Development and optimization of a DNA-based reverse genetics systems for epizootic hemorrhagic disease virus

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Abstract

Epizootic hemorrhagic disease virus (EHDV) is a member of the genus *Orbivirus*, family *Reoviridae,* and has a genome consisting of 10 linear double-stranded (ds) RNA segments. The current reverse genetics system (RGS) for engineering the EHDV genome relies on the use of *in vitro*-synthesized capped viral RNA transcripts. To obtain more-efficient and simpler RGSs for EHDV, we developed an entirely DNA (plasmid or PCR amplicon)-based RGS for viral rescue. This RGS enabled the rescue of infectious EHDV from BSR-T7 cells following co-transfection with seven helper viral protein expression plasmids and 10 cDNA rescue plasmids or PCR amplicons representing the EHDV genome. Furthermore, we optimized the DNA-based systems and confirmed that some of the helper expression plasmids were not essential for the recovery of infectious EHDV. Thus, DNA-based RGSs may offer a more efficient method of recombinant virus recovery and accelerate the study of the biological characteristics of EHDV and the development of novel vaccines.

Introduction

Epizootic hemorrhagic disease (EHD) is an infectious, non-contagious disease of wild and domestic ruminants caused by infection with epizootic hemorrhagic disease virus (EHDV). EHDV is primarily transmitted by several species of biting midges of the genus *Culicoides*. EHDV infection is widespread among members of the family Cervidae (i.e., white-tailed deer) and is associated with a high mortality rate [1,2,3]. Although most EHDV serotypes rarely cause disease in livestock, EHDV-2 (Ibaraki virus) is an exception and has caused widespread disease in cattle in Japan [4, 5]. Recently, outbreaks of EHDV in cattle have been reported in several countries around the world, including Israel, Japan, Canada, Turkey, Morocco, and Réunion [6,7,8,9,10,11,12]. Therefore, EHDV represents an important threat to livestock, requiring that greater attention be paid to this economically important viral pathogen.

EHDV is a member of the genus *Orbivirus* of the family *Reoviridae*, and its genome contains 10 linear segments of double-stranded (ds) RNA (S1-S10) that encode seven structural proteins (VP1-VP7) and four non-structural proteins (NS1, NS2 and NS3/NS3a) [13,14,15]. The structural proteins VP1, VP3, VP4, VP6, and VP7 form the virus core particle, whereas VP2 and VP5 form the outer capsid layer of the EHDV virion. VP2 and VP7 are the two major proteins that determine the serotype and serogroup, respectively [1, 2].

Reverse genetics (RG) is a powerful experimental tool that enables genetic modification and manipulation of viral genomes to generate live viruses containing engineered changes in their genomic nucleic acids. The ability to introduce defined mutations into the viral genome has not only enabled rational approaches to the molecular dissection of viral gene products but also supports the rapid generation of vaccines against infectious agents. To date, segmented RNA orbiviruses (e.g., bluetongue virus [BTV] and African horse sickness virus [AHSV]) have been recovered by transfection of permissive cells with *in vitro*-synthesized and capped RNA transcripts or plasmids containing cloned cDNAs corresponding to viral genome segments [16,17,18,19]. Although an RGS for EHDV (which relies on the use of helper viral protein expression plasmids and *in vitro*-synthesized RNA transcripts) has been established [20], an entirely DNA-based RGS for EHDV has not yet been reported.

In this study, we established DNA-based RGSs for EHDV-2 that consisted of either plasmid- or PCR-based platforms. EHDV-2, which contains a silent marker mutation in genome segment S2, was recovered by transfecting BSR-T7 cells with 10 cDNA rescue plasmids or purified PCR amplicons representing the EHDV-2 genome and different helper viral protein expression plasmid combinations. This report of DNA-based RGSs and flexible combinations of helper protein expression plasmids combinations greatly simplifies EHDV reverse genetics and provides novel platforms through which to augment the existing RNA-based RGS for EHDV.

Materials and methods

Cell lines and virus

BSR-T7 cells, which stably express bacteriophage T7 RNA polymerase, were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), 5% (v/v) fetal bovine serum (FBS, Excell), and 1 mg of Geneticin (Invitrogen) per mL at 37 °C and 5% CO_2. BHK-21 cells were cultured under the same conditions in DMEM supplemented with 5% (v/v) FBS and antibiotics (100 U of penicillin and 100 μg of streptomycin [Gibco] per mL).

The Ibaraki BK13 strain of EHDV-2 (BK13) was kindly provided by Dr. Goto Yoshiyuki (National Institute of Animal Health, Tsukuba, Ibaraki, Japan).

Plasmids

Plasmids containing full-length cDNA copies of each EHDV-2 genome segment, as well as EHDV-2 protein expression plasmids expressing VP1, VP3, VP4, VP6, VP7, NS1, and NS2, were generated as described previously [20] and were named pCI-ehdvVP1, pCI-ehdvVP3,

pCI-ehdvVP4, pCI-ehdvVP6, pCI-ehdvVP7, pCI-ehdvNS1 and pCI-ehdvNS2, respectively. The pRG15 reverse genetics vector, containing a *Bsm*B I cloning site flanked by an upstream T7 RNA polymerase promoter sequence and downstream hepatitis delta virus ribozyme and T7 RNA polymerase terminator sequences [19], was used to construct the EHDV-2 rescue plasmids.

