Generation of an Infectious Clone of VR-2332, a Highly Virulent North American-Type Isolate of Porcine Reproductive and Respiratory Syndrome Virus

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A full-length cDNA clone of the prototypical North American porcine reproductive and respiratory syndrome virus (PRRSV) isolate VR-2332 was assembled in the plasmid vector pOK12. To rescue infectious virus, capped RNA was transcribed in vitro from the pOK12 clone and transfected into BHK-21C cells. The supernatant from transfected monolayers were serially passaged on Marc-145 cells and porcine pulmonary alveolar macrophages. Infectious PRRSV was recovered on Marc-145 cells as well as porcine pulmonary macrophages; thus, the cloned virus exhibited the same cell tropism as the parental VR-2332 strain. However, the cloned virus was clearly distinguishable from the parental VR-2332 strain by an engineered marker, a BstZ17I restriction site. The full-length cDNA clone had 11 nucleotide changes, 2 of which affected coding, compared to the parental VR-2332 strain. Additionally, the transcribed RNA had an extra G at the 5′ end. To examine whether these changes influenced viral replication, we examined the growth kinetics of the cloned virus in vitro. In Marc-145 cells, the growth kinetics of the cloned virus reflected those of the parental isolate, even though the titers of the cloned virus were consistently slightly lower. In experimentally infected 5.5-week-old pigs, the cloned virus produced blue discoloration of the ears, a classical clinical symptom of PRRSV. Also, the seroconversion kinetics of pigs infected with the cloned virus and VR-2332 were very similar. Hence, virus derived from the full-length cDNA clone appeared to recapitulate the biological properties of the highly virulent parental VR-2332 strain. This is the first report of an infectious cDNA clone based on American-type PRRSV. The availability of this cDNA clone will allow examination of the molecular mechanisms behind PRRSV virulence and attenuation, which might in turn allow the production of second-generation, genetically engineered PRRSV vaccines.

Porcine reproductive and respiratory syndrome is considered one of the most economically important infectious diseases of swine (1, 15). The disease is associated with severe reproductive disorders in sows and gilts and respiratory problems in pigs (11, 13, 16). The causative agent of the disease is porcine reproductive and respiratory syndrome virus (PRRSV), which, together with equine arteritis virus, lactate dehydrogenase-elevating virus, and simian hemorrhagic fever virus, are placed in the Arteriviridae family, within the order Nidovirales (9).

The PRRSV genome consists of a 5′-capped and 3′-polyadenylated single-stranded positive-sense RNA molecule of 15.1 to 15.5 kb. The viral genome contains, in the 5′ to 3′ direction, a 5′ leader, at least nine open reading frames (ORF 1a, ORF 1b, and ORFs 2 to 7), and a 3′ nontranslated region (14, 23, 32, 39). ORF 1a and ORF 1b are located directly downstream of the 5′ leader and encode a large replicate polyprotein, which is thought to be autoproteolytically cleaved into 13 smaller non-structural proteins assumed to be involved in virus replication and transcription (4, 35, 38). ORFs 2 to 7 encode the structural proteins associated with the virion. These proteins are expressed from a 3′-coterminal nested set of subgenomic mRNAs (23, 24, 32).

PRRSV emerged almost simultaneously in North America and Europe in the late 1980s and early 1990s, respectively. Even though the same PRRSV-associated disease was observed on both continents, phylogenetic analysis has revealed two distinct genotypes of PRRSV, North American and European, with a sequence homology of only approximately 63% at the nucleotide level (2).

The establishment of infectious full-length cDNA clones has become critical in the study of viruses. The availability of such cDNA clones offers an opportunity for analysis and modification of viral genomes at the molecular level and has greatly aided research on virus replication, pathogenesis, and vaccine development (7). Yet, to date, only one infectious cDNA clone of PRRSV has been established, and this clone is unfortunately not generally available to the scientific community. Furthermore, this cDNA clone is based on the European-type Lelystad virus (25). The large genetic differences observed between the North American and European genotypes of PRRSV make it
**TABLE 1. Summary of oligonucleotides used for RT-PCR amplification**

