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Generation of recombinant pestiviruses using a full-genome amplification strategy

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Abstract

Complete genome amplification of viral RNA provides a new tool for the generation of modified viruses. We have recently reported a full-genome amplification strategy for recovery of pestiviruses (Rasmussen et al., 2008). A full-length cDNA amplicon corresponding to the Border disease virus-Gifhorn genome was generated by long RT-PCR and then RNA transcripts derived from this amplicon were used to rescue infectious virus. Here, we have now used this full-genome amplification strategy for efficient and robust amplification of three additional pestivirus strains: the vaccine strain C and the virulent Paderborn strain of Classical swine fever virus plus the CP7 strain of Bovine viral diarrhoea virus. The amplicons were cloned directly into a stable single-copy bacterial artificial chromosome generating full-length pestivirus DNAs from which infectious RNA transcripts could be also derived.

Keywords: *Pestivirus*, *Flaviviridae*, long RT-PCR, full-genome amplicon, infectious clone, pBeloBAC11

Introduction

The genus *Pestivirus* is part of the family *Flaviviridae* and contains economically important animal viruses such as Classical swine fever virus (CSFV), Bovine viral diarrhoea virus (BVDV) and Border disease virus (BDV). Pestivirus virions are enveloped and contain a positive-stranded RNA genome of about 12.3 kb which has a single long open reading frame flanked by non-translated regions (5'NTR and 3'NTR), which are important for viral replication. Generation of pestivirus cDNA clones is a general prerequisite for genetic manipulation of their genomes, a process that can be both lengthy and laborious. Several infectious cDNA clones containing complete pestivirus genomes have been described (Meyers et al., 1996; Moormann et al., 1996; Ruggli et al., 1996; Vassilev et al., 1997; Fan and Bird, 2008). These cDNA clones have provided new options for directed genetic manipulations of pestiviruses. However, construction of these infectious cDNA clones has been hampered due to the relatively large size of the viral RNA genome and because of genetic instability of the cloned cDNA in combination with the plasmid vectors in the bacterial host. Therefore, new strategies are needed to facilitate construction of stable infectious cDNA clones from a wider range of strains.

Generation of infectious cDNA clones can be facilitated by using long RT-PCR for full-genome amplification. This approach has been applied with success to a number of RNA viruses from the *Flaviviridae* (Tellier et al., 1996; Gritsun and Gould, 1998; Zhang et al., 2001). For pestiviruses, the methodology has been used to obtain the entire open reading frame for sequencing studies (Jones et al., 2006) as well as for virus rescue (Rasmussen

et al., 2008). Full-length genome amplification facilitates, in addition to virus rescue, direct full-length sequence analysis and the amplicons can also be directly inserted into suitable cloning vectors.

In the present study, we developed an optimized, efficient and streamlined method for the robust amplification of full-length cDNAs of four strains (BVDV-CP7, BDV-Gifhorn, CSFV-C and CSFV-Paderborn). This procedure was followed by the insertion of the BDV and CSFV amplicons into the stable single-copy vector pBeloBAC11 (Wang et al., 1997). This bacterial artificial chromosome (BAC) has been shown to successfully maintain the full-length cDNAs of Japanese encephalitis virus (Yun et al., 2003) and BVDV-SD1 (Fan and Bird, 2008). Using this procedure, we have generated new full-length cDNA clones of BDV and CSFV, which enlarges the range of cloned pestivirus genomes that can be manipulated.