Construction of EHDV-2 reverse genetics plasmids

To construct recombinant reverse genetics plasmids, the previously cloned cDNA copy of each EHDV-2 genome segment was amplified by PCR using KOD FX DNA polymerase (Toyobo) and the appropriate primer pairs (Table 1) containing restriction sites for *Aar*I (S5, S8, and S9), *Bbs*I (S6 and S10) or *Bsa*I (S1, S2, S3, S4, and S7). Full-length PCR products were purified from an agarose gel using an AxyPrep DNA Gel Extraction Kit (Axygen), digested with the appropriate restriction enzyme, and ligated into the *Bsm*BI-digested vector pRG15. The resulting recombinant plasmids were verified by nucleotide sequencing, and the sequences matched the GenBank database sequences (GenBank accession numbers KM509050-KM509059). The recombinant plasmids were named pRG-ehdvS1 through pRG-ehdvS10. A mutant version of the EHDV-2 S2 genome segment containing an introduced *Stu*I restriction enzyme recognition sequence mutation from nucleotides 1680 to 1685 was constructed according to previously published methods [20, 21]. The plasmid was designated pRG-mehdvS2, and the presence of the mutation was confirmed by nucleotide sequencing.

Table 1. Primers used for construction of reverse genetics plasmids

Name	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')
BK13 S1 BsaI	GGAT***GGTCTC***Cgttaaaatgcaatggtc	GGAT***GGTCTC***Cgtaagtgtaatgcgg
BK13 S2 BsaI	GGAT***GGTCTC***Cgttaaattgtccccag	GGAT***GGTCTC***Cgtaagtttgttgttcccag
BK13 S3 BsaI	GGAT***GGTCTC***Cgttaaatttccagagcg	GGAT***GGTCTC***Cgtaagtgtatttccagtgc
BK13 S4 BsaI	GGAT***GGTCTC***Cgttaaaacatgccgg	GGAT***GGTCTC***Cgtaaggtgtaacatgcagg
BK13 S5 AarI	GTT***CACCTGC***GGTTgttaaaaagttcttcgtc	GTT***CACCTGC***GGTTgtaagtgtaaagttcgaatg
BK13 S6 BbsI	GT***GAAGAC***GGgttaaaaaggaggcacg	GT***GAAGAC***GGgtaagtgtaaggagttcgcg
BK13 S7 BsaI	GGAT***GGTCTC***Cgttaaaatttggtgaaaatg	GGAT***GGTCTC***Cgtaagttgaatttgggaaaac
BK13 S8 AarI	GTT***CACCTGC***GGTTgttaaaaattccctg	GTT***CACCTGC***GGTTgtaagtgtaaatcccttc
BK13 S9 AarI	GTT***CACCTGC***GGTTgttaaaaattgcgc	GTT***CACCTGC***GGTTgtaagttttaaatcgc
BK13 S10 BbsI	GT***GAAGAC***GGgttaaaaagagatcggtaccatg	GT***GAAGAC***GGgtaagtgtgtcgagatcgg

Note: Restriction enzyme sites are indicated in italics

Recovery of EHDV-2 using the plasmid DNA system

Prior to transfection, plasmid DNA was purified using a QIAfilter Plasmid Midi Kit (QIAGEN) according to the manufacturer's instructions. Procedures for plasmid transfection and the recovery of infectious virus were performed as described previously [19], with the following modifications: Briefly, BSR-T7 cell monolayers were first transfected with different combinations of helper viral protein expression plasmids, using 700 ng of pCI-ehdvVP1, 600 ng of pCI-ehdvVP3, 500 ng of pCI-ehdvVP4, 300 ng of pCI-ehdvVP6, 300 ng of pCI-ehdvVP7, 500 ng of pCI-ehdvNS1 and 300 ng of pCI-ehdvNS2, and 18 hours later, the cell monolayers were transfected with 10 recombinant reverse genetics plasmids using Lipofectamine LTX

Reagent (Life Technologies) according to the manufacturer's instructions. Transfection was carried out using 700 ng of pRG-ehdvS1, 600 ng of pRG-mehdvS2, 600 ng of pRG-ehdvS3, 500 ng of pRG-ehdvS4, 500 ng of pRG-ehdvS5, 500 ng of pRG-ehdvS6, 450 ng of pRG-ehdvS7, 400 ng of pRG-ehdvS8, 400 ng of pRG-ehdvS9 and 350 ng of pRG-ehdvS10. Six hours later, the medium was replaced with an overlay of DMEM containing 1% (w/v) low melting-point agarose and 2% (v/v) FBS. Plaques could typically be observed three to five days after the second transfection. Individual plaques were selected for amplification of the virus in BHK-21 cells. For visualization, the monolayers were stained with 0.1% (w/v) crystal violet.

