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# Validation of a site-specific recombination cloning technique for the rapid development of a full-length cDNA clone of a virulent field strain of vesicular stomatitis New Jersey virus

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ABSTRACT

This study reports the use of a site-specific recombination cloning technique for rapid development of a full-length cDNA clone that can produce infectious vesicular stomatitis New Jersey virus (VSNJV). The full-length genome of the epidemic VSNJV NJ0612NME6 strain was amplified in four overlapping cDNA fragments which were linked together and cloned into a vector plasmid by site-specific recombination. Furthermore, to derive infectious virus, three supporting plasmid vectors containing either the nucleoprotein (N), phosphoprotein (P) or polymerase (L) genes were constructed using the same cloning methodology. Recovery of recombinant VSNJV was achieved after transfecting all four vectors on into BSR-T7/5 cells, a BHK-derived cell line stably expressing T7 RNA polymerase (PMID: 9847328). *In vitro* characterization of recombinant and parental viruses revealed similar growth kinetics and plaque morphologies. Furthermore, experimental infection of pigs with the recombinant virus resulted in severe vesicular stomatitis with clinical signs similar to those previously reported for the parental field strain. These results validate the use of site-directed specific recombination cloning as a useful alternative method for rapid construction of stable full-length cDNA clones from vesicular stomatitis field strains. The approach reported herein contributes to the improvement of previously published methodologies for the development of full-length cDNA clones of this relevant virus.

Vesicular stomatitis virus (VSV) has long been used in laboratories as a model to study the evolution of RNA viruses (Zarate and Novella, 2004; Novella et al., 2010: Velazquez-Salinas et al., 2016), as a vaccine platform for the expression of foreign proteins (Matassov et al., 2015), and as a prototype oncolytic virus for treatment of cancer in humans and dogs (Naik and Russell, 2009; LeBlanc et al., 2013; Velazquez-Salinas et al., 2017). In the field, VSV epidemic strains sporadically emerge causing economically significant outbreaks in livestock (Rodriguez, 2002). Currently, intrinsic determinants of virulence in epidemic VSV strains are the subject of experimental research (Velazquez-Salinas et al., 2018a, 2018b), and the ability to conduct rapid genetic manipulation of these strains is a key to understanding associated biological features.

VSV is an ~11 kb non-segmented negative-strand RNA virus and the prototype of the family Rhabdoviridae and the genus Vesiculovirus. VSV encodes five structural proteins: nucleocapsid (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and the large RNA-dependent RNA polymerase (L) (Wagner and Rose, 1996). The methodology of developing full-length cDNA VSV clones, involves the construction of four plasmid vectors (Whelan et al., 1995; Lawson et al., 1995; Harty et al., 2001). In applying this technique, one plasmid vector containing the full-length cDNA genome of VSV is used to drive the synthesis of a genome-length negative-sense transcript, while three supporting plasmids contain the components of the viral replication complex (the N, P and L genes). However, this approach typically requires multiple costly and time-consuming steps of cloning and sub-cloning because of the reliance on natural restriction sites in the viral genome.

Recently, recombination-based cloning methods have dramatically

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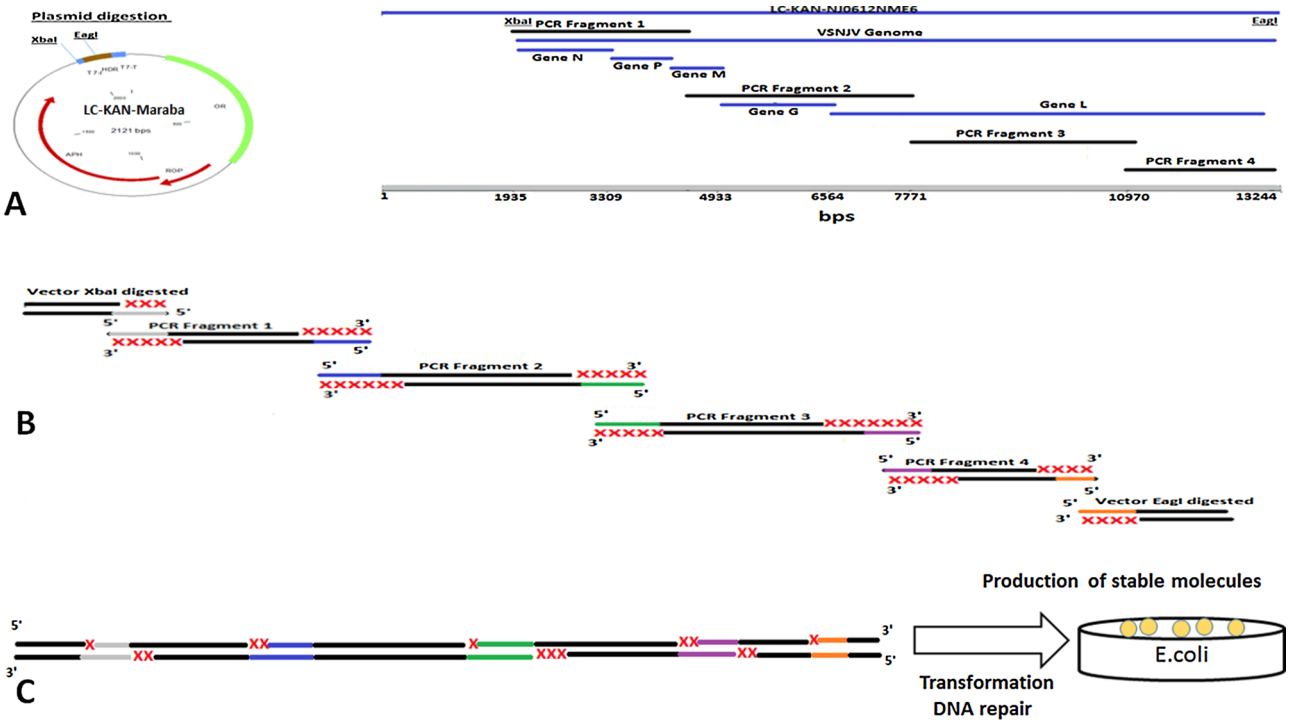