<table>
<thead>
<tr>
<th>Primera</th>
<th>Sequence (5' → 3')b</th>
<th>Position within VR-2332 genomec</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ terminus (fragment b) primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT416</td>
<td>5’ TGC GAT TGG A 3’</td>
<td>435–445</td>
</tr>
<tr>
<td>F20</td>
<td>5’ CTC GAG GCC GCG CCT AAT AGC ACT CAC TAT AGG TAT GAC GTA</td>
<td>1–21</td>
</tr>
<tr>
<td>R229</td>
<td>5’ CGT GTG CAG TAT ACT TGG CCC T 3’ (BstZ17I)</td>
<td>249–270</td>
</tr>
<tr>
<td>Fragment e primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT4890</td>
<td>5’ AAG GCT TGG A 3’</td>
<td>4910–4919</td>
</tr>
<tr>
<td>F251</td>
<td>5’ GGA GGG CCA AGT ATA CTG CAC ACG A 3’ (BstZ17I)</td>
<td>247–271</td>
</tr>
<tr>
<td>R4774</td>
<td>5’ GTG TCA TGG TTC ACC ACG A 3’</td>
<td>4794–4812</td>
</tr>
<tr>
<td>Fragment f primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT7924</td>
<td>5’ TGC ATC AGC A 3’</td>
<td>7944–7953</td>
</tr>
<tr>
<td>F4333</td>
<td>5’ ATC TTG GCT GGA GCT TAC GT 3’</td>
<td>4334–4353</td>
</tr>
<tr>
<td>R7821</td>
<td>5’ TGG TTT TGC TCA ACC GCG T 3’</td>
<td>7841–7859</td>
</tr>
<tr>
<td>Fragment d primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT13019</td>
<td>5’ AGC TGA AGG A 3’</td>
<td>13039–13048</td>
</tr>
<tr>
<td>F7406</td>
<td>5’ TCT CAG AGT TGG CGA CGA CCC T 3’</td>
<td>7408–7426</td>
</tr>
<tr>
<td>R12887</td>
<td>5’ ATC CTG CAC CAA AGA GAC CT 3’</td>
<td>12907–13026</td>
</tr>
<tr>
<td>Fragment e primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT15327</td>
<td>5’ AAT TGA AGT GATT 3’</td>
<td>15347–15358</td>
</tr>
<tr>
<td>F12513</td>
<td>5’ TTT CAG CAT CAT TGA GCC GCC A 3’</td>
<td>12515–12533</td>
</tr>
<tr>
<td>R15278</td>
<td>5’ AAT CAG TGC GTG TTA CCA ACC ATT CTT CCA 3’ (HpaI)</td>
<td>15298–15327</td>
</tr>
<tr>
<td>3’ terminus (fragment a) primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT15392</td>
<td>5’ CAG GAA ACA GCT ATG ACA CCT GAT TCC TAG A AA CTT T 3’ (XbaI, AccI)</td>
<td>Poly (A) tail</td>
</tr>
<tr>
<td>F15301</td>
<td>5’ AGA ATG TGT GGT TAA CGG CA 3’ (HpaI)</td>
<td>15302–15321</td>
</tr>
<tr>
<td>M13</td>
<td>5’ CAG GAA ACA GCT ATG AC 3’</td>
<td>M13 sequence</td>
</tr>
</tbody>
</table>

a Primer names are organized in groups. Prefixes: RT, reverse transcription primer; F, forward PCR primer; R, reverse PCR primer.
b The T7 RNA polymerase promoter sequence in primer F20 is shown in italics. Restriction sites introduced by PCR are underlined and specified in parentheses at the end of the sequence. Silent mutations within the viral sequence are shown in boldface. Fragments a through f correspond to the letters in Fig. 1.
c The nucleotide positions within the VR-2332 genome are based on GenBank accession numbers PRU87392 and AF094475 (26, 30).

highly relevant to obtain infectious cDNA clones representing both genetic types of PRRSV.

The present article describes the establishment of an infectious full-length cDNA clone of the North American-type isolate VR-2332. VR-2332 was chosen because it is the prototypical North American-type isolate, is well characterized, and has been shown to be highly virulent for sows and piglets (5, 10, 13). In addition, VR-2332 forms the basis of an attenuated live vaccine (3, 22), the complete genome sequences of both VR-2332 and the vaccine virus have been published (3, 26, 41), and both VR-2332 and the vaccine virus are freely available. Given the availability of complete genome sequences for the vaccine and VR-2332 strains, the infectious clone of VR-2332 should be immediately applicable for the identification of genetic attenuation and virulence determinants in the PRRSV genome, with a view to the development of second-generation, genetically engineered vaccines.

