RAPID COMMUNICATION

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First report of porcine parainfluenza virus 1 (species Porcine respirovirus 1) in Europe

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Abstract

Porcine respirovirus 1, also known as Porcine parainfluenza virus 1 (PPIV-1) was first identified in Hong Kong in 2013, later in the USA and most recently in Chile. Here, we report the first detection of PPIV-1 outside these three regions. We screened 22 farms in Hungary by testing 15 nasal swab samples obtained from 3-week-old piglets (3 randomly chosen piglets from 5 litters in each farm). Only one farm was found to be positive. We subsequently sampled the positive farm by taking cross-sectional 20 nasal swab samples from 2-, 4-, 6- and 8-week-old piglets. Virus detection by qRT-PCR showed that although all investigated age groups were positive to PPIV-1, a higher number of infected animals and higher viral loads were found among 4-week-old animals. Based on the phylogenetic analyses of partial F and L genes, the 3 Hungarian strains are genetically closely related to the very first PPIV-1 strain identified in Hong Kong in 2013, whereas the overall genetic difference compared to the recently described North American isolates was around 10%.

KEYWORDS

genome sequencing, phylogenetic analysis, porcine parainfluenza virus 1, respirovirus

1 | INTRODUCTION

The family *Paramyxoviridae* consists of viruses that are known to affect a wide range of species including humans, pigs, cattle, poultry and companion animals. Porcine parainfluenza virus type 1 (PPIV-1, species *Porcine respirovirus 1*) was first detected in rectal and nasopharyngeal swabs obtained from pigs in a slaughterhouse in Hong Kong (Lau et al., 2013). Upon full genome sequence analysis, Lau et al. proposed the classification of this newly discovered virus as a novel paramyxovirus. PPIV-1 has a negative sense, single-stranded RNA genome approximately 15 kilobases in length consisting of six genes (3′-N-P-M-F-HN-L-5′); these genes encode for major proteins: nucleocapsid, phosphoprotein, matrix, fusion, haemagglutinin–neuraminidase and large proteins, respectively (Henrickson, 2003; Lau et al., 2013; Park et al., 2017, 2019). The haemagglutinin–neuraminidase (HN) protein is responsible for the attachment of the virus to target cells via sialic acid receptors, suggesting a neuraminidase activity which is important for virus release from cells (Henrickson, 2003). The fusion (F) protein, however, directly mediates the fusion of the cell membrane and the viral envelope (Morrison, 2003).

Full genome sequence analyses have revealed that the closest genetic relatives of PPIV-1 are the human parainfluenza virus 1 (HPIV-1), and the Sendai virus (SeV) of mice and other rodents (Palinski et al., 2016), both of which can cause respiratory disease in their host species (Mostafa et al., 2016). Challenge trials in the United States

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revealed that inoculation of conventional, and CD/CD piglets with PPIV-1 showed no mortality and minimal morbidity despite significant viral replication (Welch et al., 2018). In a recent study, the same research group found a significant reduction in viral genome copies in BALF, tracheal swab and turbinate samples in PPIV-1-challenged animals that were previously vaccinated with an RNA particle vaccine (Welch et al., 2020).

Recent reports from Chile substantiate the presence of PPIV-1 as well. The ubiquitous presence of PPIV-1 has been proposed by Agüero et al. after finding at least one positive sample in each of the screened 6 farms, which altogether provide > 80% of the Chilean intensive swine production (Agüero et al., 2020).

Paramyxoviruses have a tendency for interspecies transmission (e.g. Hendra and Nipah virus), and almost all paramyxovirus genera (*Rubulavirus*, *Respirovirus*, Morbillivirus) include relevant human pathogens. Every newly discovered species in these genera is worth close investigation (Thibault et al., 2017).

As the presence of PPIV-1 has never been reported outside Hong Kong, the United States and Chile, the aim of our work was to screen large-scale pig herds in Hungary for the presence of this virus.

2 | MATERIALS AND METHODS

2.1 | Sample collection

We analysed nasal swab samples obtained from 3-week-old (21 ± 3 days), due-to-wean piglets (3 randomly chosen piglets from 5 randomly chosen litters) originating from 22, farrow-to-finish farms. Most of these farms were located in different regions of Hungary, 7 of them belong to the same company (without live animal movement between the farms). Participation in the sampling was voluntary upon a call to screen Hungarian herds for the presence of a neuroinvasive astrovirus (Boros et al., 2017). These samples (altogether 330) were further analysed for the presence of PPIV-1.

For the cross-sectional study in the single positive herd, 20–20 nasal swab samples were obtained from randomly chosen 2-week-old suckling, 4-, 6- and 8-week-old weaned piglets regardless of their health status.