Materials & Methods

The different viruses used in this study were; BVDV-CP7 from bovine KOP-R cells (10^6 $TCID_{50}$/ml); CSFV-Paderborn from porcine PK15 cells (10^7 $TCID_{50}$/ml; vaccine strain CSFV-C from porcine PK15 cells ($10^{4.75}$ $TCID_{50}$/ml); and BDV-Gifhorn from ovine SFT cells (10^6 $TCID_{50}$/ml). Infected cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% BVDV-free foetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. The total RNA was extracted from the cells using a combined Trizol/RNeasy protocol. This protocol was used to obtain high-quality full-length genomic RNA. Briefly, total RNA was extracted from 1 ml cell supernatant or cell lysate with 3 ml Trizol

LS reagent (Invitrogen) and 0.8 ml chloroform. After phase separation the aqueous phase was mixed with an equal volume of 75% ethanol and 0.7 ml of the resulting mix was transferred sequentially to the same RNeasy column (Qiagen). The column was washed according to the manufacturer's protocol. Total RNA was eluted from the column with 4 times 30 μl nuclease free water and stored at -80°C until use. Pestivirus genomes were amplified from total RNA preparations using long RT-PCR. It was found that the third RNA eluate empirically performed the best in long RT-PCR, presumable due to a higher proportion of full-length genomic RNA in this fraction.

The total RNA was reverse transcribed at 50°C for 90 minutes using SuperScript III reverse transcriptase kit (Invitrogen) and specific cDNA primers (Table 1). For improved long RT-PCR efficiency, the resulting cDNA was subsequently treated with RNAse H to remove the RNA template. Full-length PCR amplification was performed using primers specific for the 5'NTR and the 3'NTR of each strain (Table 1) with the Accuprime High Fidelity kit (Invitrogen) which consists of a mixture of *Taq* and proofreading *Pyrococcus GB-D* DNA polymerases. Reactions containing 2 μl cDNA were then amplified in a final volume of 50 μl using 94ºC for 30 seconds followed by 35 cycles of 94ºC for 15 seconds, 65ºC for 30 seconds and 68ºC for 12 minutes. Samples (1-10 μl) of each PCR reaction were analysed on a 1 % agarose gel in TBE buffer alongside a 1kb DNA ladder (Fermentas).

Standard techniques were used for cloning of the full-length amplicons into pBeloBAC11 (Invitrogen). Briefly, pBeloBAC11 (7507 bp) was digested with *Not*I and afterwards treated with Antarctic phosphatase (NEB).

The full-length amplicons of about 12.3 kb were also digested with *Not*I and purified using the Nucleotide removal kit (Qiagen). Equal amounts of vector and amplicons were mixed and ligated in 25 μl reactions overnight at 16ºC using DNA ligase for long fragments (Takara Bio) according to the manufacturer's recommendations. The products (1 μl) were electroporated into electrocompetent DH10B T1 phage resistant *E.coli* (Invitrogen) and transformants harbouring the cloned amplicons were isolated from selective LB plates containing 12.5 μg/ml chloramphenicol. Small scale preparations of DNA from selected transformants were obtained using the BAC miniprep protocol previously described (Warming et al., 2005) and the presence of the full-length pestivirus cDNA was determined following restriction analysis using *Not*I. Selected BAC DNAs were digested with *Not*I, *in vitro* transcribed using T7 RNA polymerase and the RNA transcripts were tested for infectivity as previously described (Reimann et al., 2004; Rasmussen et al., 2007). Immunostaining of electroporated porcine SK6 cells (CCLV RIE262) was performed with the pan-pestivirus antibody (C16) targeting the non-structural NS3 proteins (Reimann et al., 2004).

Results

Full-length cDNAs corresponding to four different pestivirus strains were successfully amplified in single long RT-PCRs using an optimized version of the full-genome amplification strategy described by Rasmussen et al. (2008). The original protocol was modified by substituting the Elongase enzyme mix with the Accuprime High Fidelity kit. This polymerase mix, in combination with the