**MATERIALS AND METHODS**

**Cells and virus.** The virus used in the present study was a third cell culture passage of the highly virulent North American-type isolate VR-2332 (American Type Culture Collection).

Marc-145 cells were grown in Eagle’s minimal essential medium (EMEM) with 5% fetal calf serum. BHK-21C cells were grown in EMEM with 10% fetal calf serum. BHK-21C cells were grown in EMEM with 10% fetal calf serum. BHK-21C cells were grown in EMEM with 10% fetal calf serum. BHK-21C cells were grown in EMEM with 10% fetal calf serum. BHK-21C cells were grown in EMEM with 10% fetal calf serum. BHK-21C cells were grown in EMEM with 10% fetal calf serum.

Marc-145 and PPAM are fully permissive for VR-2332 and were used to propagate and titrate the virus. To rescue virus from the infectious cDNA clones, cells had to be transfected with in-vitro-transcribed RNA. Pulmonary macrophages are difficult to transfect, and preliminary experiments showed a slightly better transfection efficiency with BHK-21C cells than Marc-145 cells (not shown). Although BHK-21C cells cannot be infected by VR-2332, infectious virus was generated after transfection with viral RNA, similar to the observation for Lelystad virus (25). Therefore, BHK-21C cells were used to rescue infectious virus by transfection with in-vitro-transcribed RNA.

**RNA extraction and RT-PCR.** Viral RNA was isolated by binding to silica particles in guanidine thiocyanate as previously described (28, 29) and used immediately for cDNA synthesis.

cDNA synthesis was performed with SuperScript II reverse transcriptase (RT) (Invitrogen A/S, Taasstrup, Denmark) and specific RT primers (Table 1). A total of six fragments, covering the complete VR-2332 genome (two small fragments representing the viral termini and four large fragments representing the internal part of the genome), were subsequently PCR amplified with PfuTurbo DNA polymerase according to the manufacturer’s protocol (Stratagene, Aarhus, Denmark). The cycling conditions were 94°C for 1 min, then 30 cycles of 92°C for 20 s, 58°C for 60 s, and 72°C for 6 min, followed by 25 cycles of 92°C for 20 s, 58°C for 60 s, and 72°C for 6 min with the time increased by 10 s per cycle, and finally 72°C for 10 min.