Recovery of EHDV-2 using PCR amplicons

For viral rescue, recombinant reverse genetics plasmids were replaced with EHDV-2 PCR amplicons corresponding to the viral genomic RNA segments, and virus recovery was performed as described above, using the same amount of each amplicon DNA as was used for the corresponding plasmid. A PCR amplicon of each of the 10 EHDV-2 genome segments containing a T7 promoter sequence at the 5-terminal end was generated by PCR using KOD FX DNA polymerase (Toyobo) together with the corresponding cDNA clone for that genome segment as a template and the appropriate primer pair (Table 2). The PCR amplicons were purified using an AxyPrep DNA Gel Extraction Kit (Axygen) according to the manufacturer's instructions. BSR-T7 cell monolayers were first transfected with different combinations of helper viral protein expression plasmids, and a second transfection was performed 18 h later, with the 10 EHDV-2 PCR amplicons (ehdv-T7S1, mehdv-T7S2, and ehdv-T7S3-S10) using Lipofectamine LTX Reagent (Life Technologies). Six hours later, the medium was replaced with DMEM containing 1% (w/v) low-melting-point agarose and 2% (v/v) FBS. Plaques could typically be observed 3–5 days after the second transfection. Individual plaques were selected for virus amplification in BHK-21 cells. The monolayers were stained with 0.1% (w/v) crystal violet for visualization.

Table 2. Primers used for the production of EHDV-2 PCR amplicons

Name	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')
BK13 S1 BsaI	TAATACGACTCACTATAgttaaaatgcaatggtc	gtaagtgtaatgcgg
BK13 S2 BsaI	TAATACGACTCACTATAgttaaattgtccccag	gtaagtttgttgttcccag
BK13 S3 BsaI	TAATACGACTCACTATAgttaaatttccagagcg	gtaagtgtatttccagtgc
BK13 S4 BsaI	TAATACGACTCACTATAgttaaaacatgccgg	gtaaggtgtaacatgcagg
BK13 S5 AarI	TAATACGACTCACTATAgttaaaaagttcttcgtc	gtaagtgtaaagttcgaatg
BK13 S6 BbsI	TAATACGACTCACTATAgttaaaaaggaggcacg	gtaagtgtaaggagttcgcg
BK13 S7 BsaI	TAATACGACTCACTATAgttaaaatttggtgaaaatg	gtaagttgaatttgggaaaac
BK13 S8 AarI	TAATACGACTCACTATAgttaaaaattccctg	gtaagtgtaaatcccttc
BK13 S9 AarI	TAATACGACTCACTATAgttaaaaattgcgc	gtaagttttaaatcgc
BK13 S10 BbsI	TAATACGACTCACTATAgttaaaaagagatcggtaccatg	gtaagtgtgtcgagatcgg

Note: T7 RNA polymerase promoter sequences are indicated in uppercase letters

Characterization of the mutant virus

Viral dsRNA was extracted from the supernatant of infected BHK-21 cells using TRIzol LS Reagent (Ambion) according to the manufacturer's instructions, electrophoresed in a non-

denaturing 8% polyacrylamide gel, and stained with ethidium bromide. To distinguish between the wild-type and DNA-derived EHDV-2, full-length RT-PCR products of genome segment S2 from the recovered viruses were digested with *Stu*I, and the digestion products were analyzed on a 1% (w/v) agarose gel. The nucleotide sequences of the S2 genome segment cDNA from both wild-type and DNA-derived EHDV-2 were also determined.

Electron microscopy

Recombinant viruses rescued using the different RGSs were examined by transmission electron microscopy. A 20-µL sample was applied to a carbon-coated grid that had been glow-discharged for 20 s in air, and the grids were immediately negatively stained using 2% phosphotungstic acid. The grids were examined using an H-7650 transmission electron microscope (Hitachi, Tokyo, Japan) operated at 80 kV.

Virus growth curves

Confluent monolayers of BHK-21 cells in 6-well plates were infected with the virus at a multiplicity of infection (MOI) of 0.1. After virus adsorption at 37°C for 1 h, the inoculum was removed, the cell monolayers were washed with PBS, and 2 mL of DMEM (supplemented with penicillin [100 U/mL], streptomycin [100 µg/mL], and 2% [v/v] FBS) was added to each well. The contents of each well were harvested at different time points postinfection, and the samples were clarified by low-speed centrifugation to pellet the cells, after which the supernatants were collected. Viral titers were determined using an end-point dilution assay on BHK-21 cells.