The study and all the sampling were conducted in compliance with the provisions of Directive 2010/63/EU, Hungarian Act XXVIII/1998 and the Hungarian Ministerial Decree No. 40/2013. (II.14.).

2.2 | Detection and phylogenetic analyses of PPIV-1 in clinical samples

The nasal swabs were vortexed in PBS, and RNA was isolated in a QIAcube automatic instrument using QIAmp cador Pathogen Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Endpoint PCR for the first round detection of respiroviruses was performed by amplifying a 555-bp fragment of the L gene using degenerate primers (5′-GACTCATCTACTAACGGNTAYGARA-3′ and 5′-CACAAACATCTTGCTACTWATDATNGT-3′) described in Lau et al. (2013). We chose a more general primer set in the first round of testing to increase the spectrum of detection. The reactions were performed using a One-Step RT-PCR Kit (Qiagen) in a Genesy 96T gradient PCR machine (Tianlong, China). Positive control RNA obtained from a PPIV-1 isolate was kindly provided by Phillip C. Gauger (Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University).

Real-time quantitative RT-PCR to detect the L gene of PPIV-1 and subsequent melting point analysis was performed in a Rotor-Gene Q instrument (Qiagen) using a QuantiNova SYBR Green RT-PCR Kit (Qiagen), with specific primers (5′-TACAATATATGTGGGTGATCCTTACT-3′ and 5′-GCCTGAATCTTCATGATCTTCTAAA-3′) as described previously in Lau et al., 2013. Pairwise comparison of the Cq values obtained in the different age groups was performed and plotted in R-environment, using the Wilcoxon rank-sum exact test (R Core Team, 2020).

The L gene product used for diagnostic PCR and a partial F gene segment was sequenced, the latter using primers and conditions described in Park et al., 2019. Briefly, for the F gene an internal and an outer primer pair (F-For: 5′-ACTTAGGGTACAAGTTATCCAAAAAA-3′, F-Rev: 5′-TCGTGCACCCTAAGTTTTCTTTA-3′, F-For: int 1 5′-GAGAGAAGAGCTTAACATTACAGGC-3′, F-Rev: int 1 5′-TCATAAATATCTGTYTTCCCGAGATT-3′) was used with the One-Step RT-PCR Kit (Qiagen).

The PCR products were subjected to gel electrophoresis, and amplicons with suitable length were cut out of the gel and purified by the Qiagen Gel Extraction Kit (Qiagen). Sanger sequencing was performed with a BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Ljubljana, Slovenia) with the corresponding primers in both directions; the capillary electrophoresis was carried out by a commercial provider (Hungarian Natural History Museum).

The obtained forward and reverse sequences were proofread, compared to each other and assembled using E-INS-i method of the online software mafft version 7 (Katoh & Toh, 2008) and aligned against available PPIV-1 genomes, and reference respirovirus sequences downloaded from the GenBank. Maximum-likelihood (ML) analyses were conducted using MEGA version X (Kumar et al., 2018). The GTR+ I nucleotide substitution model was used for the partitions with ML estimation of base frequencies, and an ML bootstrap analysis with 1,000 replicates was used to test the support for the branches.

3 | RESULTS AND DISCUSSION

The initial respirovirus screening of the 22 herds revealed the presence of two positive samples in the case of one herd. To gain insight

into the herd infection dynamics, we performed cross-sectional nasal swab sampling directly from the positive herd: 20–20 samples were obtained from 2-, 4-, 6- and 8-week-old piglets. The samples were analysed by a PPIV-1-specific real-time quantitative RT-PCR. The results of the cross-sectional PCR investigations are shown in Figure 1. Out of the 20 samples obtained from the 4-week-old age group, we found 13 positives, 8 in the 6-week-old group and 5 in the 8-week-old group. Only 1 positive sample was found among the 2-week-old animals. Numerically, the highest viral burden was observed among the 4-week-old animals, where the mean Cq value was 28.39 (±5.09), but by Wilcoxon rank-sum exact test there were no significant ($p < .05$) differences in the pairwise comparisons (Figure 1).

Melting curve analysis was conducted after every real-time run. Surprisingly, there was a 1.5–2.5°C difference between the melting point of the positive control (76°C) and the positive samples (73.5–74.5°C), suggesting multiple nucleotide differences between the two amplicons (Figure S1).

After sequencing three positive samples (GenBank accession numbers: MT765271–3) with low Cq values, initial BLAST analysis of the partial L and F gene sequences confirmed the presence of PPIV-1 in our samples. The sequences obtained were almost identical to each other with a less than 5 nucleotide difference (4 in the F gene, 1 in the L gene) between each other (more than 99% similarity). Maximum-likelihood trees constructed with a 1,636 nucleotide long part of the F gene and the 555 nucleotide long part of the L gene revealed almost identical topology (Figure 2). When grouped with other PPIV-1 sequences, our sequences were supported by relative high bootstrap values. Pairwise similarities were calculated with every available PPIV-1 sequence. The highest values were found in the case of two sequences originating from Hong Kong submitted by Lau et al.: 95.6% (acc. no.: JX857410) and 92.2% (acc. no.: JX857409) (Lau et al., 2013). All other PPIV-1 sequences, including the recently submitted ones from the USA, were found to be less than 92% similar.