Superscript III reverse transcriptase, efficiently produced, from each of the different viral RNAs, amplicons of approximately 12.3 kb, corresponding to the full-length genomes (Fig. 1). The Accuprime High Fidelity polymerase mix increased the specificity and efficiency of the long RT-PCR compared to the Elongase enzyme mix and produced distinct products of the expected size without smears and aberrant amplification products (Fig. 1). High yields (several micrograms) of DNA were obtained in each long RT-PCR. For amplification of complete genomes in a single RT-PCR, specific primers were designed based on nucleotide sequences of the terminal 5'NTR- and 3'NTR- genomic ends (Table 1). The primers for amplification of BDV-Gifhorn were described in Rasmussen et al. (2008). The primers for BVDV-CP7 (Meyers et al., 1996, Accession number BVU63479), and for the CSFV-C (Accession number AY259122) were based on published sequences. Similarly, the 5'NTR primer for CSFV-Paderborn was designed from Accession number AY072924 (Oleksiewicz et al., 2003) but this sequence lacks approximately 75 nucleotides from the terminus of the 3'NTR. Therefore, the 3'Paderborn reverse primer was designed using a nucleotide alignment of known full-genome CSFV sequences. Generally, knowledge of less than 50 nucleotides of the terminal sequences from each of the strains was needed for successful amplification of the whole genome.

By incorporating unique *Not*I restriction sites within the primers (Table 1), the amplicons were designed for subsequent insertion into pBeloBAC11. Full-length amplicons of the two strains CSFV-C and CSFV-Paderborn, and also the BDV-Gifhorn were obtained using the respective *Not*I

159 primers (Fig. 2). Amplification and cloning of BVDV-CP7 was not attempted by
160 this strategy due to an internal *Not*I site in the cDNA sequence.

161 The amplified genomes were digested with *Not*I and ligated directly
162 into the *Not*I site in pBeloBAC11. Electroporation of DH10B *E.coli* cells with the
163 ligated products yielded small and large bacterial colonies (from a few to
164 several hundreds) after incubation for 24 hours at 37ºC. Both small and large
165 colonies were tested for the presence of full-genome amplicons. Generally, two
166 thirds of the tested transformants carried a BAC containing an insert of the
167 expected size of approximately 12.3 kb (Fig. 3). One third of the transformants
168 either carried BACs with smaller inserts, empty BACs or no visible BAC vector.
169 There was no correlation between the size of the BAC inserts and the size of
170 the bacterial colonies.

171 Testing of the BAC clones for their ability to generate virus
172 infectivity in cells is ongoing in our laboratories. The preliminary results of this
173 evaluation show that infectious pestiviruses can be rescued from the DNAs
174 generated by direct insertion of full-length genomes into the BAC vector. For
175 example, selected BAC clones harbouring the CSFV-Paderborn full-length
176 cDNA were *Not*I digested, *in vitro* transcribed and the RNA transcripts were
177 tested for infectivity by electroporation into porcine cells. RNA derived from one
178 out of two tested BAC clones (pBeloPader10) was shown to efficiently replicate
179 in porcine cells. The cells electroporated with RNA transcripts from
180 pBeloPader10 showed a high number of NS3-positive cells (as judged by
181 immunofluorescence, Fig. 4) 24 hours later and infectious virus could be
182 recovered after 1st and 2nd cell passages. In addition, RNA transcripts derived

183 from three BAC clones harbouring BDV-Gifhorn full-length cDNA have been
184 tested for infectivity in ovine cells. Following electroporation, viral RNA derived
185 from each of these three BAC clones was found to be replicating in the cells
186 (data not shown). However, only rather few cells were scored as positive after
187 the 1st cell passage indicating a possible growth defect in these constructs.
188

189 **Discussion**

190 The use of the full-length genome amplification strategy for amplification of
191 pestivirus genomes has several advantages; (i) the same optimized protocol
192 can be used for amplification of a wide selection of strains; (ii) only limited
193 terminal nucleotide sequence information of a given strain is needed for
194 amplification of the whole genome; (iii) multiple cloning steps can be
195 circumvented since full-length amplicons can be directly *in vitro* transcribed or
196 inserted into plasmid vectors; and (iv) construction of genetically modified
197 viruses can be facilitated using stable cDNA clones.

