Arch Virol (1998) 143: 1683–1700

Archives of Virology

Sequence analysis of porcine reproductive and respiratory syndrome virus of the American type collected from Danish swine herds

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Accepted April 15, 1998

Summary. Vaccine-like viruses of American type of porcine reproductive and respiratory syndrome virus (PRRSV) were detected in serum samples by RT-PCR. The viruses were analysed by nucleotide sequencing of the genomic region encoding open reading frames 2 to 7. During the ongoing study of Danish isolates of PRRSV by means of nucleotide sequencing, RT-PCR reactions and subsequent nucleotide sequencing showed the presence of American type PRRSV in Danish breeding herds. Most likely, these atypical viruses originated from boars vaccinated with live vaccine of American type (MLV RespPRRS), which were taken to artificial insemination centres and there brought together with unvaccinated boars already at the centres. The nucleotide sequences of three Danish viruses of American type PRRSV were compared to those of known PRRSV isolates. The nucleotide sequence identities of the atypical Danish isolates were between 99.2–99.5% to the vaccine virus RespPRRS and 99.0–99.3% to VR2332 which are the parental virus to the vaccine virus. Phylogenetic analysis including field isolates of American type supports the conclusion that the introduction of American type PRRSV in Denmark was due to spread of vaccine virus.

Introduction

The porcine reproductive and respiratory syndrome was first reported in the United States in 1987 [18]. The causal agent of the disease was first described in Europe in the Netherlands in 1991 and was named the Lelystad virus [38]. In US a virus related to the Lelystad virus was found in 1992 [2, 10]. PRRS was first described in Denmark in 1992 [3]. The virus causing the syndrome is now termed PRRS virus (PRRSV) and classified within the single genus *Arterivirus* in the family *Arteriviridae* placed in the order *Nidovirales* [5].

In order to control the disease in Denmark, a control progamme was worked out by the Federation of Danish Pig Producers and Slaughterhouses. All breeding herds were to be screened for antibodies against PRRSV, and antibody positive herds should be vaccinated. An element of the programme was vaccination of all boars entering the artificial insemination (AI) centres, in order to minimize infections at the centres and thereby minimise the risk of spread of PRRSV to noninfected herds through semen. The vaccination was given at least 8 weeks before the boars entered the AI centres in a special quarantine facility. The programme for vaccination of boars at AI centres started in November 1995, and was based on a dispensation given by the Danish authorities. The vaccination of breeding and fattening herds started on the 1st of July 1996, after final approval of the vaccine by Danish Health (DH) authorities for use in 3–18 week old pigs. The vaccine used was the RespPRRS vaccine (MLV RespPRRS) [13], which is registrered in Europe as Ingelvac PRRS MLV. It is a live vaccine and is a derivative of the virulent American field isolate VR2332 [10] and is also referred to as vaccine strain JJ1882 [29]. The vaccine was licenced in the United States in July 1994, for use in 3–18 week old pigs against the respiratory form of the disease and was approved in US in October 1996 for use in breeding animals against the reproductive syndrome.

Since the first appearance of PRRSV in Denmark in 1992 we have collected sequence data from Danish strains of PRRSV in order to determine genetic variation and phylogenetic relationships of Danish viruses. These data indicated the presence of two distinctly genomic types of PRRSV in Denmark (data not shown). As a follow up to these preliminary studies of earlier isolates, it was decided to collect further reference material of circulating Danish PRRSV from different parts of the country by means of nucleotide sequences. Due to the application of a live vaccine, we also wanted to monitor the selective pressure that the use of vaccine might put on wildtype PRRSV. No introduction into Denmark or other European countries of the American type of virus had been reported in the spring of 1996. Reverse transcription polymerase chain reaction (RT-PCR) methods were tested for their ability to detect either type of virus (European type and American type) and to discriminate beween the types. Blood samples collected from breeding herds with antibody profiles indicating recent infection were used as source of field virus.

From the first eight herds tested, European type PRRSV was detected, but in herd number 9 tested (given the identity number 3506) American type virus was surprisingly detected. Subsequently, further RT-PCR reaction against American type virus was observed in another herd (referred to as number 5163). The samples from herd 3506 were collected before the vaccine programme was approved in Denmark [4]. The present paper compares sequence data of these Danish isolates of American type virus with a Danish isolate of PRRSV isolated previously (referred to as isolate DK111-92), the MLV RespPRRS and other American strains of PRRSV.

The possibility of the herds being infected with vaccine-derived PRRSV through semen received from the AI centres is discussed.

Materials and methods

Serology and source of virus

The serum samples selected for testing in RT-PCR, had a profile of low or negative antibody level in blocking ELISA [35] combined with high level antibody titre in IPMA [3] indicative of recent infection with PRRSV.

RNA extraction

For the detection of PRRSV in serum samples, RNA was extracted from 500 μl of serum by the acid-guanidinium-thiocyanate-phenol-chloroform method essentially as described by Chomczynski [6]. After precipitation with isopropanol RNA was resuspended in 100 μl of DEPC-treated water. For each set of serum samples, one sample containing 500 μl of water and serving as a negative control in the subsequent RT-PCR, were included in the RNA extraction. Viral RNA used for cloning and sequencing of orf2 to orf7 of the Danish isolate DK111-92 was extracted from 500 μl of the supernatant from a culture of porcine pulmonary alveolar macrophages (PPAM) [3] and resuspended in 50 μl of DEPC-treated water.

RT-PCR

Primers designed to detect European type PRRSV and to amplify fragments of the six individual open reading frames orf2 to orf7 of the Danish isolate DK111-92, were based on the sequence of Lelystad virus [25]. Primers to amplify North American type PRRSV were based upon the sequence of VR2332 [27]. Primers to be universal for PRRSV were based on an alignment of available sequences from the EMBL database, using the Pileup program from the GCG software package [11]. Consensus primers were degenerate in a few positions. Primers used are listed in table 1. Primers were obtained from DNA Technology (Science Park, Aarhus, DK).

Reverse transcription and PCR to provide fragments of orf2 to orf7 of DK111-92 were performed with 2 μl of extracted RNA as template and using an one-enzyme and single reaction tube process [28] employing rTth DNA Polymerase (Perkin Elmer) following the recommendations of the manufacturer. Annealing and extension temperatures were held at 60 °C for 1 min and 15 sec.