Results

Recovery of infectious EHDV-2 from plasmid- and PCR-based reverse genetics platforms

To generate recombinant EHDV-2, BSR-T7 cells were first transfected with seven helper viral protein expression plasmids. After 18 h, the cells were subjected to a second transfection with either the 10 reverse genetics plasmid constructs or 10 PCR amplicons corresponding to the viral genome segments (Fig. 1). The transfected cells were covered with low melting-point agarose, and plaques could be observed at three to five days after the second transfection after staining with crystal violet (Fig. 2a). Virus rescue was performed at least three times independently, each time resulting in clearly visible plaques. To confirm virus recovery, individual plaques were selected and propagated in BHK-21 cells, and the viral dsRNA was extracted and analyzed by non-denaturing polyacrylamide gel electrophoresis. The electropherotype of plasmid-DNA-derived EHDV-2 (termed mut-rBK13-1) and PCR-amplicon-derived EHDV-2 (termed mut-rBK13-2) was identical to that of wild-type EHDV-2 derived from infected cells (termed wtBK13) (Fig. 2b).

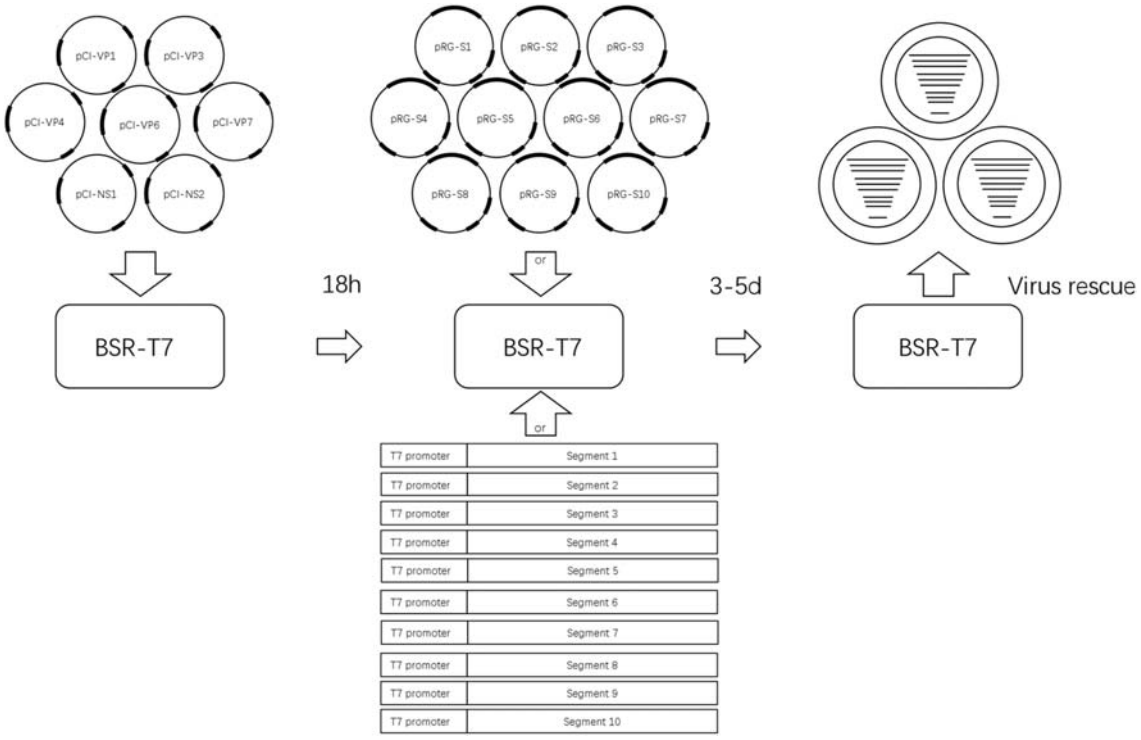

Fig. 1. Strategy for recovery of EHDV-2 from plasmids or PCR amplicons. BSR-T7 cells were first transfected at 80% confluence in 6-well plates with several protein expression plasmids. A second transfection was performed 18h later with 10 recombinant plasmids or 10 PCR amplicons. The rescued EHDV-2 was recovered from BSR-T7 cells with protein expression and genome replication occurring within 3-5 days after the second transfection