198 In the present study, four different pestiviruses representing each of
199 three main types (CSFV, BVDV and BDV) were amplified by long RT-PCR. This
200 demonstrates that the recently described full-length genome amplification
201 strategy (Rasmussen et al., 2008) is of a generic nature since it can be utilized
202 for amplification of diverse pestivirus strains. Furthermore, the direct cloning of
203 the amplified genomes into the stable single-copy BAC vector, pBeloBAC11,
204 shows that we have created an efficient, streamlined strategy for direct
205 preparation of new pestivirus cDNA clones. Full-length cDNAs corresponding to
206 other members of the *Flaviviridae,* cloned in pBeloBAC11, have previously been

shown to be stable after multiple generations in *E.coli* cells (Yun et al., 2003; Fan and Bird, 2008) and we anticipate that our constructs will display similar stability in the bacterial host but this does need to be determined.

Our new strategy allows construction of stable infectious BAC DNAs from a single full-length PCR product, which gives an increased flexibility in the design of new genetically modified pestiviruses. Since long RT-PCR preserves the genetic variants present in a virus population (Chumakov, 1996) many independent full-length cDNA clones can be obtained and tested which increases the probability of finding infectious constructs. For targeted design of genetically modified pestiviruses, the efforts can now be expedited and focused on, in principal, any pestiviral strain and is hence not limited by the availability of existing infectious cDNAs. Furthermore, our full-genome amplification protocol obviates the time-consuming and costly process of construction and screening of infectious clones by traditional methods**.** Therefore, the full-genome amplification strategy significantly simplifies and streamlines the workflow for the generation of new recombinant pestiviruses and also facilitates direct full-length sequence analysis.

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231 **Conflict of interest statement**

232 None

233

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Table 1: Primers used for long RT-PCR[a]

Primers for CSFV-C and CSFV-Paderborn	
5'Paderborn_T7 (forward)	TAATACGACTCACTATAGTATACGAGGTTAGCTCGTCCTCGTGTACAACATT
5'Paderborn_T7_NotI (forward)	ATAT*GCGGCCGC*TAATACGACTCACTATAGTATACGAGGTTAGCTCGTCCTCGTGTACAACATT
3'Paderborn (reverse)	GGGCCGTTAGGAAATTACCTTAGTCCAACTGTGGA
3'Paderborn-NotI (reverse)	ATAT*GCGGCCGC* GGGCCGTTAGGAAATTACCTTAGTCCAACTGTGGA
3'Paderborn_cDNA (reverse)	GGGCCGTTAGGAAATTACCTTAGT
5'C-strain_T7 (forward)	TAATACGACTCACTATAGTATACGAGGTTAGTTCATTCTCGTATACACGATTGGACAAATC
5'C-strain_T7_NotI (forward)	ATAT*GCGGCCGC*TAATACGACTCACTATAGTATACGAGGTTAGTTCATTCTCGTATACACGATTGGACAAATC
Primers for BVDV-CP7	
5'BVDV CP7_T7 (forward)	TAATACGACTCACTATAGTATACGAGAATTAGAAAAGGCAC
3'BVDV CP7 (reverse)[b]	GGGTGACGTCGGGTGTACCCTCATAC
Primers for BDV-Gifhorn	
5'T7Gif-NotI (forward)	ATAT*GCGGCCGC*TAATACGACTCACTATAGTATACGAGAGTAGTTCAGGCTCGTATGCAAAATTGGGTGTTTC
3'Gif-NotI (forward)	ATAT*GCGGCCGC*GGGGCTGTTAGGGTTTTTCCTTAATCCAACTATGGACTTCAG

[a] T7 promoter is underlined, and *Not*I sites are shown in italics.

[b] Same primer was also used as cDNA primer.

Fig. 1. Full-genome amplification of pestiviruses by long RT-PCR using T7-5'NTR and 3'NTR primers. The pictures show agarose gel electrophoresis of the full-length amplicons of BDV-Gifhorn (A, 1 μl), BVDV-CP7 (B, 1 μl), CSFV-C (C, 10 μl) and CSFV-Paderborn (D, 10 μl).