To detect European type PRRSV in serum samples, primers P6254 and P6257 generating a fragment comprising the open reading frame 4 were used. In brief, 15 μl of the extracted RNA was denatured at 70 °C for 10 min in the presence of 25 pmol reverse primer. Reverse transcription was performed in a volume of 30 μl with 50 units of M-MuLV-RT enzyme (Expand-RT, Boehringer Mannheim), 1 × RT buffer, 10 mM DTT and 2.5 mM of each dNTP at 42 °C for 1 h. Five μl of the reverse transcription product was used as template in the subsequent PCR reaction in a final volume of 100 μl including 25 pmol each primer, 1 × Pfu polymerase buffer, 200 μM of each dNTP and 2.5 units of Pfu DNA polymerase (Stratagene). Two drops of mineral oil (Sigma) was overlaid the mix and the PCR reactions were performed in capped 0.5 ml polypropylene microcentrifuge tubes (Perkin Elmer) in a DNA Thermal Cycler 480 (Perkin Elmer). After initial heating for 1 min at 94 °C, the reaction were followed by 35 cycles of 94 °C for 45 sec, 60 °C for 30 sec, 72 °C for 1 min and finally terminated by 5 min at 72 °C. Ten μl of PCR products were run on 1% agarose gels (FMC bioproducts) containing 0.5 μg/ml ethidium bromide and visualized under UV transillumination. In cases of only few or none positive samples, a nested PCR were performed. Five μl of the product from the first round of PCR were used as template together with orf4 internal primers P23891 and P23892. Conditions for the nested PCR were the same as for the initial PCR reaction, except that elongation time at 72 °C were 45 sec.

Table 1. Oligonucleotides used for amplification and sequencing of European and American type PRRSV

Code	Sequence (5′-3′)	Location[a]	Application
P5293	+GGGTGATGCAATGGGGTCACTGTGGAG	11781–11807	Eur orf2: A[b] and S[c]
P5300	−GATGGTCAGCTCGAATGATGTGTTGCCG	12512–12540	Eur orf2: A and S
P5294	+CAGACAATGGCTCATCAGTGTGCACGC	12388–12414	Eur orf3: A and S
P5301	−GCCGTTATCGTGATGTACTGGGGAGTAC	13168–13195	Eur orf3: A and S
P6257	+GGAATGGCTGCGGCCACTCTTTTCTTC	12933–12959	Eur orf4: A, S and D[d]
P6254	−GAGAACATCTCATATTGCCAAGAGAATGGC	13467–13496	Eur orf4: A, S and D
P6256	+GCAATATGAGATGTTCTCACAAATTGGGG	13479–13507	Eur orf5: A and S
P5253	−CGTCTAGGCCTCCCATAGCTCAGCCG	14067–14092	Eur orf5: A and S
P6255	+GCAATGGGAGGCCTAGACGATTTTTGC	14084–14100	Eur orf6: A and S
P6252	−CTGGTTTTTACCGGCCATACTTGACGAGG	14577–14605	Eur orf6: A and S
P5296	+CGTCAAGTATGGCCGGTAAAAACCAGA	14580–14606	Eur orf7: A, S and D
P5295	−GTCAAATTAACTTGCACCCTGACTGGCGG	14952–14980	Eur orf7: A, S and D
P9726	+CCGTTCGCGCTCTGCCATTC	11955–11974	Eur: S
P9727	+GGTCAAGCGGCCTGGAAGC	12143–12261	Eur: S
P9728	+GCATGCCCTGTTCTACCAG	12554–12572	Eur: S
P9729	+CCTACGCGGCCAATTCCATC	12740–12760	Eur: S
P8708	+CCTCACGGGGTCTCAGC	13098–13114	Eur: S
P10547	+CACGACCGCGGCTGCCGGT	13046–13064	Eur: S
P8707	+GGTTGGGCAGTCGAGACC	13670–13687	Eur: S
P8706	+GCATGGCCTGCCGCTATGC	13881–13899	Eur: S
P8705	+CGGATACATGACATATGTGC	14244–14263	Eur: S
P21252	+CTCCGTWCGCGCYCTGCCATTCACTCT	11953–11979	Eur and Am: consensus primer
P21253	−CTCBGGATGGAAYTGGGCCGYGTAG	12741–12765	Eur and Am: consensus primer
P21254	−GGAATTCGAAGCCAAAGCACTATGAAC	12451–12477	Eur: specific primer for semi-nested PCR
P21255	−CACAACAAAAAGAGTACAAGAAGCTGCA	747–774	Am: specific primer for semi-nested PCR

P16533	+AGAATGGCTTCGTCCCTTCTTTTCCTC	1250–1276	Am orf4: A, S and D
P16534	−CATTCAAATTGCCAACAGAATGGCAAAAAG	1763–1792	Am orf4: A, S and D
P23891	+CAGATATTAAGACCAACACGACCGC	13030–13054	Am and Eur orf4 nested PCR: D and S
P23892	−GCATAGAAAAGGCAAGAAGAAAGCAT	13239–13264	Am and Eur orf4 nested PCR: D and S
P24925	+CCTGGATCCAAATGAAATGGGGTCCATGCAAAGCC	88–108	Am orf2-7: A and S
P15279	−CAAGAATGCCAGCCCATCATGCTGAG	3263–3288	Am orf2-7 and orf7: A, S and D
P15280	+GTAGATCTTGTTAAATATGCCAAATAACACCGGC	2885–2918	Am orf7: A, S and D
P25226	+GGGGRATGGCCAGCCAGTCAATCA	2939–2962	Am and Eur orf7 nested PCR: D and S
P25225	−ACGCGRATCAGGCGCACWGTATGA	3227–3250	Am and Eur orf7 nested PCR: D and S
P25386	+GGGTCCATGCAAAGCC	93–108	Am: S-1pt]
P25387	+GGTGTCAACCCTGATTGATG	390–409	Am: S
P25388	+GGCCAAAGCTTCATGATTTTCAG	683–705	Am: S
P25389	+CGAGCTAGGGTTTATGATAC	971–990	Am: S
P25390	+CTTGGTTTCTCAGGCGTTCG	1297–1316	Am: S
P15278	+CTCATGCTTTCTTCTTGCCTTTTCTATGC	1541–1569	Am: S
P25391	+GGTGGTATTTGGCAATGTGT	1597–1616	Am: S
P25392	+GCTATGTGAGCTGAATGGCA	1937–1956	Am: S
P25393	+GACACTAAGGGCAGACTC	2238–2255	Am: S
P25394	+CCTCAATTGTGCTTTCACCT	2536–2555	Am: S
P25395	+GGTTAAAAAGCCTCGTGTTG	2835–2854	Am: S
P101877	−CATCAATCAGGGTTGACACC	390–409	Am: S
P101878	−GCGGTGGTGTTGGTCTTAATATC	1349–1371	Am: S
P15277	−CTGTGCCATTCAGCTCACATAGCGTCAAG	1931–1959	Am: S
P101879	−GTACTTGCGGCCTAGCAAGC	2691–2710	Am: S
P101880	−CACAGCTGATTGACTGGCTGG	2948–2968	Am: S

+: Genomic sense −: antisense primer

[a]Locations are from the sequences of Lelystad virus (nt $_{11781-14980}$) [25] and VR2332 (nt$_{88-3288}$) [27]

[b]Primers used for amplification (A) of individual orfs of European (Eur) or American (Am) type PRRSV

[c]Primers used for sequencing (S)

[d]Primers used for detection (D)

An RT-PCR method followed by a "semi-nested" PCR (snPCR) was used to discriminate beween heterogenous types of PRRSV. Fifteen μl of extracted RNA was used in the first round of RT-PCR, employing primers P21252 and P21253 based upon conserved regions of European and North American genotype. Two separate reactions of snPCR were performed using 5 μl each of the product from the first round RT-PCR as template. The snPCR using type-specific primers P21255 and P21254, respectively, together with consensus primer P21252 were able to differentiate between either type of virus. Buffering and enzyme conditions for RT and PCR were the same as described above. Following heating for 2 min at 94 °C, 35 cycles of 94 °C for 45 sec, 55 °C for 45 sec and 72 °C for 1 min was performed, terminated by 5 min at 72 °C.