Interestingly, the partial F and L sequences obtained from our samples showed the highest similarity values to the very first PPIV-1 genomes submitted from Hong Kong in 2013, whereas the overall genetic difference compared to the recently described North American isolates was around 10%. The genetic difference was already apparent during the real-time PCR investigations where the melting curve analyses revealed a 2.5°C difference between our samples and the positive control originating from the USA. The pairwise alignment of the amplicons' sequences revealed 6 mismatches, 5 of which resulted in smaller GC content in our samples, explaining the decreased melting point.

To our knowledge, this is the first report of porcine parainfluenza virus 1 in Europe. The cross-sectional sampling revealed significant virus circulation in young animals. Both the number of positive animals and the viral copy amounts were highest among 4-week-old weaned piglets. Elder animals had fewer positive samples and lower viral amounts. However, the lowest viral load was found in the suckling age group which suggests that PPIV-1 circulates mostly among weaned piglets, spreading rapidly in the nursery units. This distribution is supported by previously published data from the Veterinary Diagnostic Laboratory of Iowa State University where 37.2% of all the PPIV-1-positive cases found originated from nursery units (Gauger et al., 2018).

Among the 22 herds screened, we only found a single one (4.5%) infected with the virus. Our results show a notably lower infection rate as compared to the USA where among the 842 porcine samples submitted to the Iowa State University Veterinary Diagnostic Laboratory during 2016–2017, 43.3% were positive for PPIV-1 by qPCR; these USA results suggest that PPIV-1 may be common in US swine, especially in animals with respiratory pathologies (Park et al., 2019). On the other hand, when lung homogenate, oral fluid or nasal swab samples of unknown infection status were tested, only 6.1% was qPCR positive for PPIV-1 (Palinski et al., 2016). Though the latter value seems comparable to our results, there were major differences in sample selection that do not

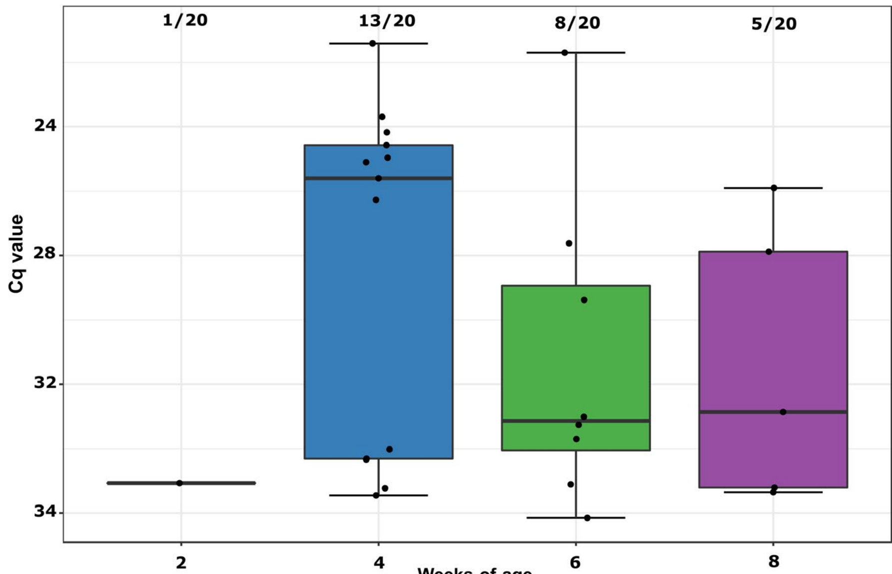

FIGURE 1 Boxplots of qPCR Cq values of the different age groups. Each sample is represented by a dot with horizontal jitter for visibility. The horizontal box lines represent the first quartile, the median and the third quartile. Whiskers denote the range of points within the first quartile—1.5 × the interquartile range and the third quartile + 1.5 × the interquartile range. The numbers above the boxes indicate the number of positives among the 20 sampled animals [Colour figure can be viewed at wileyonlinelibrary.com]