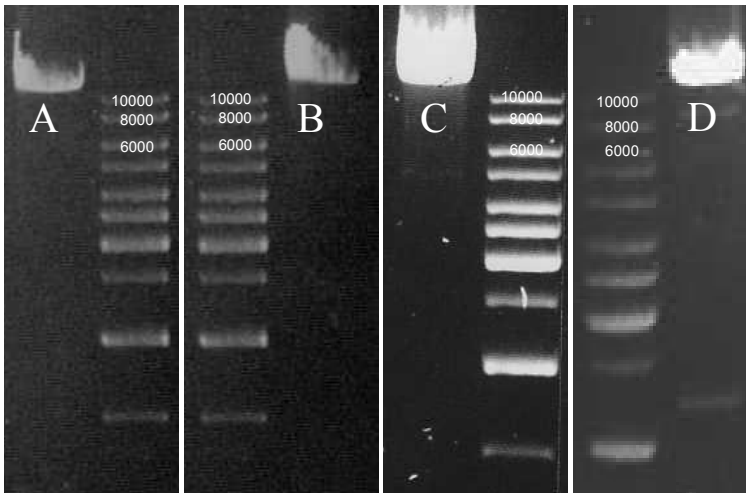
Fig. 2. Full-genome amplification of pestiviruses by long RT-PCR using T7-5'NTR and 3'NTR primers containing *Not*I restrictions sites. The picture shows agarose gel electrophoresis of the full-length amplicons of BDV-Gifhorn (A, 10 μl), CSFV-C (B, 10 μl) and CSFV-Paderborn (C, 10 μl). The non-specific bands in A and C are most likely caused by the increased complexity of the primers (extra *Not*I sites).

Fig. 3. *Not*I digestion of BAC transformants. DNA preparations from BAC transformants were digested with *Not*I and analysed by agarose gel electrophoresis. Four have the correct size insert (A-C, E), whereas one (D) has a smaller insert, and another (F) contained no detectable BAC vector.

Fig. 4. Recovery of infectious CSFV-Paderborn derived from *in vitro* transcribed pBeloPader10 RNA electroporated into porcine SK6 cells. The pictures show immunofluorescence (IF) staining of cells expressing the NS3 proteins after the 1st (Panel A) and 2nd (Panel B) cell culture passage. Panels C and D are control cells electroporated without any RNA transcripts.

332 Figure 1.

333



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337 Figure 2.

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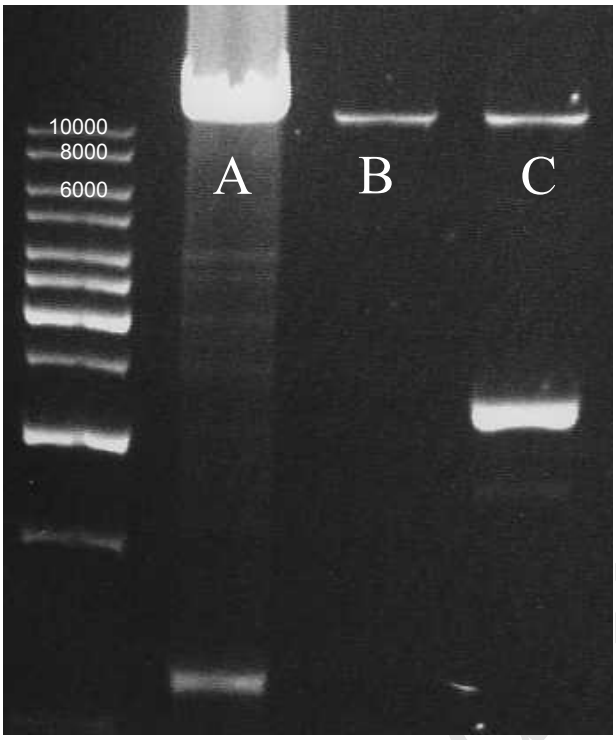

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341 Figure 3.