To amplify a PCR-fragment covering the 3188 basepair region encoding orf 2-7 of American type PRRSV antisense primer P15279 and sense primer P24925 were used. Conditions of reverse transcription and PCR were the same as those used to amplify European type orf4, except for a prolonged time of elongation at 72 °C for 3 min. Prior to sequencing, PCR products chosen for sequence analysis were purified using a Qiagen PCR spin kit (Qiagen, Inc.).

Sequence analysis

RT-PCR products containing the six individual orfs 2 to 7 of the Danish reference strain (DK111-92) were cloned into the pBluescript-SK(+) vector (Stratagene) using standard procedures [28]. Double stranded DNA inserts were sequenced by dideoxy chain termination [32] employing Redivue [(γ-^{32}P]-dATP (Amersham) using an AmpliCycle Sequencing kit (Perkin Elmer). At least two clones of each open reading frame were sequenced using both universal and reverse primers together with specific internal sequencing primers (Table 1) for the European type PRRSV based on the sequence of Lelystad virus [25].

Sequencing of orf 2-7 PCR-fragments of American type PRRSV were performed by flourescent dye terminator cycle sequencing (AmpliTaq DNA polymerase FS, Perkin Elmer) and analysed on an ABI 310 Genetic Analyzer (Perkin Elmer). Specific primers with orientations in both directions were made according to the VR2332 sequence (Table 1).

Alignment of sequences were made by use of the PILEUP program from the GCG software package [11] and percents of identity and genetic distances were calculated using the DNADIST program from the PHYLIP software package [12]. To construct a phylogenetic tree, sequences were aligned by use of the CLUSTAL V method [16] and branched according to the neighbor-joining method [30].

Nucleotide sequence data reported here have been deposited with the EMBL database under the accession numbers AJ223078 (DK111-92), AJ223079 (DK3506-12), AJ223080 (DK5163-17), AJ223081 (DK5163-23) and AJ223082 (MLV RespPRRS).

Virus isolation

PPAM cultures and the MARC-145 cell line [19] were used to propagate viruses. Fifty μl serum samples were added to monolayers of cells in 24-well plates containing 200 μl of Eagle's medium. After incubation for 2 h at 37 °C, 800 μl of Eagle's +10% FCS were added to the wells and incubated until CPE was observed. The primary culture was freezed and thawed and a second passage was made by adding 200 μl of the suspension to monolayers of PPAM and MARC-145 cells, respectively. Identification of virus was made by IPMA as described [3]. Vaccine virus was propagated by inoculation of MARC-145 cells with Ingelvac PRRS MLV.

Results

Serum samples from herds with serological profiles indicating recent infection, were initially subjected to RT-PCR with primers specific for the European type PRRSV. Usually, antibody-negative sera from such herds were found positive for PRRSV in RT-PCR using primers based upon the sequence of Lelystad virus. However, all samples from such a herd (identity number 3506) reacted negative in the RT-PCR using primers based upon the sequence of Lelystad virus and were therefore tested again, this time using primer pairs designed from the VR2332 sequence. This retesting showed three positive serum samples. From another herd (identity number 5163) four samples were also positive in RT-PCR using the VR2332 derived primers.

PRRSV was isolated in MARC-145 cell cultures from three out of the seven PCR-positive samples, and PCR products amplified directly from these three serum samples were chosen for the further investegation. No virus was isolated in PPAM.