A fragment representing the 5’ end of the viral genome immediately preceded by a T7 RNA polymerase site was made by PCR with the F20 sense and R229 antisense primers (Table 1, fragment b). The F20 primer contained, in the 5’ to 3’ direction, an XhoI site, an AscI site, the T7 promoter, and nucleotides 1 to 21 of the VR-2332 sequence (based on GenBank accession number AF094475) (30). The R229 primer introduced a silent mutation at nucleotide 259 (based on GenBank accession numbers PRU87392 and AF094475) (26, 30). The R229 primer contained a silent mutation at nucleotide 259 (based on GenBank accession numbers PRU87392 and AF094475) (26, 30). The R229 primer contained a silent mutation at nucleotide 259 (based on GenBank accession numbers PRU87392 and AF094475) (26, 30). The R229 primer contained a silent mutation at nucleotide 259 (based on GenBank accession numbers PRU87392 and AF094475) (26, 30). The R229 primer contained a silent mutation at nucleotide 259 (based on GenBank accession numbers PRU87392 and AF094475) (26, 30). The R229 primer contained a silent mutation at nucleotide 259 (based on GenBank accession numbers PRU87392 and AF094475) (26, 30).
pOK12 plasmid (37) was prepared by inserting the above-mentioned stuffer fragment containing all the unique restriction sites shown in the top cartoon in Fig. 1. A full-length cDNA clone of VR-2332 was assembled by following the multistep strategy illustrated in Fig. 1. First, the complete viral genome was divided into six fragments flanked by unique restriction sites naturally found in the viral sequence. As shown in the bottom cartoon, these fragments were individually cloned into the pOK12 vector in the order indicated by the letters a to f. Prior to viral genome assembly, pOK12 was prepared by inserting a stuffer fragment containing all the unique restriction sites shown in the top cartoon in Fig. 1 and Table 1 (fragments c through f) were designed to allow assembly with unique restriction sites naturally found in the viral genome. The primers used for RT-PCR amplification of these four internal fragments of the heart based (nuclease) were cycle sequenced with a T7 RNA promoter were fused to the genome. The asterisk indicates the transcription start site of T7 RNA polymerase, resulting in the sequence 5'-m7G(5')ppp(5')G cap analog-TA TGA CGT ATA GGT...3' as the predicted 5' terminus of RNA transcribed in vitro with the T7 mMessage Machine kit. Downstream of the 3' nontranslated region, a poly(A) tail of 38 A's and the restriction sites AciI and XhoI were inserted. The complete viral genome was divided into six fragments flanked by unique restriction sites, represented by the horizontal lines labeled a through f. The length of each fragment is indicated in parentheses below the horizontal lines (in nucleotides). As shown in the bottom cartoon, these fragments were individually cloned into the pOK12 vector in the order indicated by the letters a to f. The length of each fragment is indicated in parentheses below the horizontal lines (in nucleotides). As shown in the bottom cartoon, these fragments were individually cloned into the pOK12 vector in the order indicated by the letters a to f. Prior to viral genome assembly, pOK12 was prepared by inserting a stuffer fragment containing all the unique restriction sites shown in the top cartoon in the XhoI and XbaI sites.

For transfection, BHK-21C cells were seeded in six-well plates (20,000 cells/well in 2 ml of medium) and grown overnight to approximately 80% confluence. Then 5 μg of in vitro-transcribed RNA was mixed with 10 μl of DMRIE-C (1,2-dimyristoyl-sn-glycero-3-phosphocholine with 2% DMRIE-C and added directly to the medium. As a negative control, DMRIE-C without RNA was added to BHK-21C cells. As a positive control, BHK-21C cells were transfected with viral RNA from the parental VR-2332 isolate. This control RNA was extracted by acid phenol-chloroform purification followed by binding of the RNA to silica particles in guanidine thiocyanate (29). After 4 h of exposure to DMRIE-C and RNA, the monolayers were washed, and fresh medium was added. Supernatants from cells at 24 h posttransfection were serially passaged first on Marc-145 cells (four passages, each for 5 days) and then on PPAM (one passage, 3 days).

FIG. 1. Multistep strategy used to assemble full-length cDNA clone of VR-2332. In the top cartoon, the organization of the viral genome is shown, as are the positions of the unique restriction sites used for cloning purposes. The numbers 1a, 1b, and 2 through 7 indicate the PRRSV restriction sites and a T7 RNA promoter were fused to the genome. The asterisk indicates the transcription start site of T7 RNA polymerase, resulting in the sequence 5'-m7G(5')ppp(5')G cap analog-TA TGA CGT ATA GGT...3' as the predicted 5' terminus of RNA transcribed in vitro with the T7 mMessage Machine kit. Downstream of the 3' nontranslated region, a poly(A) tail of 38 A's and the restriction sites AciI and XhoI were inserted. The complete viral genome was divided into six fragments flanked by unique restriction sites, represented by the horizontal lines labeled a through f. The length of each fragment is indicated in parentheses below the horizontal lines (in nucleotides). As shown in the bottom cartoon, these fragments were individually cloned into the pOK12 vector in the order indicated by the letters a to f. Prior to viral genome assembly, pOK12 was prepared by inserting a stuffer fragment containing all the unique restriction sites shown in the top cartoon in the XhoI and XbaI sites.
Titration and detection of virus were performed with an immunoperoxidase monolayer assay essentially as described by Bottrn et al., with the monoclonal antibody SDOW17 directed against the PRRSV nucleocapsid protein (27).