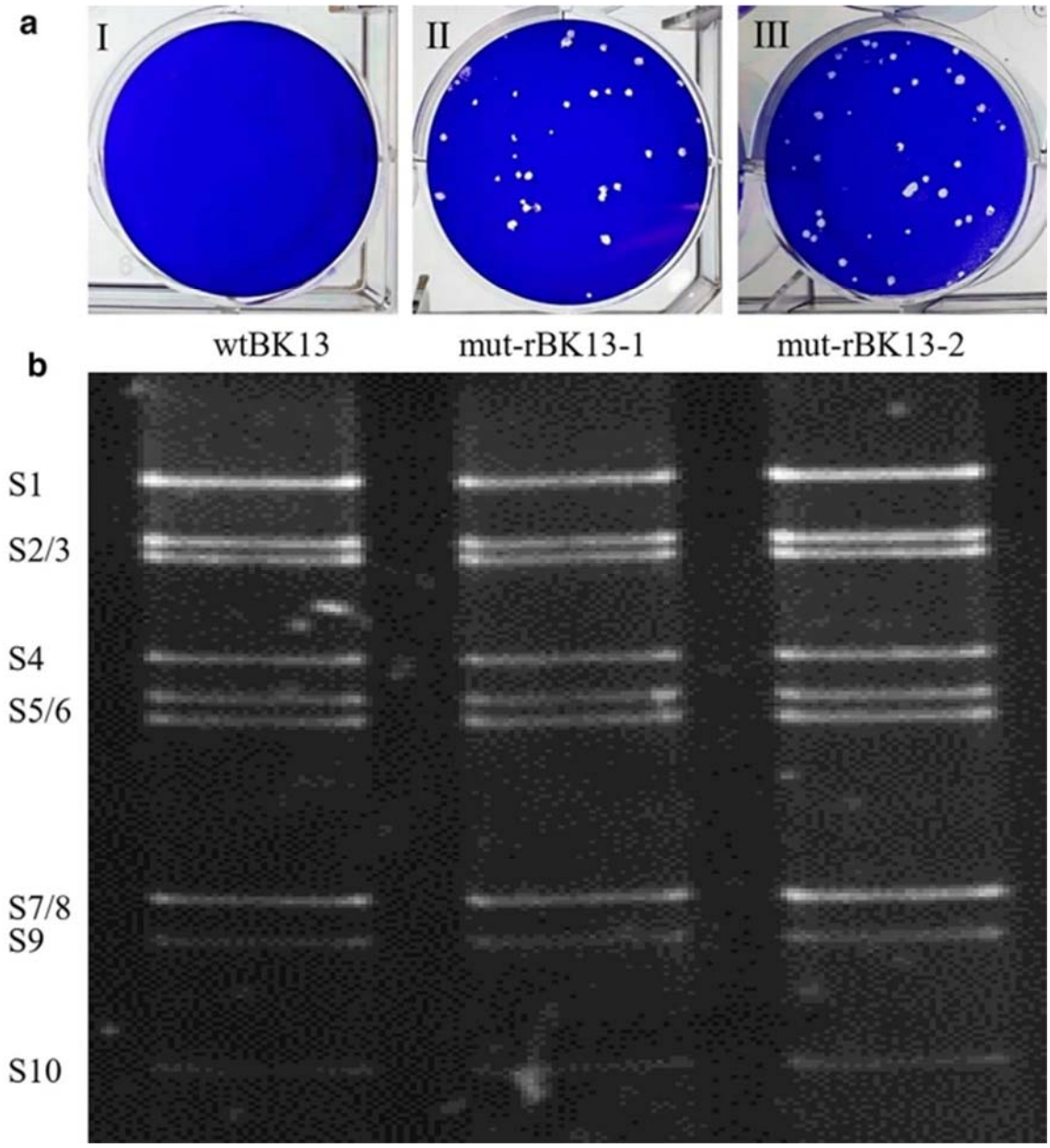

Fig. 2. a) Recovery of infectious EHDV-2 by DNA-based reverse genetics. In well I, BSR-T7 cells were not transfected with plasmids or PCR amplicons as a control. In well II, BSR-T7 cells were transfected with seven helper viral protein expression plasmids and 10 recombinant reverse genetics plasmids; and in well III, BSR-T7 cells were transfected with seven viral protein expression plasmids and 10 PCR amplicons. The monolayers were fixed and stained with crystal violet five days after the second transfection to visualize the plaques. **b)** Electropherotypes of wild-type EHDV-2 and the DNA-derived recombinant EHDV-2 viruses. Viral dsRNA was electrophoresed in an 8% non-denaturing polyacrylamide gel, followed by staining with ethidium bromide to visualize the viral genome segments (S1-S10). Lane 1, wtBK13; lane 2, mut-rBK13-1; lane 3, mut-rBK13-2