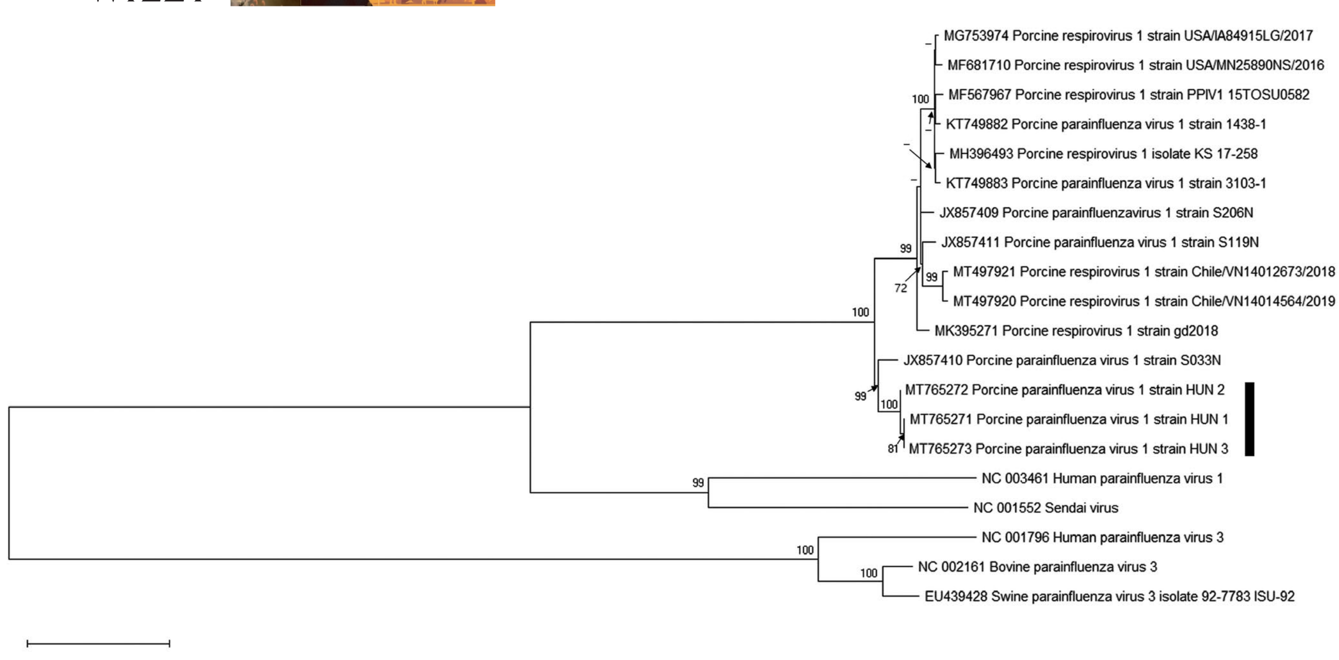

FIGURE 2 Phylogenetic tree displaying relatedness of the Fusion polyprotein coding gene of representative respirovirus and PPIV-1 sequences from GenBank and Hungarian strains (marked with a black rectangle). ML bootstrap support values (≥70) are shown as percentages above branches. The scale bar indicates 0.20 expected changes per site per branch. Strains displayed on the phylogenetic tree are coded as the accession number/name of strain

allow for direct comparison. Park et al. analysed respiratory or oral fluid samples that were already submitted to a diagnostic laboratory which suggests a pre-existing, most likely respiratory condition, whereas our samples were obtained randomly, regardless of the health status of the pigs.

In a recently published study from Chile, 31 out of 164 (18.9%) (oral fluid, nasal swab and lung) samples were PCR positive for PPIV-1. Interestingly, the authors confirmed the presence of the virus in every farm they tested. Their samples were collected during a swine influenza surveillance programme from animals showing respiratory symptoms. Even though the results are again hardly comparable due to the different sampling strategies, these differences in results suggest that the prevalence of PPIV-1 in Hungary is far less than in Chile, and probably less than in the USA.

Summarizing our findings in light of these results, even though the challenge studies failed to confirm any significant disease development upon PPIV-1 infection, the fact that the percentage of positive samples is generally higher in the ones obtained from respiratory pathologies might suggest that the virus belongs to a myriad of pathogens involved in the porcine respiratory disease complex.

A limitation of our study is the relatively small number of farms screened with a rather small sample size from each site. In order to obtain more relevant information regarding the actual prevalence of PPIV-1 infection in Hungary, we plan to conduct widespread, systematic and representative oral fluid testing in our country along with virus isolation and full genome sequence determination.

We could not successfully identify the source of infection in the positive herd, as the farm is breeding its own replacement gilts, the semen is obtained from a high health status boar stud in Hungary, and they had no prior contact to personnel coming from either USA, Hong Kong or Chile.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest regarding the research, authorship and/or publication of this article.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The study was conducted in compliance with the provisions of Directive 2010/63/EU, Hungarian Act XXVIII/1998 and the Hungarian Ministerial Decree No. 40/2013. (II.14.).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/genbank/.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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