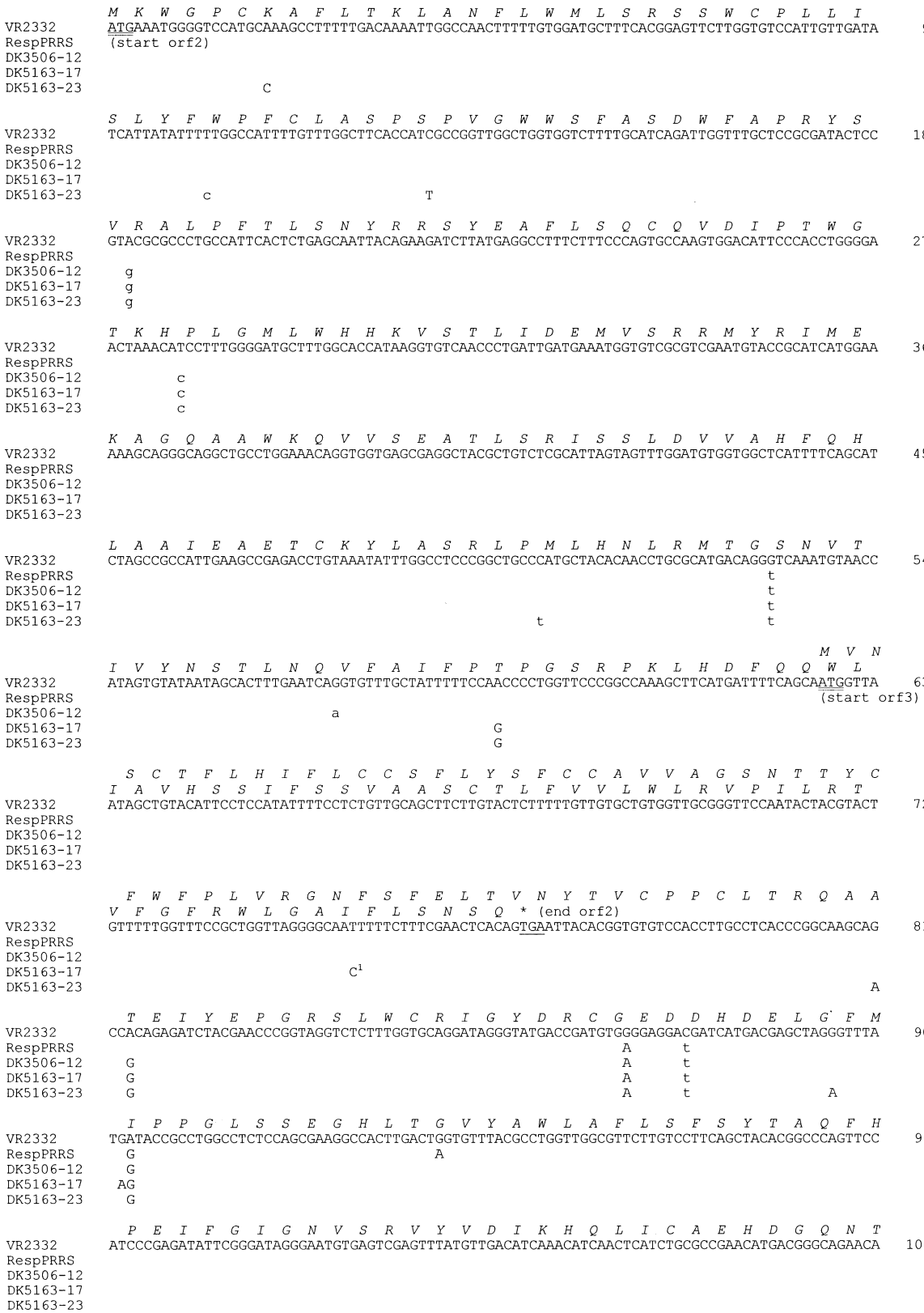
Determination of identity

To determine the identity of the PRRSV that failed to react in RT-PCR with European type specific primers, the entire region of orf2-7 from virus found in serum sample #12 from herd 3506 and #17 and #23 from herd 5163 (given the virus identity numbers DK3506-12, DK5163-17 and DK5163-23) were subjected to sequence analysis (Fig. 1). Nucleic acid identities between these atypical Danish viruses were from 99.4 to 99.6% (Table 2) indicating a common origin.

A comparison to the sequences obtained with the Danish isolate (DK 111-92) and with the Lelystad virus (LV) showed only distant nucleic acid identities at 64–65% (Table 2). In contrast, comparison of DK3506-12, DK5163-17 and DK5163-23 with American type PRRSV showed a much higher identity at the nucleotide level (92.6–99.3%). The highest identity was observed to the ISU22 and VR2332 isolates with nucleotide identities in the orf2-7 region between 97.8% and 99.3% (Table 2). Since no nucleotide sequence data of the vaccine virus were available, MLV RespPRRS was also amplified by RT-PCR and directly sequenced in the entire orf2-7 region (Fig. 1). A comparison between the vaccine virus and VR2332 showed 11 nucleotide changes giving a identity of 99.7%, reflecting only little variation in the compared region, which covered approximately 20% of the PRRSV genome. Comparison of DK3506-12, DK5163-17 and DK5163-23 with MLV RespPRRS showed relative identities between 99.1–99.5%, values higher than those observed with VR2332 (Table 2).

Nucleotide and amino acid changes

Nucleotide changes of MLV RespPRRS and Danish viruses compared to VR2332 and other American type PRRSV are summarised in Fig. 2. The viruses found in the samples from herd 3506 and 5163 shared nucleotide changes at seven positions (nt528, 871, 878, 903, 1396, 2663 and 2665) with the MLV RespPRRS in comparison to VR2332, indicating a direct phylogenetic relationship of the virus


M K W G P C K A F L T K L A N F L W M L S R S S W C P L L I
VR2332 ATGAAATGGGGTCCATGCAAAGCCTTTTTGACAAAATTGGCCAACTTTTTGTGGATGCTTTCACGGAGTTCTTGGTGTCCATTGTTGATA 90
RespPRRS (start orf2)
DK3506-12
DK5163-17
DK5163-23 C
S L Y F W P F C L A S P S P V G W W S F A S D W F A P R Y S
VR2332 TCATTATATTTTTGGCCATTTTGTTTGGCTTCACCATCGCCGGTTGGCTGGTGGTCTTTTGCATCAGATTGGTTTGCTCCGCGATACTCC 180
RespPRRS
DK3506-12
DK5163-17
DK5163-23 c T
V R A L P F T L S N Y R R S Y E A F L S Q C Q V D I P T W G
VR2332 GTACGCGCCCTGCCATTCACTCTGAGCAATTACAGAAGATCTTATGAGGCCTTTCTTTCCCAGTGCCAAGTGGACATTCCCACCTGGGGA 270
RespPRRS
DK3506-12 g
DK5163-17 g
DK5163-23 g
T K H P L G M L W H H K V S T L I D E M V S R R M Y R I M E
VR2332 ACTAAACATCCTTTGGGGATGCTTTGGCACCATAAGGTGTCAACCCTGATTGATGAAATGGTGTCGCGTCGAATGTACCGCATCATGGAA 360
RespPRRS
DK3506-12 c
DK5163-17 c
DK5163-23 c
K A G Q A A W K Q V V S E A T L S R I S S L D V V A H F Q H
VR2332 AAAGCAGGGCAGGCTGCCTGGAAACAGGTGGTGAGCGAGGCTACGCTGTCTCGCATTAGTAGTTTGGATGTGGTGGCTCATTTTCAGCAT 450
RespPRRS
DK3506-12
DK5163-17
DK5163-23
L A A I E A E T C K Y L A S R L P M L H N L R M T G S N V T
VR2332 CTAGCCGCCATTGAAGCCGAGACCTGTAAATATTTGGCCTCCCGGCTGCCCATGCTACACAACCTGCGCATGACAGGGTCAAATGTAACC 540
RespPRRS t
DK3506-12 t
DK5163-17 t
DK5163-23 t t
M V N
I V Y N S T L N Q V F A I F P T P G S R P K L H D F Q Q W L
VR2332 ATAGTGTATAATAGCACTTTGAATCAGGTGTTTGCTATTTTTCCAACCCCTGGTTCCCGGCCAAAGCTTCATGATTTTCAGCAATGGTTA 630
RespPRRS (start orf3)
DK3506-12 a
DK5163-17 G
DK5163-23 G
S C T F L H I F L C C S F L Y S F C C A V V A G S N T T Y C
I A V H S S I F S S V A A S C T L F V V L W L R V P I L R T
VR2332 ATAGCTGTACATTCCTCCATATTTTCCTCTGTTGCAGCTTCTTGTACTCTTTTTGTTGTGCTGTGGTTGCGGGTTCCAATACTACGTACT 720
RespPRRS
DK3506-12
DK5163-17
DK5163-23
F W F P L V R G N F S F E L T V N Y T V C P P C L T R Q A A
V F G F R W L G A I F L S N S Q * (end orf2)
VR2332 GTTTTTGGTTTCCGCTGGTTAGGGGCAATTTTTCTTTCGAACTCACAGTGAATTACACGGTGTGTCCACCTTGCCTCACCCGGCAAGCAG 810
RespPRRS
DK3506-12
DK5163-17 C1
DK5163-23 A
T E I Y E P G R S L W C R I G Y D R C G E D D H D E L G F M
VR2332 CCACAGAGATCTACGAACCCGGTAGGTCTCTTTGGTGCAGGATAGGGTATGACCGATGTGGGGAGGACGATCATGACGAGCTAGGGTTTA 900
RespPRRS A t
DK3506-12 G A t
DK5163-17 G A t
DK5163-23 G A t A
I P P G L S S E G H L T G V Y A W L A F L S F S Y T A Q F H
VR2332 TGATACCGCCTGGCCTCTCCAGCGAAGGCCACTTGACTGGTGTTTACGCCTGGTTGGCGTTCTTGTCCTTCAGCTACACGGCCCAGTTCC 990
RespPRRS G A
DK3506-12 G
DK5163-17 AG
DK5163-23 G
P E I F G I G N V S R V Y V D I K H Q L I C A E H D G Q N T
VR2332 ATCCCGAGATATTCGGGATAGGGAATGTGAGTCGAGTTTATGTTGACATCAAACATCAACTCATCTGCGCCGAACATGACGGGCAGAACA 1080
RespPRRS
DK3506-12
DK5163-17
DK5163-23

