Characterization of a circulating PRRSV strain by means of random PCR cloning and full genome sequencing

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Abstract
PRRS is a pig disease of major economic importance that causes respiratory and reproductive problems in pigs. Over the last years it has become clear that PRRSV heterogeneity is increasing. Consequently, this has a potential impact on diagnosis and strategies to counter this disease. The use of sequence-independent PCR techniques for the detection and characterization of PRRSV could be useful to bypass problems associated with the heterogeneity of this virus.

A random PCR cloning approach was tested for the characterization of PRRSV strain 07V063 of unknown genetic background that circulated on a Belgian farm. By using this approach, 7305 bp of sequence data were obtained, distributed randomly across the genome. Using RT-PCR with strain-specific primers, the full length sequence (15014 nt) was obtained. Phylogenetic relationships using ORF5 and ORF1a (NSP2) sequences showed that 07V063 was classified in type 1 subtype 1 and that 07V063 was genetically different from prototype Lelystad Virus (LV). 07V063 showed 87-93% aa identity with LV ORFs coding for structural proteins. Most variation (compared to LV) was noticed in Nsp2 (81% identity) with a deletion of 28 aa. This deletion was different from other known deletions in this ORF. In conclusion, it is shown that this random PCR cloning approach can be used for the characterization of new PRRSV strains of unknown genetic background.

Findings
Porcine reproductive and respiratory syndrome (PRRS) is an economically important viral pig disease in swine producing countries worldwide. The virus can cause reproductive disorders and can give rise to respiratory problems in pigs of all ages [1]. Prevention of the disease is based on a combination of management and vaccination. Evidence is accumulating that PRRSV heterogeneity is affecting the vaccination efficiency. It is suggested that vaccines are only efficacious when the vaccine virus and the challenge virus share a sufficiently high homology [2-6]. PRRSV heterogeneity was originally considered mainly to occur between European (genotype 1) and American type (genotype 2) PRRSV, but current understanding shows a more complex situation with considerable genetic variability within genotypes [7-9]. Since such variability may affect the efficacy of vaccination programs and pose an obstacle for PRRSV prevention and control, knowledge on the PRRSV strains circulating on a farm may be essential for choosing an appropriate vaccine [10].

PRRSV diagnosis is mainly based on detection of PRRSV antibodies, Reverse Transcriptase (RT) PCR or virus isolation. Detection of antibodies by ELISA or IPMA is not sufficient to establish the level of PRRSV heterogeneity [11]. RT-PCR allows rapid detection and genotyping of PRRSV, but the high degree of sequence variation observed for PRRSV can influence results obtained by (real-time) RT-PCR and primers and/or probes should be carefully designed based on conserved regions [8,12]. The development of sequence-independent PCR techniques could be useful for the diagnosis and genotyping of unknown PRRSV isolates and for assessment of the PRRSV heterogeneity of field isolates. Several methods have been developed for the identification of viruses without prior sequence knowledge [13]. For instance, whole genome amplification and random PCR are relatively simple. In both these methods, viral
particles (from biological samples or cell culture) are treated with DNase and RNase to remove contaminating nucleic acids. RNA and/or DNA from the viral particles is extracted and RNA is reverse transcribed to cDNA using a primer with a random 3’ end. Subsequently, cDNA or viral DNA is amplified using a shorter primer (without the 3’ random end). This results in DNA fragments of varying size (e.g. 0.5 - 2 Kb) and these fragments can be cloned and sequenced. For instance Allander et al. [14] used random PCR on human respiratory tract samples which allowed identification of several unknown viruses.

The aim of this study was to test a random PCR cloning technique [14] for the detection and genotyping of a PRRSV strain of unknown genetic background.

Random PCR cloning for the identification of PRRSV 07V063

PRRSV 07V063 was isolated from an aborted foetus from a Belgian farm, by inoculation of porcine alveolar macrophages. On this farm, vaccination with Porcilis™ was in place. PRRS diagnosis was confirmed upon detection of cytopathic effect (CPE), and detection of PRRSV antigens by IPMA staining with the nucleocapsid specific mAb. PRRS diagnosis was confirmed upon detection of PRRSV antigens by IPMA staining with the nucleocapsid specific mAb

The viral pellet was treated with DNAseI and RNAse. MARC-145 cells and concentrated as described [16] and the viral pellet was treated with DNaseI and RNase. RNA was extracted using commercial kits and used in reverse transcription and random amplification using the tagged random hexanucleotide 5’-GCCGGAGCTCTG-CAGATATCNNNNNNN-3’ for both first- and second strand cDNA synthesis and subsequent amplification of the cDNA with primer 5’-GCCGGAGCTCTGCGAG-CATC-3’ [14]. Random PCR fragments ranging between 500 and 1200 bp were cloned in pCR-Blunt II-TOPO (Invitrogen). Twenty nine clones were sequenced as described [17]. Twenty three clones (80% of the clones) contained PRRS sequences (Table 1). The six other clones showed no match when performing BlastN [16]. Random PCR fragments ranging between 622 and 2072 bp) were obtained (Figure 1). Thus, without prior knowledge of the sequence it was possible to obtain 7305 bp sequence data using a random PCR cloning approach, hereby confirming PRRS identity.