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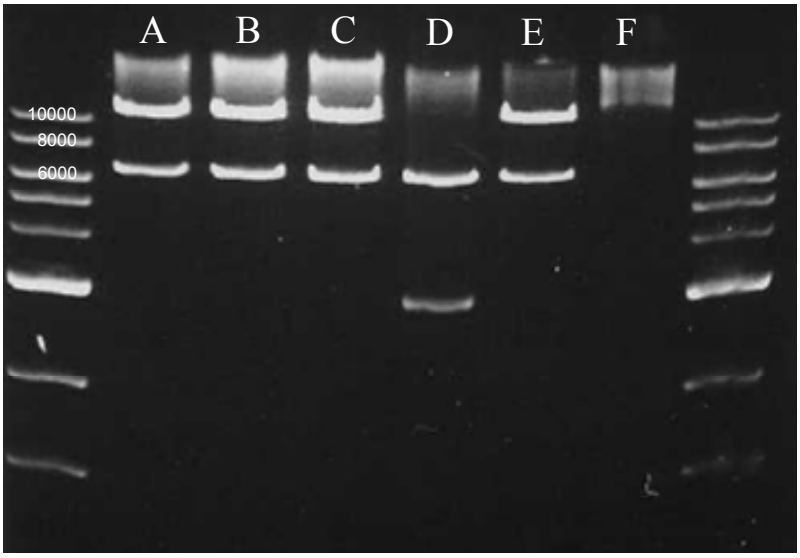

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345 Figure 4.

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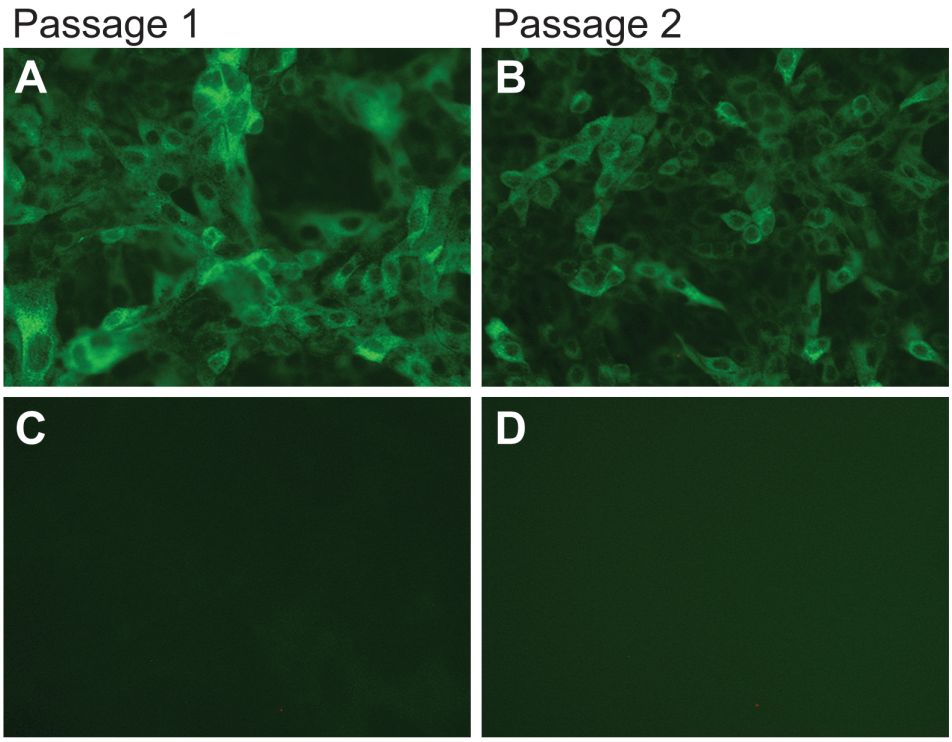

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