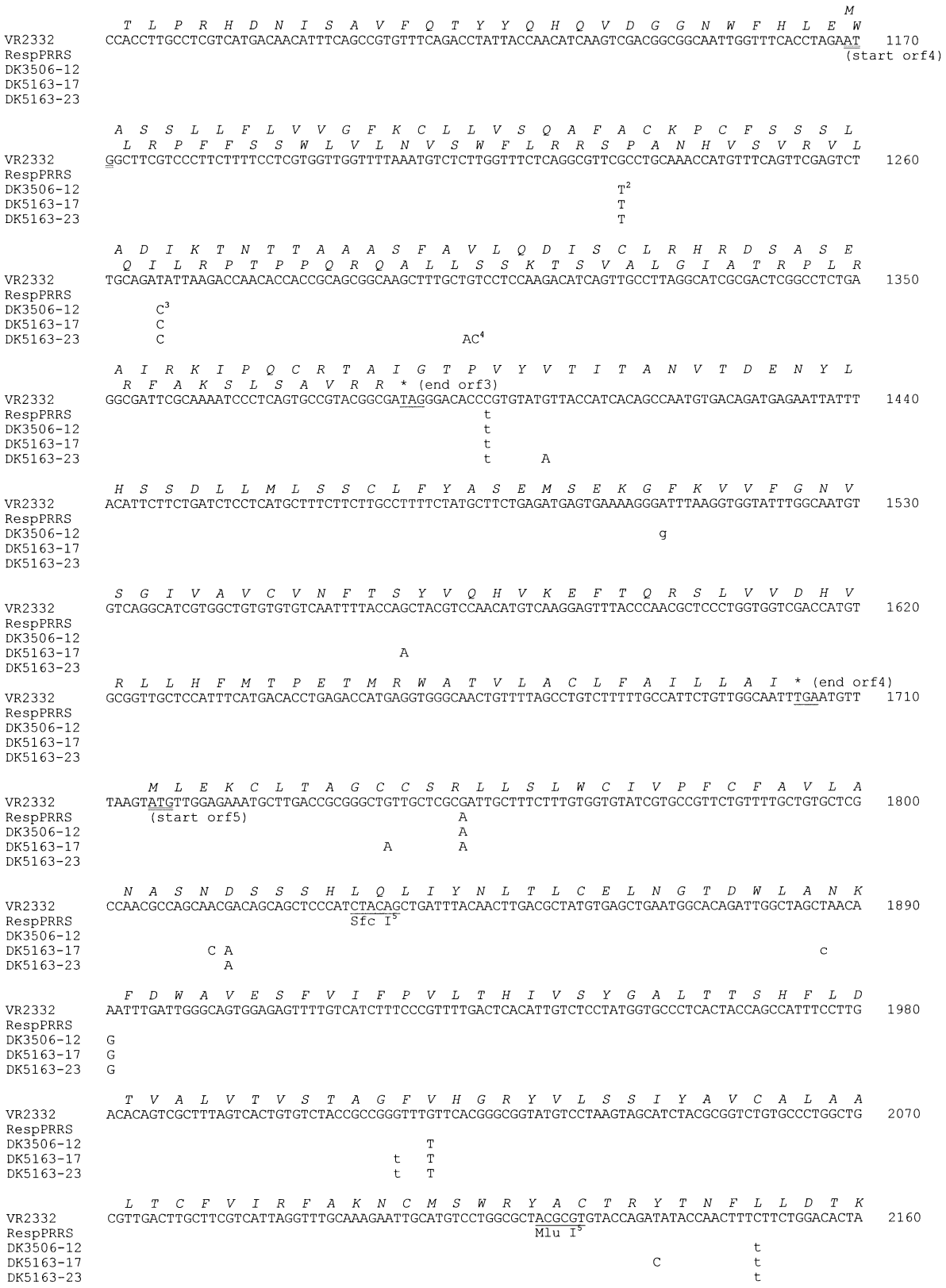


Fig. 1 (*continued*)

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                G  R  L  Y  R  W  R  S  P  V  I  I  E  K  R  G  K  V  E  V  E  G  H  L  I  D  L  K  R  V
VR2332      AGGGCAGACTCTATCGTTGGCGGTCGCCTGTCATCATAGAGAAAAGGGGCAAAGTTGAGGTCGAAGGTCATCTGATCGACCTCAAAAGAG   2250
RespPRRS        G
DK3506-12        T                                                                                  g
DK5163-17        T                                                                                  g
DK5163-23        T                                                                                  g

                                                                                M  G  S  S  L  D  D  F  C  H  D  S  T
              V  L  D  G  S  V  A  T  P  I  T  R  V  S  A  E  Q  W  G  R  P  * (end orf5)
VR2332      TTGTGCTTGATGGTTCCGTGGCAACCCCTATAACCAGAGTTTCAGCGGAACAATGGGGTCGTCCTTAGATGACTTCTGTCATGATAGCAC   2340
RespPRRS                                                                        (start orf6)
DK3506-12                                                                                    G
DK5163-17                                                                                    G
DK5163-23                                                                                    G

             A  P  Q  K  V  L  L  A  F  S  I  T  Y  T  P  V  M  I  Y  A  L  K  V  S  R  G  R  L  L  G
VR2332      GGCTCCACAAAAGGTGCTTTTGGCGTTTTCTATTACCTACACGCCAGTGATGATATATGCCCTAAAGGTGAGTCGCGGCCGACTGCTAGG   2430
RespPRRS          G
DK3506-12
DK5163-17
DK5163-23

              L  L  H  L  L  I  F  L  N  C  A  F  T  F  G  Y  M  T  F  A  H  F  Q  S  T  N  K  V  A  L
VR2332      GCTTCTGCACCTTTTGATCTTCCTGAATTGTGCTTTCACCTTCGGGTACATGACTTTCGCGCACTTTCAGAGTACAAATAAGGTCGCGCT   2520
RespPRRS
DK3506-12
DK5163-17
DK5163-23