Table 1 Overview of the sequences from 07V063 obtained by random PCR cloning

| Clone | Size (nt) | Position | % nt identity |
|-------|----------|----------|---------------|
| 49    | 671      | 774-1444 | 81            |
| 73    | 198      | 1692-1889| 89            |
| 104   | 826      | 1808-2633| 89            |
| 20    | 798      | 2616-3413| 86            |
| 105   | 375      | 3069-3443| 88            |
| 88    | 429      | 3420-3847| 93            |
| 33    | 332      | 3957-4288| 91            |
| 92    | 316      | 6198-6512| 93            |
| 61    | 713      | 6367-7079| 93            |
| 103   | 312      | 6768-7079| 94            |
| 35    | 364      | 6500-6863| 93            |
| 12    | 247      | 8132-8378| 89            |
| 51    | 627      | 8931-9557| 86            |
| 80    | 358      | 9200-9557| 87            |
| 82    | 622      | 11225-11846| 87          |
| 11    | 258      | 11225-11482| 86         |
| 81    | 601      | 11928-12528| 92         |
| 70    | 189      | 12336-12524| 94         |
| 40    | 277      | 12364-12640| 90         |
| 78    | 395      | 12991-13385| 90         |
| 57    | 935      | 13195-14129| 91         |

The position of the sequences is indicated relative to LV. % nt identity is with LV.

The full length genome sequence of 07V063 and comparison with prototype LV

To allow a more detailed evaluation of the PRRSV isolate 07V063, the full length genome sequence was obtained using primers that were based on the 07V063 sequences from the random PCR cloning approach (Table 2). Overlapping amplicons (spanning the complete genome) were obtained using RT-PCR. Both strands of these fragments were directly sequenced. For the amplification of the 3’ end oligodT was used in combination with ORF7fw. A 5’ end primer (5’endfw) was designed based on the alignment of genotype 1 strains LV (M96262), EuroPRRS (AY366525), SD01-08 (DQ489311), KNU-07 (FJ349261) and HKEU16 (EU076704). This primer was used in combination with primer Lavgsprev to amplify the 5’ end of LV with 92.3% identity and 17 nt dispersion over the genome. The contigs are shown with a line.

Figure 1 PRRSV genome and position of the contigs. The sequences obtained in the random PCR cloning approach were assembled in six contigs (with sizes between 622 and 2072 bp) dispersed over the genome. The contigs are shown with a line.

Table 1: 07V063 sequence information and in combination with small scale shotgun sequencing, this can result in viral sequences. Virus 07V063 was grown on MARC-145 cells and concentrated as described [16] and the viral pellet was treated with DNaseI and RNase. RNA was extracted using commercial kits and used in reverse transcription and random amplification using the tagged random hexanucleotide 5’-GCCGGAGCTCTG-CAGATATCNNNNNNN-3’ for both first- and second strand cDNA synthesis and subsequent amplification of the cDNA with primer 5’-GCCGGAGCTCTGCGAG-CATC-3’ [14]. Random PCR fragments ranging between 500 and 1200 bp were cloned in pCR-Blunt II-TOPO (Invitrogen). Twenty nine clones were sequenced as described [17]. Twenty three clones (80% of the clones) contained PRRS sequences (Table 1). The six other clones showed no match when performing BlastN.
differences. Several motifs such as the transcription regulatory sequence (UUAACC) and CACCC stretches (involved in binding of host cell transcription factors) are conserved in 07V063 [18]. Table 3 gives an overview of all ORFs in the 07V063 genome and comparison with ORFs from prototype LV. Most variation with LV was