**Discrimination between the cloned virus and VR-2332.** Viral RNA was extracted with the QiaAmp viral RNA minikit (Qiagen, Hilden, Germany) and reverse transcription with Ready-to-go RT beads (Amersham Bioscience, Hørsholm, Denmark) and random hexamers was performed as described previously (29). A 990-bp fragment containing the BstZ171 site engineered in the cloned virus was PCR amplified with the primers 5'CCT TTT GGA TGA TTA TCA ACA GCA GCC TAA GGA GAT CCA CAT GTA CAG AAG TCA GGC AAC ACT GGA CAC TCC ACC CC-3' and 5'GAA TCC AAA CAA GCT CCA CCA GCA GCT-3' and for PCR amplification were digested with BstZ171 and analyzed on a 2.5% agarose gel.

**Inoculation in pigs.** Twelve 5.5-week-old pigs from a specific-pathogen-free and PRRSV-seronegative herd were divided into three groups, each consisting of four animals. The pigs were Landrace/Yorkshire crossbred. The first group received 10^4.8 TCID50 of the parental VR-2332 isolate (third passage on Marc-145 cells) per ml, the second group received 10^5 TCID50 of the parental virus isolate VR-2332 (fourth passage on Marc-145 cells) per ml, and the third group was mock inoculated with EMEM. All the animals received 1 ml of inoculum in each nostril. The animals were kept in separate rooms throughout the experiment and observed daily for clinical signs of disease. Blood samples were collected on days 0, 7, 14, and 21 postinfection and tested for antibodies by blocking enzyme-linked immunosorbent assay and the immunoperoxidase monolayer assay (6, 33). All pigs were euthanized on day 21 postinfection.

**Nucleotide sequence accession number.** The complete genomic sequence for the infectious full-length cDNA clone of VR-2332 described in this report has been deposited as GenBank accession number AY150564.

**RESULTS**

**Assembly of full-length cDNA clone of VR-2332.** Marc-145 cells were infected with the VR-2332 isolate (third passage on Marc-145 cells) at a multiplicity of infection of 1.3 TCID50/cell, and virus was harvested 5 days postinfection. From this material, a full-length cDNA clone covering the entire genome of the pathogenic North American isolate VR-2332 was assembled from overlapping PCR fragments flanked by unique restriction sites (Fig. 1). In order to minimize the number of PCR mutations, the amplifications were performed with a proofreading thermostable DNA polymerase (12). Nevertheless, even a proofreading polymerase would be expected to introduce mutations in a target of this size (Fig. 1; the North American-type PRRSV genome is 15.4 kb long). Therefore, to minimize PCR artifacts, the individual PCR fragments (labeled a through f in Fig. 1) were verified by sequencing before being selected for the final assembly. Finally, to ensure that the infectious clone was as well characterized as possible, the completely assembled VR-2332 sequence in the full-length clone (complete 15.4-kb construct flanked by XhoI and XbaI sites in pOK12, as shown in Fig. 1) was DNA sequenced.

In total, 11 nucleotide differences were identified (Table 2) when the DNA sequence of the full-length cDNA clone was compared to previously published full-length sequences of North American isolates (GenBank accession numbers AF094475 and PRU87392, 26,30). Three of these differences were silent mutations that were introduced intentionally to generate the BstZ171 and HpaI restriction sites (Tables 1 and 2). The remaining eight nucleotide differences were either PCR artifacts or the result of genetic variation in the VR-2332 isolate. Only two of the nucleotide mutations resulted in amino acid changes. These were at nucleotides 5520 (Tyr → Ile) and 6854 (Asp → Asn), both located in ORF 1a (Table 2).

Capped RNA was in vitro transcribed from the AcI-linearized full-length cDNA clone with T7 RNA polymerase, and the quality of the RNA was verified by gel electrophoresis (data not shown). At the 5′ end, the in vitro-transcribed RNA had a nonviral G corresponding to the transcription initiation site of the T7 RNA polymerase (Fig. 1).

To recover infectious virus from the full-length cDNA clone, BHK-21C cells were transfected with the capped RNA with the transfection reagent DMRIE-C. Supernatants from the transfected BHK-21C cells obtained 24 h posttransfection were serially passaged four times on Marc-145 cells. Positive staining for nucleocapsid protein was detected in Marc-145 cells inoculated with supernatant from passage 1 (Fig. 2). As nucleocapsid production is a late event in PRRSV replication and requires the production of subgenomic mRNA, nucleocapsid staining strongly indicated that the infectious clone was not grossly impeded in any step of intracellular replication (Fig. 2). Similar results were observed after transfection with viral RNA from the VR-2332 isolate. However, no obvious cytopathic effect was detected earlier than passage 3 after transfection with the in vitro-transcribed RNA. This observation deviated from the results obtained when BHK-21C cells were transfected with viral RNA from the parental VR-2332 strain. In this case, cytopathic effect was observed as early as passage 1 on Marc-145 cells.