              T  M  G  A  V  V  A  L  L  W  G  V  Y  S  A  I  E  T  W  K  F  I  T  S  R  C  R  L  C  L
VR2332      CACTATGGGAGCAGTAGTTGCACTCCTTTGGGGGGTGTACTCAGCCATAGAAACCTGGAAATTCATCACCTCCAGATGCCGTTTGTGCTT   2610
RespPRRS
DK3506-12
DK5163-17
DK5163-23

              L  G  R  K  Y  I  L  A  P  A  H  H  V  E  S  A  A  R  F  H  P  I  A  A  N  D  N  H  A  F
VR2332      GCTAGGCCGCAAGTACATTCTGGCCCCTGCCCACCACGTTGAAAGTGCCGCACGGTTTCATCCGATTGCGGCAAATGATAACCACGCATT   2700
RespPRRS                                                          G c[6]
DK3506-12                                                         G c
DK5163-17                                                         G c
DK5163-23                                                         G c

              V  V  R  R  P  G  S  T  T  V  N  G  T  L  V  P  G  L  K  S  L  V  L  G  G  R  K  A  V  K
VR2332      TGTCGTCCGGCGTCCCGGCTCCACTACGGTCAACGGCACATTGGTGCCCGGGTTAAAAAGCCTCGTGTTGGGTGGCAGAAAAGCTGTTAA   2790
RespPRRS
DK3506-12
DK5163-17
DK5163-23                                                               g

                                M  P  N  N  N  G  K  Q  Q  K  R  K  K  G  D  G  Q  P  V  N  Q  L
              Q  G  V  V  N  L  V  K  Y  A  K  * (end orf6)
VR2332      ACAGGGAGTGGTAAACCTTGTCAAATATGCCAAATAACAACGGCAAGCAGCAGAAGAGAAAGAAGGGGGATGGCCAGCCAGTCAATCAGC   2880
RespPRRS                        (start orf79
DK3506-12
DK5163-17
DK5163-23

              C  Q  M  L  G  K  I  I  A  Q  Q  N  Q  S  R  G  K  G  P  G  K  K  N  K  K  K  N  P  E  K
VR2332      TGTGCCAGATGCTGGGTAAGATCATCGCTCAGCAAAACCAGTCCAGAGGCAAGGGACCGGGAAAGAAAAATAAGAAGAAAAACCCGGAGA   2970
RespPRRS
DK3506-12
DK5163-17
DK5163-23

              P  H  F  P  L  A  T  E  D  D  V  R  H  H  F  T  P  S  E  R  Q  L  C  L  S  S  I  Q  T  A
VR2332      AGCCCCATTTTCCTCTAGCGACTGAAGATGATGTCAGACATCACTTTACCCCTAGTGAGCGGCAATTGTGTCTGTCGTCAATCCAGACCG   3060
RespPRRS
DK3506-12
DK5163-17
DK5163-23

              F  N  Q  G  A  G  T  C  T  L  S  D  S  G  R  I  S  Y  T  V  E  F  S  L  P  T  H  H  T  V
VR2332      CCTTTAATCAAGGCGCTGGGACTTGCACCCTGTCAGATTCAGGGAGGATAAGTTACACTGTGGAGTTTAGTTTGCCTACGCATCATACTG   3150
RespPRRS
DK3506-12
DK5163-17
DK5163-23