### Table 2 Oligonucleotide primers used in RT-PCR amplification and nucleotide sequencing of 07V063

| Primer    | Sequence                  | Position |
|-----------|---------------------------|----------|
| S'endFW   | atgatgcaggggtttccccccc   | 1-22     |
| Orf1uniFW | cccctagaaccatgcctggc     | 111-130  |
| Orf1-1fw  | cattcccgttctgctgctttt    | 336-355  |
| Orf1-2fw  | gggagccacccacctgtgctt    | 681-701  |
| Lav49fw   | acaatgtgcaggggtttccccccc| 1072-1091|
| Orf1-3fw  | tcaatgacccgagttcccaggcc  | 1631-1650|
| Orf1-4fw  | ctggtagctgctgctgtttt     | 1988-2007|
| Orf1-5fw  | acaacgcaggggtttccccccc   | 2472-2490|
| Lav73fw   | tcaatgacccgagttcccaggcc  | 3012-3121|
| Orf1-6fw  | gttctctgctgctgctgtttt    | 3451-3469|
| Orf1-7fw  | cttgaccctgctgctgctgtttt  | 3686-3705|
| Lav33fw   | ggtctctgctgctgctgtttt    | 4129-4147|
| Orf1-8fw  | gttctctgctgctgctgtttt    | 4538-4557|
| Orf1-9fw  | tcttcggagtccctgctgctgtttt| 4859-4878|
| Orf1-10fw | gttctctgctgctgctgtttt    | 5364-5383|
| Orf1-11fw | gttctctgctgctgctgtttt    | 6819-6911|
| Orf1-12fw | gttctctgctgctgctgtttt    | 7001-6819|
| Orf1-13fw | gttctctgctgctgctgtttt    | 7280-7299|
| Orf1-14fw | gttctctgctgctgctgtttt    | 7765-7783|
| Orf1-15fw | gttctctgctgctgctgtttt    | 8172-8191|
| Orf1-16fw | gttctctgctgctgctgtttt    | 8442-8461|
| Lav51fw   | gttctctgctgctgctgtttt    | 8817-8835|
| Orf1-17fw | gttctctgctgctgctgtttt    | 9316-9335|
| Orf1-18fw | gttctctgctgctgctgtttt    | 9764-9783|
| Orf1-19fw | gttctctgctgctgctgtttt    | 10136-10155|
| Orf1-20fw | gttctctgctgctgctgtttt    | 10633-10652|
| Orf1-21fw | gttctctgctgctgctgtttt    | 11132-11150|
| Orf1-22fw | gttctctgctgctgctgtttt    | 11577-11598|
| Orf1-23fw | gttctctgctgctgctgtttt    | 12234-12253|
| Orf1-24fw | gttctctgctgctgctgtttt    | 12661-12280|
| Orf1-25fw | gttctctgctgctgctgtttt    | 12672-12691|
| Orf1-26fw | gttctctgctgctgctgtttt    | 13320-13337|
| Orf1-27fw | gttctctgctgctgctgtttt    | 13838-13856|
| Orf1-28fw | gttctctgctgctgctgtttt    | 14328-14345|
| Orf1-29fw | gttctctgctgctgctgtttt    | 14966-14986|

### Table 2 Oligonucleotide primers used in RT-PCR amplification and nucleotide sequencing of 07V063 (Continued)

| Primer    | Sequence                  | Position |
|-----------|---------------------------|----------|
| Orf1-30fw | gttctctgctgctgctgtttt    | 15686-15705|
| Orf1-31fw | gttctctgctgctgctgtttt    | 16254-16273|
| Orf1-32fw | gttctctgctgctgctgtttt    | 16817-16835|
| Orf1-33fw | gttctctgctgctgctgtttt    | 17382-17400|
| Orf1-34fw | gttctctgctgctgctgtttt    | 17950-17968|
| Orf1-35fw | gttctctgctgctgctgtttt    | 18516-18534|
| Orf1-36fw | gttctctgctgctgctgtttt    | 19082-19100|
| Orf1-37fw | gttctctgctgctgctgtttt    | 19658-19676|
| Orf1-38fw | gttctctgctgctgctgtttt    | 20214-20232|
| Orf1-39fw | gttctctgctgctgctgtttt    | 20780-20798|
| Orf1-40fw | gttctctgctgctgctgtttt    | 21346-21364|
| Orf1-41fw | gttctctgctgctgctgtttt    | 21902-21920|
| Orf1-42fw | gttctctgctgctgctgtttt    | 22468-22486|