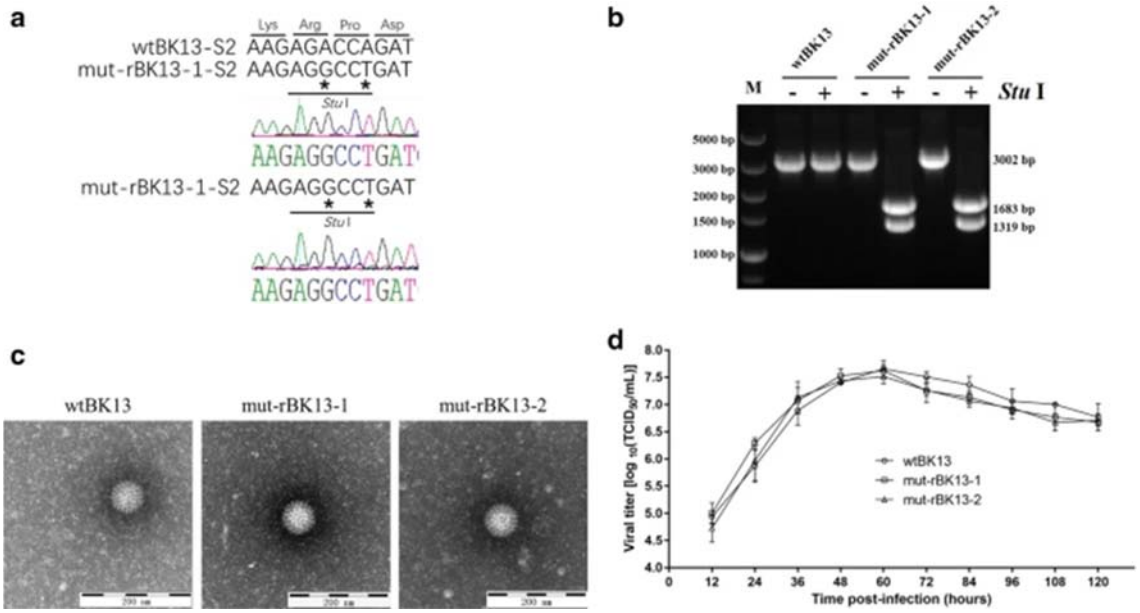

Fig. 3. Characterization of recovered recombinant EHDV-2. **a)** The nucleotide sequence of the S2 genome segment RT-PCR products were determined and compared. The sequence chromatogram shows the A-to-G and A-to-T mutations introduced at nucleotides 1682 and 1685 to create a new *Stu* I restriction site without changing the encoded amino acid sequence. The introduced nucleotide changes are indicated by an asterisk. **b)** RT-PCR products of the S2 genome segment of wtBK13, mut-rBK13-1, and mut-rBK13-2 were digested with *Stu*I, and the restriction products were analyzed by agarose gel electrophoresis. Size markers are indicated in base pairs. **c)** Electron micrographs of wtBK13, mut-rBK13-1, and mut-rBK13-2 virus particles. Bar: 200 nm. **d)** Growth kinetics of wtBK13, mut-rBK13-1, and mut-rBK13-2 in BHK-21 cells. Cells were infected with viruses at an MOI of 0.1 and incubated for the indicated lengths of time. Viral titers were determined by endpoint dilution assays in BHK-21 cells. The results are presented as the mean virus titer from three independent experiments and the error bars indicate the standard deviation

Characterization of recombinant EHDV-2

The complete genome sequences for mut-rBK13-1 and mut-rBK13-2 were determined and found to match the wild-type EHDV-2 sequence, except for the marker mutation that was introduced into the S2 genome segment of the recombinant viruses (Fig. 3a). To confirm that the recombinant viruses contained the introduced marker mutation, the S2 from wtBK13, mut-rBK13-1, and mut-rBK13-2 was amplified by RT-PCR and digested with *Stu*I restriction enzyme, and the digestion products were analyzed on an agarose gel (Fig. 3b). The S2 product derived from wtBK13 was not digested with *Stu*I, whereas the S2 product from the DNA-derived viruses produced the expected 1319-bp and 1683-bp DNA fragments upon digestion. The introduced marker mutation remained stable and could be detected after 20 passages in BHK-21 cells (data not shown). The rescued virus particles were visualized by electron microscopy and found to have a similar morphology to the wild-type EHDV-2 particles (Fig. 3c). The replicative capacity of wtBK13, mut-rBK13-1, and mut-rBHK13-2 was also evaluated in BHK-21 cells. The cells were infected with each virus at an MOI of 0.1, and the viral titers were determined at different time points postinfection. No significant differences were observed in the growth kinetics of wtBK13, mut-rBK13-1, and mut-rBK13-2, and the titers of the respective DNA-derived EHDV-2 and wild-type EHDV-2

were similar at all time points (Fig. 3d). The wtBK13 virus reached a peak titer of $10^{7.66}$ $TCID_{50}$/mL at 60 h postinfection, whereas the peak titers of mut-rBK13-1 and mut-rBK13-2 were $10^{7.51}$ and $10^{7.46}$ $TCID_{50}$/mL, respectively. Taken together, the data indicate that EHDV-2 could be recovered using different DNA-based reverse genetics platforms and that the DNA-derived viruses retained the properties of the wild-type EHDV-2.

Optimization of the DNA-based RGS of EHDV-2

To optimize the newly established plasmid-based RGS of EHDV-2, the contribution of individual helper viral protein expression plasmids in the successful recovery of EHDV-2 was assessed. To this end, BSR-T7 cells were transfected with a mixture of the viral protein expression plasmids, each lacking one of the plasmids, followed by a second transfection after 18 h with a complete set of the 10 recombinant reverse genetics plasmids. The results of these assays (Table 3 and Fig. 4) indicated that plasmid-driven expression of VP4 and NS1 was not essential for the recovery of EHDV-2 (Fig. 4 IV, VII, and IX); however, the number of plaques was typically reduced in the absence of the VP4-expressing plasmid, but not when the NS1 expression plasmid was omitted from the transfection mixture. In contrast, no infectious EHDV-2 could be recovered when plasmids expressing VP1, VP3, VP6, VP7, or NS2 were omitted from the first transfection mixture (Fig. 4), despite three passages of the viruses prior to performing the plaque assays. Identical results were obtained in experiments where the 10 recombinant reverse genetics plasmids were replaced with the 10 EHDV-2 PCR amplicons (data not shown).