              R  L  I  R  V  T  A  S  P  S  A  * (end orf7)
VR2332      TGCGCCTGATCCGCGTCACAGCATCACCCTCAGCATGA                                                     3188
RespPRRS
DK3506-12           t
DK5163-17           t
DK5163-23           t
```

Table 2. Identity between selected American strains of PRRSV and the atypical Danish isolates DK3506-12, DK5163-17 and DK5163-23 in the 3188 basepair region encoding orf2-7

	VR-2332	RespPRRS	3506–12	5163–23	5163–23	ISU22	ISU3927	DK111–92	[a]LV
VR-2332	–	99,7[b]	99,3	99,1	99,0	98,4	92,9	64,6	65,0
RespPRRS	11[c]	–	99,5	99,2	99,1	98,3	92,8	64,6	64,9
3506-12	22	17	–	99,6	99,4	98,1	92,8	64,6	64,9
5163-17	30	25	12	–	99,5	97,8	92,6	64,6	64,8
5163-23	32	29	16	18	–	97,9	92,7	64,6	64,8
ISU22	52	55	62	70	68	–	93,2	64,5	64,8
ISU3927	226	229	231	237	235	221	–	63,9	64,6
DK111-92	1128	1130	1131	1130	1130	1132	1150	–	92,9
LV	1116	1128	1120	1121	1123	1122	1130	227	–

[a]Sequences for DK3506-12, DK5163-17, DK5163-23, DK111-92 and RespPRRS are presented in the study, VR2332 was reported by Murtaugh et al. [27], ISU22 and ISU3927 was reported by Meng et al. [23], [24] and the sequence of Lelystad virus (LV) by Meulenberg et al. [25]. ISU22 and ISU3927 represents genotypes close (ISU22) and distant (ISU3927) to VR2332

[b]Percentages of nucleotide identity are presented above the diagonal

[c]Absolute distances expressed as number of polymorphic sites are shown below the diagonal

from herds 3506 and 5163 and the vaccine virus. $C_{2663} \rightarrow G$ and $G_{2665} \rightarrow C$ are found in all other American field strains and may represent a sequencing error of VR2332. Of the remaining five changes, three (T_{528}, A_{871} and T_{878}) are unique for MLV RespPRRS and the isolates from herd 3506 and 5163, in the sense that they are not found in other sequenced American type PRRSV strains. Three changes (A_{939}, G_{2166} and G_{2348}) are unique for MLV RespPRRS compared to DK3506-12, DK5163-17 and DK5163-23. The three Danish isolates have in these positions identical nucleotides compared to VR2332 which may point at VR2332 as the source of the American type PRRSV emerging in Denmark. The G at position nt_{939} is found solely in VR2332 and in the Danish isolates, whereas both G_{2166} and G_{2348} are unique for MLV RespPRRS. Therefore the coding changes $A_{2166} \rightarrow G$ and $C_{2348} \rightarrow G$ observed in MLV RespPRRS, may represent nucleotide changes

Fig. 1. Alignment of nucleotide sequences of orf2-7 of American field isolate VR2332 [27], Ingelvac PRRS MLV (abbreviated RespPRRS) and Danish isolates DK3506-12, DK5163-17 and DK5163-23. Start and stop codons of individual orf's 2 to 7 are underlined. Nucleotide changes resulting in amino acid changes are marked with capital letters. Translationally silent nucleotide changes are marked with small letters. Translations of reading frames orf2 to orf7 of VR2332 into amino acid sequences are added above the nucleotide sequence. [1]Nucleotide change results in aa change $Ile_{250} \rightarrow Thr$ in orf2. No change in orf3. [2]Nucleotide change results in aa change $Ser_{203} \rightarrow Leu$ in orf3. No change in orf4. [3]Nucleotide change results in aa change $Ile_{215} \rightarrow Thr$ in orf3. No change in orf4. [4]Nucleotide change results in aa change $Ser_{228} \rightarrow Pro$ in orf3. $Val_{46} \rightarrow Thr$ in orf4. [5]Sites for restriction enzymes Sfc I and Mlu I, markers for differentiation of vaccine strain and field strains [39]. [6]G_{2663} and C_{2665} are present in all other sequences of American type PRRSV except in VR2332

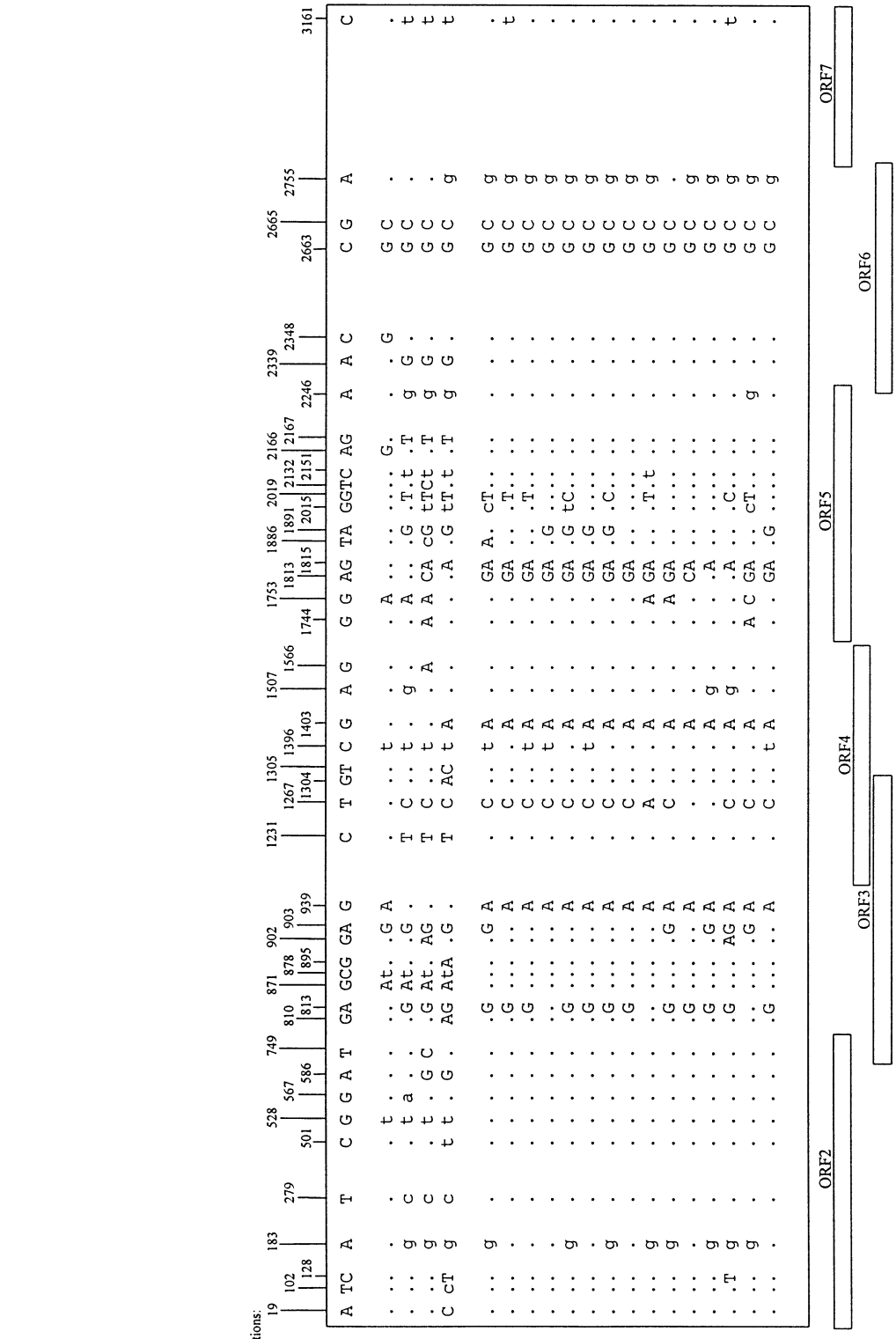

Fig. 2. Summary of the nucleotide changes presented in Fig. 1. Included for comparison are all available sequences of American type PRRSV covering the orf2-7 region. IA-1, IA-6, IL-1, KS-1, KY-1, MN-1, MO-1, NE-1 and SG-1 [17], VR-2385 [22, 26] and ISU1894, ISU22, ISU3927, ISU55 and ISU79 [23, 24]. Nucleotide positions relative to the region encoding orf2 to orf7 are indicated at the top. The extent of individual open reading frames is presented by the shaded bars at the bottom. Capital letters indicate coding changes and small letters indicate translationally silent changes

reflecting cell culture adaptation of MLV RespPPRS that reverted upon passage in vivo. Changes which were common to all three Danish isolates and which were not found in either VR2332 or MLV RespPRRS were found in 12 positions ($nt_{183,279,813,1231,1267,1891,2019,2151,2167,2246,2339,3161}$). Five of these changes were translationally silent, which indicated a common origin of the three isolates. Three additional changes ($A_{586} \rightarrow G$, $G_{1815} \rightarrow A$ and $G_{2015} \rightarrow T$) were common for the two viruses (DK5163-17, DK5163-23) originating from serum samples from the same herd, indicating that a further development away from MLV RespPRRS had occurred.

In addition to these mutations away from MLV RespPRRS, randomly occurring nucleotide changes were observed in each of the Danish isolates (Fig. 1). DK3506-12 had two additional nucleotide changes in positions nt_{567} and nt_{1507} compared to VR2332 and MLV RespPRRS while DK5163-17 and DK5163-23 underwent three common changes before they further split up by 7 and 10 additional changes in positions $nt_{749,902,1566,1744,1813,1886,2132}$ and $nt_{19,102,128,501,810,895,1304,1305,1403,2755}$, of which 6 and 7 changes were coding changes, respectively.

A phylogenetic tree (Fig. 3) was constructed showing the proportional relation of the Danish isolates to MLVRespPRRS and to VR2332 expressed by means of nuleotide diversity in the coding region for orf2-7.

Development of VR2332 into vaccine virus MLV RespPRRS resulted in 11 nucleotide changes in orf2-7 of which 7 changes resulted in amino acid changes (Fig. 2). MLV RespPRRS underwent additional 15 changes when found in herds 3506 and 5163 of which three changes represented reversions to the sequence of VR2332. Of the remaining 12 changes 7 were coding changes. The total number of nucleotide and amino acid changes were used to calculate the ratios of translationally silent to coding substitutions referred to as the K_S/K_A ratio [1, 14, 34]. When compared to MLV RespPRRS, individual K_S/K_A ratios for DK3506-12, DK5163-17 and DK5163-23 were 1.11, 0.65 and 0.94 respectively. An average ratio for 42 eucaryotic genes were calculated to be 5.28 [20, 34]. Thus, compared

