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Full genome sequence analysis of a wild, non-MLV-related type 2 Hungarian PRRSV variant isolated in Europe

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is a widespread pathogen of pigs causing significant economic losses to the swine industry. The expanding diversity of PRRSV strains makes the diagnosis, control and eradication of the disease more and more difficult. In the present study, the authors report the full genome sequencing of a type 2 PRRSV strain isolated from piglet carcasses in Hungary. Next generation sequencing was used to determine the complete genome sequence of the isolate (PRRSV-2/Hungary/102/2012). Recombination analysis performed with the available full-length genome sequences showed no evidence of such event with other known PRRSV. Unique deletions and an insertion were found in the nsp2 region of PRRSV-2/Hungary/102/2012 when it was compared to the highly virulent VR2332 and JXA-1 prototype strains. The majority of amino acid alterations in GP4 and GP5 of the virus were in the known antigenic regions suggesting an important role for immunological pressure in PRRSV-2/Hungary/102/2012 evolution. Phylogenetic analysis revealed that it belongs to lineage 1 or 2 of type 2 PRRSV. Considering the lack of related PRRSV in Europe, except for a partial sequence from Slovakia, the ancestor of PRRSV-2/Hungary/102/2012 was most probably transported from North-America. It is the first documented type 2 PRRSV isolated in Europe that is not related to the Ingelvac MLV.

1. Introduction

Porcine reproductive and respiratory syndrome emerged at the same time in Europe (early 1990s, Wensvoort et al., 1991) and North America (late 1980s, Keffaber, 1989), and since then, the virus (porcine reproductive and respiratory syndrome virus, PRRSV) has rapidly spread throughout the world, and became endemic in almost every major swine producing country.

PRRSV is a member of the Arteriviridae family within the order Nidovirales (Cavanagh, 1997; Faaberg et al., 2012). It has a positive-sense single stranded RNA genome of 15 kb in length that encodes 10 open reading frames (Snijder and Meulenberg, 1998; Firth et al., 2011; Johnson et al., 2011). Comparative nucleotide sequence analyses revealed that PRRSV strains can be classified into two distinct genotypes: type 1 (formerly named as European) and type 2 (formerly named as North American). Remarkably, the two genotypes have only 50–60% nucleotide identity (Allende et al., 1999).

A comprehensive phylogenetic study of the North American type 2 PRRSV strains has recently been published that was based on the Bayesian analysis of 8624 ORF5 sequences (Shi et al., 2010b). Based on their results, the authors defined 9 monophyletic lineages within this genotype and established a set of reference sequences representing the principal diversity of type 2 sequences.

Type 2 strains were first introduced to Europe in 1996 by the use of a modified live virus (MLV) vaccine in Denmark (Bøtner et al., 1997). Soon after its introduction into the population, the MLV strain, a cell culture adapted variant of the type 2 prototype VR2332 strain, spread horizontally and vertically among pigs and herds

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as well, and showed multiple genetic mutations (Nielsen et al., 2001). The vaccine is currently registered in Germany, Poland, The Netherlands, Belgium, Denmark, Spain and Lithuania, and under special import agreements in Slovakia. According to latest results, confirmed by full genome sequence analyses in Denmark, the vast majority of the type 2 strains found in Europe are genetically related (>95% ORF5 nucleotide identity) to the aforementioned vaccine (Kvisgaard et al., 2013a). A more recent study involving numerous type 2 ORF5 sequences from throughout Europe revealed a small group of sequences that are 91–94% similar to the Ingelvac MLV, and cannot unequivocally be attributed to the vaccine (Stadejek et al., 2014).

The aim of our study was to characterize a member of the third group of European type 2 sequences, that are 88% or less similar to the Ingelvac strain on ORF5, confirming the wild type nature of these strains. These sequences were first identified in 2005 in multiple sites of a swine breeding company with mild clinical signs of PRRS (Balka et al., 2008).

2. Materials and methods

2.1. Origin of the isolate

In 2012 lung tissue and lymph node samples were obtained from the carcass of a young growing pig originating from an endemically PRRS positive herd, where our previous investigations verified the presence of type 2 PRRSV (Balka et al., 2008). No signs of an acute outbreak were present. Only mild to moderate respiratory symptoms were observed among the young fatteners. No significant reproductive disorders were reported at the time of sampling.