### Table 3 Comparison of proteins from 07V063 and prototype LV

| ORF    | Protein | Size 07V063 | Size LV | % identity | % similarity |
|--------|---------|------------|---------|------------|--------------|
| 1a     | Nsp1    | 385        | 385     | 85         | 91           |
|        | Nsp2    | 833        | 861     | 81         | 85           |
|        | Nsp3    | 447        | 447     | 93         | 96           |
|        | Nsp4    | 203        | 203     | 92         | 96           |
|        | Nsp5    | 170        | 170     | 96         | 97           |
|        | Nsp6    | 16         | 16      | 100        | 100          |
|        | Nsp7    | 269        | 269     | 97         | 97           |
|        | Nsp8    | 45         | 45      | 100        | 100          |
| 1b     | Nsp9    | 645        | 645     | 98         | 98           |
|        | Nsp10   | 442        | 442     | 94         | 97           |
|        | Nsp11   | 224        | 224     | 95         | 97           |
|        | Nsp12   | 152        | 152     | 96         | 97           |
| 2a     | GP2     | 249        | 249     | 93         | 94           |
|        | E       | 70         | 70      | 95         | 97           |
| 2b     | GP3     | 265        | 265     | 89         | 92           |
|        | GP4     | 183        | 183     | 87         | 93           |
| 3      | N       | 173        | 173     | 93         | 94           |
noticed in Nsp1 (85% identity/91% similarity) and Nsp2 (81% identity/85% similarity). A major difference is a deletion of 28 aa in a variable region of Nsp2 (at positions 683-710). Similar deletions in this region are known e.g. EuroPRRS has a 17 aa deletion (Figure 2A; [18]). The deletion in Nsp2 in 07V63 could be a unique marker for this strain.

Strain 07V063 showed 87 - 95% aa identity with LV for the structural ORFs 2 - 7. We compared GP4 and GP5 proteins from 07V063 and LV since it has been shown that these proteins are the main target for neutralizing antibodies. Figure 2 shows an alignment of ORF4 proteins. Notably is the high variation in the region 50-70. It has been shown that a neutralizing epitope in LV (57-68) is underlined. Figure 2B shows an alignment of ORF5 proteins. A neutralizing epitope in North American strains (37-45) is underlined.

Figure 2 Alignment of Nsp2, ORF4 and ORF5 proteins from 07V063 with LV (ORF4 and ORF5) and a selection of genotype 1 strains (Nsp2). A. Alignment of Nsp2 proteins from genotype 1 strains. Only aa positions 636-755 (LV) are shown. The deletion in 07V063 is located at aa positions 683-710. B. Alignment of GP4 from 07V063 and LV (only the first 120 aa are shown). A neutralizing epitope in LV (57-68) is underlined. C. Alignment of GP5 from 07V063 and LV. A neutralizing epitope in North American strains (37-45) is underlined.
epitope is present in LV at positions 57-68 [19] and that this region is under antibody-mediated pressure in vitro and in vivo [20,21]. Pigs infected with 07V063 produce neutralizing antibodies against the 57RVTAAQGRIYTR68 epitope. However, these antibodies do not cross-protect against LV [22]. Similarly, antibodies against the same region in LV, do not cross-protect against 07V063. Interestingly, this lack of cross-neutralization is in agreement with the finding that strain 07V063 was able to replicate and cause disease on a farm where animals were vaccinated with the LV-like Porcilis™ vaccine.

GP5 has been described as the main target for virus-neutralizing antibodies in North American PRRSV strains. A neutralizing epitope has been identified at positions 37-45 [23]. Figure 2C shows that 07V063 and LV have an identical sequence from 37-45 with the exception of an extra glycosylation site at position 37 in 07V063. It has been shown that several strains are glycosylated at this position but the significance of this glycosylation is not known. Other amino acid changes occur throughout the sequence and several of these positions have been described as variable [24]. No other differences in glycosylation pattern of the structural proteins between 07V063 and LV was observed.

**Phylogenetic relationship of 07V063**

Since ORF5 is frequently used as a marker for the study of genetic relationships [8], we constructed phylogenetic trees using ORF5 sequences from a selection of genotype 1 strains (Table 4). In addition genotype 1 strains for which the full length sequence was available in Genbank were included. VR-2332 (genotype 2) was used as out-group.

Figure 3A shows a phylogenetic tree of ORF5 DNA sequences based on the Neighbour Joining (NJ) method. Several clusters are evident and supported by high bootstrap values. It can be concluded that 07V063 clusters within the pan-European subtype 1 [8]. Within subtype 1, a cluster with LV- and Olot/91-like strains can be distinguished. Although both LV and Olot/91 belong to the earliest PRRSV isolates, still LV and Olot/91-like strains such as SD01-08 are circulating. Strain 07V063 is genetically different from LV- and Olot/91-like strains. Apparently 07V063 clusters together with isolates from different geographical locations e.g. isolates from Spain (16/2000), Denmark (361-4), China (BIEU06-1) and South-Korea (IV3140) although this clustering is not supported by high bootstrap values. A similar tree topology was obtained using ORF5 protein sequences (data not shown). The sub-clustering of type 1 is complex and cannot always be explained by geographic isolation of the strains [8]. The sequence of 07V63 adds to the increase of genetic diversity of type 1 strains and is an example of continuous genetic drift within PRRSV [24].