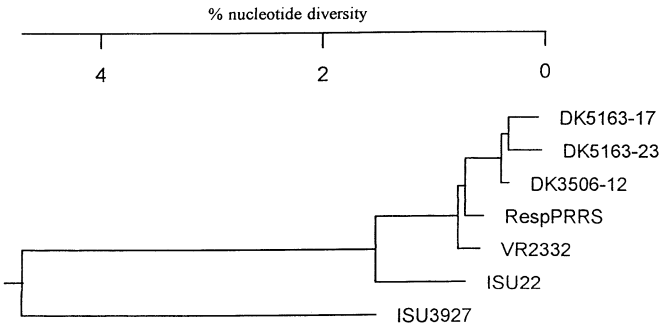

Fig. 3. Phylogenetic tree of the American type viruses included in Table 1; ISU22, ISU3927, VR2332, MLV RespPRRS and the Danish vaccine-like viruses DK3506-12, DK5163-17 and DK5163-23. The tree is based upon an alignment of the entire region encoding orf2-7 using the CLUSTAL V method [16] and branched according to the neighbor-joining method [30]

to ratios for genes which are under stringent constraint to preserve amino acids, the ratios for the Danish isolates indicates a selection in favour of coding changes, reflected in the low K_S/K_A ratios.

Discussion

Atypical Danish PRRSV isolates were identified in three blood samples from the herds designated 3506 and 5163 by sequence analysis of PCR products. A comparison of the data obtained, was made with available sequences of American type PRRSV covering orf2-7. This comparison clearly indicated a close relationship between the atypical Danish isolates and the vaccine virus MLV RespPRRS and its origin VR2332, and less relationship to other American type PRRSV (Table 2 and Fig. 2).

Prior to the findings of these vaccine-like and atypical Danish PRRSV isolates, orf2-7 of an isolate from an early case of the disease in Denmark [3] was sequenced for comparison to the sequence of Lelystad virus [25]. This comparison showed variations in 227 nucleotide positions between the Danish isolate DK111-92 and Lelystad virus resulting in a similarity between these two isolates of 92.9% in the compared region (Table 2).

VR2332 was one of the first isolated and characterized American strains of PRRSV and considering that the closely related type ISU22, was isolated during the same early period, it is unlikely that the virus found in the two Danish herds could be introductions of any of these two field strains. However, the MLV RespPRRS vaccine which is based upon VR2332, had at this point been used in Denmark for vaccination of boars entering AI centres. Therefore, spread of vaccine virus through semen delivered from the AI centres was a likely explanation of the high level of identity to VR2332.

The two herds 3506 and 5163 were previously free of PRRSV and were not vaccinated with the live vaccine. Furthermore, the first set of blood samples from herd 3506, was collected in June 1996 before the vaccination of 3–18 weeks old pigs was authorized in Denmark. Furthermore, both herds routinely received semen from the same AI centre, and an analysis of the data presented here clearly suggested an epidemiological connection between the viruses isolated from both herds. Therefore, we concluded from the data, that the initial spread of vaccine-like virus in Denmark was linked to vaccination of boars at AI centres with the MLV RespPRRS vaccine. Vaccinated boars taken into AI centres were kept under quarantine facilities before transfer for a period of at least 8 weeks after vaccination. This means that boars in some cases were able to transmit infectious virus to nonvaccinated seronegative boars at the centres more than 8 weeks after vaccination. Examination of all the nonvaccinated boars at the AI centres, showed that more than 50% had become seropositive to vaccine virus [4], thereby strongly supporting the hypothesis, that these acutely infected boars were a likely source of virus.

Studies using the American wildtype virus have shown that duration of virus shedding through semen can vary from a few days to more than 13 weeks [8, 36].

Furthermore, experimental investigations, although based on small groups of animals, showed that PRRSV may persist in the genitals for prolonged periods [7, 8, 9, 33, 36, 40]. The Equine Arteritis Virus (EAV), another virus of the Arterivirus family, can persists lifelong in stallions and cause a permanent spread of virus from semen of 30–60% of seropositive animals [15, 37].

The high degree of similarity between VR2332 and MLV RespPRRS in the genomic area encoding orf2-7 suggested that major and stable attenuating events have not taken place during development of vaccine virus. However, the K_S/K_A ratio of RespPRRS compared to VR2332, which was as low as 0.57, suggests a strong adaption of RespPRRS to cell cultures, in contrast to genes under constraint conditions to maintain their amino acid sequences reflected in high K_S/K_A ratios. Therefore, considering the development of virus in the early phase away from cell culture adapted vaccine virus, one would also expect low ratios of K_S/K_A for the Danish isolates compared to the vaccine virus, and this was actually found. The ratios for DK3506-12, DK5163-17 and DK5163-23 compared to MLV RespPRRS were 1.11, 0.65 and 0.94 respectively.

Positions G_{2166} and G_{2348}, were unique for the vaccine virus in comparison to other American type of PRRS viruses including the Danish isolates (Fig. 2), indicating sites which may influence virulence of virus. Nt_{2166} and nt_{2348}, are positioned in orf5 and orf6 respectively. These orfs encodes the heterodimeric complex of the M and E membrane proteins [21]. Nucleotide changes at these positions may therefore possibly affect virus/host or virus/cell interactions. However, more drastic changes may have occured in the attenuation of virulent VR2332 into non-virulent vaccine virus MLV RespPRRS, in orf 1a/b encoding the nonstructural proteins. Therefore sequence data of this large genomic region may be valuable in this respect.

The distance between DK5163-17 and DK5163-23 suggested a separation between these two viruses either due to different batches of semen from the A. I. center reflecting the genetic drift at the center or the separation of pigs in the breeding herd, causing independent selection of virus.

Because of the known origin and time of introduction of the American type of PRRSV in Denmark, this situation gives a future opportunity to study the evolution of just one virus and furthermore the study of reversion to virulence of this particular virus.

Acknowledgements

The authors would like to thank Preben Normann and Brian Jørgensen for their excellent technical assistance and Torben Storgaard and Per Have at DVIVR, Lindholm for their critical and inspiring review of the manuscript. This work was supported by a grant from the Foundation of Norma and Frode Jacobsen.

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Received December 24, 1997