Finally, the supernatant from the fourth Marc-145 cell passage was passaged once on PPAM. Positive antinucleocapsid staining in the immunoperoxidase monolayer assay confirmed the presence of virus replication in PPAM. These results indicated that the cloned virus possessed the ability to replicate not only in Marc-145 cells but also in PPAM, as observed for the parental VR-2332 virus isolate.

**Discrimination between cloned virus and VR-2332.** To exclude that the results obtained from the cloned virus were artifactual, for example, due to laboratory contamination with the parental VR-2332 isolate, we assayed the cloned virus

### TABLE 2. Nucleotide differences between the parental VR-2332 isolate and the full-length cDNA clone

<table>
<thead>
<tr>
<th>Nucleotide position within VR-2332 genome*</th>
<th>“Consensus” nucleotide of North American isolates†</th>
<th>Nucleotide in cDNA clone</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>259</td>
<td>G</td>
<td>A</td>
<td>Silent</td>
</tr>
<tr>
<td>1075</td>
<td>C</td>
<td>T</td>
<td>Silent</td>
</tr>
<tr>
<td>5551</td>
<td>T</td>
<td>A</td>
<td>Silent</td>
</tr>
<tr>
<td>6854</td>
<td>G</td>
<td>A</td>
<td>D → N</td>
</tr>
<tr>
<td>0967</td>
<td>T</td>
<td>C</td>
<td>Silent</td>
</tr>
<tr>
<td>7555</td>
<td>T</td>
<td>C</td>
<td>Silent</td>
</tr>
<tr>
<td>10644</td>
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<td>T</td>
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<td>15314</td>
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</tr>
<tr>
<td>15318</td>
<td>T</td>
<td>C</td>
<td>Silent</td>
</tr>
</tbody>
</table>

* Nucleotide positions within the VR-2332 genome are based on GenBank accession numbers AF094475 and PRU87392 (26,30).
† After final assembly in pOK12, the infectious clone (15.4-kb fragment flanked by XhoI and XbaI sites in pOK12; Fig. 1) was sequenced in total (GenBank accession number AY150564). This sequence was compared to a “consensus” sequence of previously published full-length sequences of North American isolates (GenBank accession numbers PRU87392, AF094475, AF066183, AF176348, AF066869, and AF159149) (2,26,38,41). In total, 11 nucleotide differences were observed, as shown in the table. The mutations at nucleotide positions 259, 15314, and 15318 were introduced intentionally to create BstZ171 and HpaI restriction sites, respectively. The sites were used for cloning purposes (see Fig. 1). Furthermore, the BstZ171 site was used as a genetic marker for the cloned virus.
FIG. 2. Detection of cloned virus replication in Marc-145 cells. Supernatants from BHK-21C cells transfected with either (a) transfection reagent DMRIE-C without RNA as a negative control reaction or (b) RNA transcribed in vitro from the full-length cDNA clone were used to infect Marc-145 cultures. At day 3 after infection, the Marc-145 cultures were ethanol fixed and stained with monoclonal antibody SDOW17, directed against PRRSV nucleocapsid protein (a late marker of viral replication), and a horseradish peroxidase-conjugated secondary antibody (immunoperoxidase monolayer assay).
Inoculation in pigs. In order to investigate the infectivity of the cloned virus in vivo and to compare it to the infectivity of the parental VR-2332 isolate, we performed an inoculation experiment in young pigs. As VR-2332 is a highly virulent isolate, we could gauge infectivity by clinical signs. Additionally, the seroconversion kinetics were monitored.