**Table 4 Overview of strains used for phylogenetic analysis**

| Strain | Genotype | Genbank Accession ORF5 | Genbank Accession ORF1a (nsp2) |
|--------|----------|------------------------|-------------------------------|
| VR-2332 | 2 | U87392 | U87392 |
| Lelystad | 1 (subtype 1) | M96262 | M96262 |
| EuroPRRS | 1 | AY366525 | AY366525 |
| 01-CB1 | 1 (subtype 1) | DQ864705 | DQ864705 |
| Amervac | 1 (subtype 1) | GU067771 | GU067771 |
| HKEU16 | 1 (subtype 1) | EU076704 | EU076704 |
| KNU-07 | 1 (subtype 1) | FJ349261 | FJ349261 |
| SHE | 1 (subtype 1) | GQ461593 | GQ461593 |
| SD01-08 | 1 (subtype 1) | DQ489311 | DQ489311 |
| BIEU06-1 | 1 (subtype 1) | GUO47344 | GUO47344 |
| NMEU09-1 | 1 (subtype 1) | GUO47345 | GUO47345 |
| 07V063 | 1 (subtype 1) | GU737264 | GU737264 |
| PysVac | 1 (subtype 1) | DQ324681 | ND |
| Porcilis | 1 (subtype 1) | AAW78001 | ND |
| Olot/91 | 1 (subtype 1) | X92942 | ND |
| Yuz-34 | 1 (subtype 3) | DQ324692 | ND |
| Bel-42 | 1 (subtype 3) | DQ324669 | ND |
| Obu-1 | 1 (subtype 3) | DQ324671 | ND |
| Soz-6 | 1 (subtype 3) | DQ324686 | ND |
| Dzi-62 | 1 (subtype 1) | DQ324675 | ND |
| Cresa11 | 1 (subtype 1) | DQ009626 | ND |
| IV3140 | 1 (subtype 1) | DQ355821 | ND |
| 28639/98 | 1 (subtype 1) | AO35912 | ND |
| 361-4 | 1 (subtype 1) | AO35915 | ND |
| Sna-4 | 2 (subtype 2) | DQ324683 | ND |
| Sid | 2 (subtype 2) | DQ324682 | ND |
| Aus | 2 (subtype 2) | DQ324667 | ND |
| Okt-35 | 1 | DQ324677 | ND |
| 16/2000 | 1 | DQ345743 | ND |
| SO02-11 | 1 (subtype 1) | AO359078 | AY383634 |
| SO01-07 | 1 (subtype 1) | AO359079 | AY383632 |
| SO03-12 | 1 (subtype 1) | AO359074 | AY383635 |
| SO03-15 | 1 (subtype 1) | AO359076 | AY383636 |
| It-22 | 1 (subtype 1) | AYT39978 | ND |
| It-39 | 1 (subtype 1) | AYT39995 | ND |
| It-44 | 1 (subtype 1) | AYT40000 | ND |
| It-35 | 1 (subtype 1) | AYT39991 | ND |
| It-13 | 1 (subtype 1) | AYT39969 | ND |
| Lena | 3 (subtype 3) | EU909691 | ND |

The type of the strains is according to Stadejek et al (2008). ND = no data. VR-2332 is genotype 2. Eleven genotype 1 isolates for which full length sequences were obtained are listed first.

A recent PRRSV study in Spain [25] demonstrated that Spanish isolates from different years show continuous evolution and increase in heterogeneity and that different genotypes and variants within the genotypes co-circulate.
Also, phylogenetic trees using Nsp2 were constructed (Figure 3B). Sequences from all known full length genotype 1 strains (Table 4) were included. Essentially, the same topology can be observed as for ORF5. A cluster of LV-like strains is evident and supported by high bootstrap values. As was already observed from the ORF5 phylogenetic tree, Amervac and SHE are very closely related as is the case for strains 01-CB1 and LV. 07V063 clusters apart from LV and is genetically distinct from the LV prototype.

Conclusions
By using a simple random PCR cloning approach we obtained PRRSV sequence data from a recent European PRRSV isolate of unknown genetic background. This approach can be used to obtain partial genome sequences from for instance East-European type strains (for which until present, no full length genomes are available) and to get a better knowledge of the increasing PRRSV variability. We also showed that the isolate sequenced in this study is genetically different from prototype LV.

List of abbreviations
PRRSV: porcine reproductive and respiratory syndrome virus; RT-PCR: reverse transcriptase polymerase chain reaction.

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