Table 3. Helper viral protein expression plasmids required for EHDV-2 recovery using plasmid DNA

Expression plasmids with ORFs							Ten cDNA plasmids	Virus Recovery
VP1	VP3	VP4	VP6	VP7	NS1	NS2		
+	+	+	+	+	+	+	+	+
-	+	+	+	+	+	+	+	-
+	-	+	+	+	+	+	+	-
+	+	-	+	+	+	+	+	+
+	+	+	-	+	+	+	+	-
+	+	+	+	-	+	+	+	-
+	+	+	+	+	-	+	+	+
+	+	+	+	+	+	-	+	-
+	+	-	+	+	-	+	+	+
-	-	-	-	-	-	-	+	-

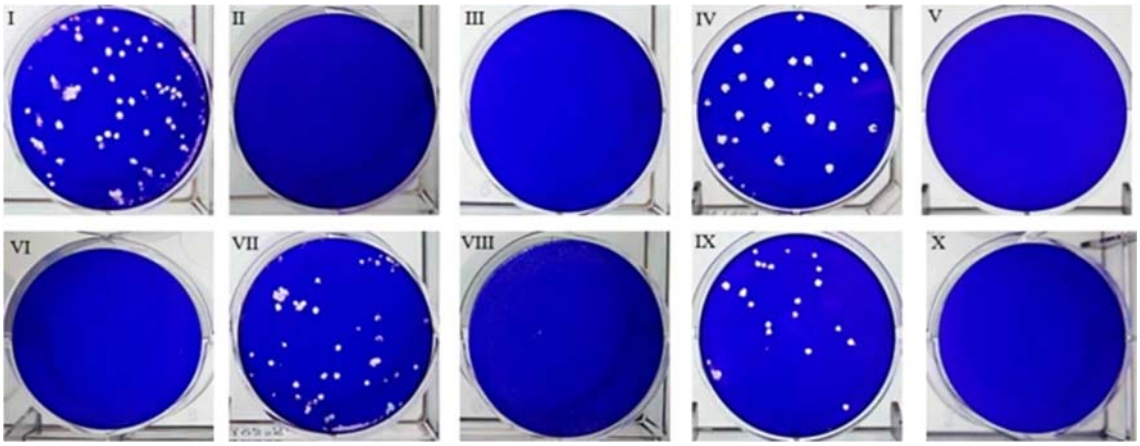

Fig. 4. Optimization of the plasmid-DNA-based RG system for EHDV-2. Monolayers of BSR-T7 cells were transfected with helper viral protein expression plasmids in which plasmids expressing VP1 (well II), VP3 (well III), VP4 (well IV), VP6 (well V), VP7 (well VI), NS1 (well VII), or NS2 (well VIII) were omitted from the first transfection mixture. The cell monolayers were transfected with the 10 recombinant reverse genetics plasmid constructs representing the cloned EHDV-2 genome. The monolayers were fixed and stained with crystal violet five days after the second transfection to visualize the plaques. Also shown are a BSR-T7 cell monolayer transfected with the full complement of seven helper viral protein expression plasmids and 10 recombinant reverse genetics plasmids (well I), a BSR-T7 cell monolayer transfected with only the 10 recombinant reverse genetics plasmids (well X), and a BSR-T7 cell monolayer transfected with five helper viral protein expression plasmids (without NS1 and VP4), as well as 10 recombinant reverse genetics plasmids (well IX)

Discussion

The RGSs initially developed for orbiviruses (e.g., BTV, AHSV, and EHDV) were limited to RNA-based systems in which a virus is either recovered from only *in vitro*-synthesized capped RNA transcripts, or alternatively, by first transfecting cells with helper protein expression plasmids to form the primary viral replication complex, followed by transfection with the full complement of synthetic RNA transcripts [16, 18, 20]. Recently, plasmid-DNA-only-based RGSs have also been established for BTV and AHSV, which allows the recovery of an infectious virus solely from cDNA clones [17, 19]. In an effort to expand the utility of EHDV reverse genetics, we aimed to develop DNA-based reverse genetics platforms to more efficiently and cost-effectively rescue recombinant EHDV compared to the RNA-based reverse genetics approach. To this end, we constructed recombinant pRG15 reverse genetics plasmids into which cDNA copies of each EHDV-2 genome segment were cloned. In contrast to previous studies on BTV [19], transfection of BSR-T7 cells with the 10 EHDV-2 rescue plasmids did not result in the recovery of recombinant EHDV-2, even though the transfected cells had been passaged three times in BHK-21 cells prior to performing the plaque assays (Fig. 4, well X). Similarly, it has been shown that AHSV cannot be rescued using a reverse genetics system developed for BTV [22] and that the recovery of BTV through reverse genetics is more efficient than that for AHSV [23]. Based on reports indicating that the plasmid-driven expression of several viral proteins followed by RNA transfection is more efficient at rescuing viruses than transfection with only RNA transcripts [18, 20, 24], we modified our approach by first transfecting BSR-T7 cells with helper viral protein expression plasmids followed by a second transfection with the 10 EHDV-2 rescue

plasmids (Fig. 4, well I). This modification consistently resulted in the recovery of recombinant EHDV-2 and suggests that higher levels of protein expression from the helper expression plasmids (expressing VP1, VP3, VP4, VP6, VP7, NS1, and NS2) contributed to the successful recovery of EHDV-2.