A blue discoloration of the ears was detected at day 9 postinfection in two of four pigs inoculated with the cloned virus (Fig. 5). Also at day 9, four of four pigs inoculated with the parental VR-2332 isolate exhibited a blue discoloration of the ears (Fig. 5). This discoloration was transient, lasting approximately 6 days. All four pigs in the VR-2332 group exhibited inappetence and appeared lethargic on days 7, 8, and 9 postinfection. Furthermore, two of four pigs in the VR-2332 group exhibited lameness from day 10 postinfection. Postmortem results revealed hemopurulent infection of the knee joint in one pig and the elbow joint in the other. These findings indicated that the lameness was most likely caused by a bacterial infection, in agreement with the known predisposition of PRRSV-infected animals to secondary bacterial infections. Both these pigs appeared lethargic throughout the experiment. The four negative control pigs remained vigorous, had good appetites, and did not at any time show clinical signs of disease.

All pigs were seronegative prior to infection (day 0). In the group infected with the cloned virus, three of four pigs had seroconverted on day 7 postinfection and four of four pigs on day 14 postinfection (Table 3). In the VR-2332-infected group, all four pigs had seroconverted on day 7 postinfection (Table 3). PRRSV-specific antibodies were not detected in pigs from the negative control group (Table 3).

DISCUSSION

In the current work, we established an infectious cDNA clone of the highly virulent, prototypical North American PRRSV isolate VR-2332 (5, 10, 13). While the construction of infectious viral clones is becoming commonplace, the technical
FIG. 5. Development of blue ears in pigs inoculated with cloned virus. A classical clinical sign of PRRSV infection, blue discoloration of the ears, was detected at day 9 postinfection in pigs experimentally infected with (a) the cloned virus or (b) the parental VR-2332 strain.
challenge involved in cloning the genomes of mammalian viruses in bacterial plasmids remains significant (7, 18, 19, 40). In particular, it is impossible to predict in advance how stable a given infectious clone will be during propagation in *E. coli*. Plasmid toxicity is not completely understood but may result from vector-specific as well as insert-specific causes (7, 18, 19, 40). Thus, a key parameter in infectious clone construction is the plasmid backbone. We used the low-copy pOK12 plasmid (37), which has been used previously for the assembly of an infectious clone of European-type PRRSV (25), and found that pOK12 also provided an appropriate backbone for an infectious clone of American-type PRRSV. Given the relatively large level of genetic differences between European-type and American-type PRRSV (2, 21, 26), we consider this a nontrivial technical achievement which paves the way for further infectious clones of North American-type as well as European-type PRRSV, for example, based on isolates differing in virulence.

The main design feature of our infectious clone is the division of the viral genome into six cassettes flanked by unique restriction sites, making future manipulation of the cloned virus very easy (Fig. 1, cassettes labeled a through f). Furthermore, the clone was completely sequenced (GenBank accession number AY150564), i.e., it has been fully genetically characterized. Finally, the clone is based on a highly virulent PRRSV strain which produces pronounced clinical and histopathological changes that are reproducible and easy to monitor during experimental infection of pigs. We expect that these features will make the infectious clone useful for the rapid identification of attenuation and virulence determinants in the PRRSV genome.

To rescue infectious virus from the pOK12 clone, full-length PRRSV genomic RNA was generated by in vitro transcription and transfected into BHK-21C cells. Since previous results indicated that a cap structure enhances the specific activity of transfected PRRSV genomic RNA (25), the cap analog 5′ppp(5′)G was included during in vitro transcription. By passaging supernatants from transfected BHK-21C cells on Marc-145 cells, stocks of infectious virus at a reasonable titer (10^4.8 TCID\textsubscript{50}/mL, sufficient for experimental infections) could be obtained. Identification of a marker *Bst*Z171 site introduced in the cDNA clone confirmed that the recovered virus did not represent contamination by the parental VR-2332 strain.