2.2. Cells and viruses

Porcine alveolar macrophages (PAMs) obtained from PRRSV-free piglets were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Sigma–Aldrich, Saint Louis, MO, USA) at 37 °C and 5% CO_2. To culture PRRSV-2/Hungary/102/2012, approximately 0.5 g pieces of lung tissue were taken from dead pigs. The lung and tracheobronchial lymph node samples originating from the endemically infected farm were homogenized with Tissue Lyser (Qiagen, Hilden, Germany) in sterile phosphate-buffered saline (PBS) containing antibiotics and antimycotics, to obtain a 50% (w/v) suspension. After complete homogenization, the samples were centrifuged at 5000 × *g* for 10 min to remove cellular debris. Cell-free supernatants were frozen at −80 °C for RNA isolation. PAMs were inoculated with 100 μl of supernatant and incubated for five days. Besides the periodic examination of the cell cultures for the presence of cytopathic effects, real-time RT-PCR analysis (Balka et al., 2009) was also applied on the supernatants to confirm the growth of the virus. Cell-free supernatants were stored at −80 °C for RNA isolation.

2.3. RNA isolation and cDNA synthesis

RNA was isolated with QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) from the original tissue homogenates and PAM cell culture supernatants. In order to improve the quantity and quality of the template RNA, next generation sequencing has been performed on inoculated PAM cell culture supernatants of the first passage after confirming viral growth by real-time PCR. cDNA was generated using Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) kit and a T_{20} primer, according to the protocol of the manufacturer.

Table 1
Oligonucleotide primers used for RT-PCR amplification of PRRSV-2/Hungary/102/2012. Primers with the same numbers at the third letter position were used as primer pairs.

Primer	Sequence	Position
PR_USA_1F	ATGACGTATAGGTGTTGGCTCTATG	1–25
PR_USA_3358R	CAAGCTTAGTCGCATCACATGCCTC	3334–3358
PR_USA_3248F	ACTCAGCTCAAGCCATCATCGACTC	3248–3272
PR_USA_6709R	CAGAGAACACTCCATCGCCAACAAG	6685–6709
PR_USA_6408F	GTCTGCGCAAGTTCTGATGATCAGG	6408–6432
PR_USA_9230R	ATACAGCACGAGGTCGTCCGAATAG	9206–9230
PR_USA_9018F	GTGACTAAGAGAGGTGGCCTGTCGT	9018–9042
PR_USA_12972R	GGAATCCTAGCTCGTCATGATCGTC	12,948–12,972
PR_USA_12827F	CTTCGAGCTCACGGTGAATTACACG	12,827–12,851
Pr15_USA_15397R	GGTTCTCGCCAATTAAATCTCACCC	15,373–15,397

2.4. Overlapping PCR products for next generation sequencing

The genome of PRRSV-2/Hungary/102/2012 was amplified in five overlapping parts (similarly to Kvisgaard et al., 2013b) using the Phusion II HotStart PCR kit (Thermo Scientific, Waltham, MA, USA), in 25 μl final volume with 1 μl of cDNA template, in 1× GC buffer in the presence of 4% DMSO. Amplification was performed using the following gradient PCR program: 98 °C 1′, 35 × [98 °C 20″, 54–72 °C (gradient ramp: 2 °C) 20″, 72 °C 5′], 72 °C 5′. The primer pairs used for the amplification of the different fragments are listed in Table 1. Primers were designed using Primer3Plus (Untergasser et al., 2007). PCR fragments were purified from agarose gel slices by the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). For determination of both ends of the genome, the 5′ RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen, Carlsbad, CA, USA) as well as a forward ORF7 primer (Balka et al., 2008) and the T_{20} primer were applied.

2.5. Next generation sequencing (NGS)

An equimolar mixture of the overlapping PRRSV PCR products was used as template for next generation sequencing. In brief, a DNA library was prepared using the NEBNext® Fast DNA Fragmentation & Library Prep Set for Ion Torrent (New England Biolabs, Beverly, MA, USA) with the Ion Torrent Xpress barcode adapters (Life Technologies, Carlsbad, CA, USA) according to the protocol recommended by the manufacturers. The emulsion PCR and subsequent template enrichment were carried out with the Ion OneTouch™ Template Kit on a OneTouch v1 instrument and an Ion OneTouch™ ES pipetting robot, respectively. Sequencing was carried out on a 316 chip using the Ion Torrent semiconductor sequencing equipment (Ion Personal Genome Machine® (PGMTM); Life Technologies). Sequences were assembled and aligned with SeqMan Ngen software (Lasergene, Madison, WI, USA).

2.6. Phylogenetic analysis

PRRSV whole genome sequences in GenBank, including 16 type 1 and 199 type 2 field, vaccine and laboratory strains, were obtained from the continents of Europe, Asia, and North America ($n = 215$ [suppl. Table 2a]). PRRSV whole genome alignment was done with the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm (Edgar, 2004) in Geneious Pro 6.1.7 using default settings. The evolutionary history was inferred using the maximum likelihood method based on the Tamura–Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (−266,940) is shown. Initial trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 215