Having established a DNA-based RGS for EHDV-2, we next sought to simplify the system by evaluating the contribution of each individual helper expression plasmids in the virus recovery process. The results of these experiments indicated that the plasmid-driven expression of VP4 or NS1 was not essential for virus recovery. Indeed, BSR-T7 cells transfected with a mixture of helper protein expression plasmids that lacked both the VP4 and NS1 expression plasmids followed by transfection with the 10 reverse genetics constructs resulted in recovery of EHDV-2. In contrast to the absence of pre-expressed NS1, which did not have any apparent effect on virus recovery, the lack of pre-expressed VP4 moderately decreased the efficiency of virus rescue, and fewer plaques were observed after staining of the transfected cell monolayers.

As indicated above, the pre-expression of some structural and non-structural viral proteins was required for the recovery of EHDV-2 from cloned cDNA. Specifically, the pre-expression of VP1, VP3, VP6, VP7, and NS2 was essential for successful virus recovery, and pre-expression of VP4, although not essential, enhanced the recovery of EHDV-2. We speculate that the increase in recovery efficiency when the VP4 protein expression plasmid was included in the transfection mixture may have been due to *in vivo* capping of the transcripts, which enhances their stability, as well as the subsequent pool of ssRNA that is available for translation and packaging. The requirement for pre-expressed viral proteins may suggest that the *in vivo* transcription of the cloned cDNA copies of the EHDV-2 genome segments is not sufficient to enable viral recovery and that higher levels of some viral proteins are required to efficiently recover the virus. In all RNA-based RGSs described for orbiviruses to date, it has been reported that pre-expression of VP1 is essential for successful virus recovery. Therefore, this may suggest that high levels of VP1 are required to facilitate the interactions required to form the initial replication complexes. The VP6 protein has been reported to be essential for BTV replication [25] and has recently been suggested to play a role in genome packaging [26], and this could therefore explain why VP6 pre-expression is required for virus recovery. The pre-expression of VP3 may enhance EHDV-2 recovery through stabilization of the premature replication complexes and the formation of subcore particles. Once subcore proteins have been assembled into transcriptionally active core particles, the VP7 protein is likely required to stabilize the assembled cores, as has been suggested previously [23, 24]. Among the non-structural proteins, the pre-expression of NS2, but not NS1, is essential for EHDV-2 recovery. The NS2 protein is the major component of virus inclusion bodies (VIBs) in virus-infected cells and also has ssRNA-binding activity [27]. Thus, higher levels NS2 may be required to recruit viral transcripts from the cytoplasm to the replication complexes. Moreover, VIBs may serve to protect the transcripts from turnover prior to their assembly into a replication complex. Although NS1 is not an essential component of the primary replication machinery, it may play a role in enhancing BTV protein synthesis [28]. Since plasmid-driven protein expression results in high levels of viral proteins required to initiate virus recovery, it follows that NS1 may not be required as a translational enhancer of the viral proteins.

During the construction of the recombinant reverse genetics plasmids, we found that cDNA copies of some EHDV-2 genome segments were problematic to clone into the pRG15 reverse genetics vector. In addition, the recombinant plasmids were either unstable or cytotoxic when cultured in *Escherichia coli*, resulting in low yields of purified plasmid DNA. Such instability of the cloned cDNA sequences from different members of the genus *Orbivirus* has been reported previously [17, 23]. To overcome these challenges, we developed a PCR-based RGS for EHDV-2. Transfection of BSR-T7 cells with the helper viral protein expression plasmids and PCR amplicons of the 10 EHDV-2 genome segments, each equipped with a 5'-terminal T7 RNA polymerase promoter sequence, resulted in efficient virus recovery. In addition, the recombinant virus retained the biological properties of the parental virus. As such, the use of PCR amplicons instead of plasmids represents an effective and viable alternative to the plasmid-based RGS. A PCR-based reverse genetics approach has not yet been described for any of the orbiviruses and may provide a new platform to complement the existing RNA- and DNA-based RGSs. A significant advantage of this system was that it did not require cloning steps, which accelerated the recovery of infectious virus. In addition, this approach could be particularly important for orbiviruses that are occasionally associated with cloning difficulties or plasmid instability.

In conclusion, we have established a plasmid-DNA-based RGS for EHDV-2 and introduced a modification to this system based on PCR amplicons. These novel EHDV reverse genetics platforms will facilitate basic research into virus replication, pathogenesis, and immunity. Moreover, these platforms will also aid in the engineering of new recombinant viruses as potential vaccine candidates.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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