An infectious clone should ideally be genetically completely identical to the parental virus. In practice, this is difficult to achieve. First, the sequence for the parental virus, especially when dealing with RNA viruses, may be ambiguous due to the heterogeneity (sometimes referred to as quasispecies structure) often seen in RNA viruses (20, 31). Second, during clone construction, some mutations will invariably occur during RT-PCR amplification (8, 12), other mutations have to be introduced for cloning purposes, and viral 5’ termini are sometimes difficult to reproduce faithfully with the T7 RNA polymerase system. Thus, comparing the biological properties of an infectious clone-derived virus and its parental strain is important. We found that, compared to the parental VR-2332 strain, the cloned virus exhibited the same cell tropism (replicated in Marc-145 cells as well as PPAM), exhibited essentially the same growth kinetics in Marc-145 cells (Fig. 4), and induced seroconversion in experimentally infected pigs (Table 3). Most importantly, experimental infection with the cloned virus induced a classical clinical sign of PRRSV infection, blue discoloration of the ears, which was also observed following infection with the parental VR-2332 strain. Thus, the cloned virus was qualitatively very similar to the parental VR-2332 strain. However, some quantitative differences were observed. First, the cloned virus replicated to slightly lower titers in Marc-145 cells (Fig. 4). Second, the seroconversion kinetics in pigs were marginally slower for the cloned virus (Table 3).

The quantitative biological differences between the cloned virus and VR-2332 mentioned above were minor and have as yet not been confirmed in repeated experiments. Thus, the slightly lower titers in Marc-145 cells (Fig. 4) might be due to experimental variability, and differences in the virus dose used for the animal experiments could account for the marginally quicker seroconversion kinetics (Table 3) and more pronounced clinical changes (see results) in the VR-2332-infected group. Alternatively, the quantitative biological differences observed between the cloned virus and VR-2332 might be real and reflect mutations and genetic defects in the cloned virus, which potentially include (i) 11 nucleotide mutations (Table 2), (ii) an extra 5’ nonviral G, derived from the T7 promoter (Fig. 1 and Table 1), and (iii) a relatively short poly(A) tail (Fig. 1 and Table 1).

The 11 mutations in the cloned virus might potentially influence replication. Only two of these mutations affected coding (Table 2). Both amino acid changes are conservative (17). However, the function of the ORF 1a protein segment, where quantitative biological differences between the cloned virus and VR-2332 are pronounced, affect replication. For example, to obtain the fragment covering the 3’ end of the viral genome (Fig. 1, fragment a), an *Hpa*I restriction site was created by introduction of two silent mutations in the 3’ non-coding region.
translated region (Table 1, primer F15301). With the infectious cDNA clone of the European strain Lelystad, Verheije et al. (37) showed that a highly conserved region in the 3′ nontranslated region folds into a stem-loop, which is engaged in a seven-nucleotide kissing interaction with a similar domain in ORF 7. These domains, which are highly conserved among PRRSV isolates, may play a critical role in virus replication. The mutations introduced by primer F15301 (Table 1) are both situated in this 3′ nontranslated region stem-loop (36). Even though none of the mutations are situated within the seven-nucleotide sequence essential for the kissing interaction, mutations anywhere in this domain might weaken the interaction, thereby reducing viral replication. Experiments are planned to evaluate whether the mutations introduced by primer F15301 (Table 1) interfere with virus replication.

Finally, it should be mentioned here that extension of the 5′ terminus is considered to have a substantial effect on virus replication (7). However, the presence of an additional 5′ nonviral G in the infectious cDNA clone of Lelystad virus did not seem to impair its growth (25). Furthermore, 5′ rapid amplification of cDNA ends of the European-type PRRSV isolate 111/92 revealed a further extension of two nucleotides at the 5′ end compared to the published Lelystad virus sequence (23, 30). Thus, it is possible that there is some natural heterogeneity in the 5′ end of the PRRSV genome without this having a major effect on viral replication.

As mentioned, there are substantial genetic differences between American-type and European-type PRRSVs. Additionally, the two virus types are antigenically very different, and there is only limited cross protection against reinfection with the heterologous type (20). That is, a genetically engineered vaccine based on, for example, an infectious clone of Lelystad virus would not be expected to offer full protection against infection with American-type PRRSV. Thus, the infectious clone of American-type PRRSV generated in the present study represents a significant new opportunity for recombinant vaccine development. Furthermore, previous studies on PRRSV have mainly focused on the functional role of the structural proteins. Nonstructural proteins, important information concerning their individual roles remains to be addressed. The availability of an infectious clone of VR-2332, supplementing the infectious clones of Lelystad virus (25) and the related equine arteritis virus (34), may broaden the questions that can be examined experimentally in the field of Arterivirus replication and pathogenesis.

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