**Fig. 1.** Site-specific recombination cloning strategy to produce LC-Kan-NJ0612NME6. A) LC-Kan-Maraba plasmid was double digested, while the full-length genome of VSV was PCR amplified in four overlapping fragments. B) To promote site-specific recombination, plasmids and PCR fragments were incubated in the presence of VVpol. The 3′-5′ exonuclease activity of VVpol removes nucleotides at the 3′ends (represented with red X) of different overlapping fragments, promoting single-stranded alignment of complementary sequences (represented with similar colors). C) The final metastable non-covalently recombinant molecules of LC-Kan-NJ0612NME6 were produced. In theory, multiple nucleotide gaps or short overhangs (represented as red X) might be expected after the recombination reaction. Metastable molecules were repaired after being transformed into competent *Escherichia coli* (Hamilton et al., 2007; Irwin et al., 2012).

simplified the construction of full-length cDNA clones, mainly because of their lack of dependence on unique restriction sites in the target sequence, thus saving multiple steps of cloning and sub-cloning (Marsischky and LaBaer, 2004; Zhu et al., 2007). These methods have been used for the successful development of full-length cDNA clone systems for some important human and animal RNA viruses including dengue virus (Gibson assembly method), the porcine reproductive respiratory syndrome virus (Gibson assembly method), classical swine fever virus (In-fusion assembly method) and rabies virus (linear to-linear RedE/T recombination method) (Siridechadilok et al., 2013; Suhardiman et al., 2015; Kamboj et al., 2015; Nolden et al., 2016). The availability of novel recombination cloning methodologies, as well as the necessity to quickly and efficiently manipulate the genome of important RNA viral pathogens, encourages the continued improvement and validation of these methods.

This communication reports the validation of the In-fusion assembly method for the rapid development of a full-length cDNA clone derived from a highly virulent field strain of vesicular stomatitis New Jersey virus (VSNJV). This method is based on site-specific recombination catalyzed by the vaccinia virus DNA polymerase (VVpol) (Irwin et al., 2012).

The epidemic NJ0612NME6 strain was used as parental virus to derive the full-length cDNA clone. This highly virulent strain (GenBank accession #MG552609, Velazquez-Salinas et al., 2018a) was isolated from an epithelial lesion of a naturally infected equine in New Mexico during the 2012 VSNJV outbreak in the U.S (Velazquez-Salinas et al., 2014). To support the construction of different vector plasmids, multiple overlapping PCR products were synthesized (supplementary file 1). Total RNA was extracted from a high titer viral stock (HTVS) (titer $1 \times 10^{9.8}$ $TCID_{50}$/ml) using the RNeasy Mini Kit (QIAGEN). cDNA was synthesized by reverse transcription using random hexamers (In-vitrogen) and SuperScript ™ II RNase H-Reverse Transcriptase (In-vitrogen) following the manufacturer's instructions.

PCR's were conducted using the Platinum™ Hot Start PCR Mater mix (2X) kit in a final volume of 25 µl following manufacturer specifications using multiple sets of primers based on the NJ0612NME6 genomic sequence. Reactions were gel purified using ZYMORESEARCH DNA™ & Clean Concentrator ™ following manufacturer recommendations. PCR products were resuspended in a final volume of 6 µl in DNase/RNase-free Distilled Water. The overlapping PCR products were cloned by site-specific recombination using the VVpol (In-Fusion HD Cloning plus Kit, TaKaRa) into a previously described LC-KAN vector (Lucigen, Middleton, WI) (PMID: 20551913). Plasmids expressing the N, P or L proteins were derived from the pTIT vector as previously described (Finke and Conzelmann, 1999).

LC-Kan-Maraba and pTIT plasmids were doubled linear digested using XbaI- EagI-HF, and EcoRI-HF- BlpI respectively. Digestions were gel purified (LC-Kan-Maraba = ˜2057 bp, and pTIT = ˜3056 bp) as described above, and resuspended in DNase/RNase-free Distilled Water.

Specific reactions for the construction of each plasmid can be found in supplementary file 1. Fig. 1 illustrates the recombination cloning strategy used in this study.

The identification of stable vector plasmids was done by evaluating individual colonies of each reaction. Colonies were inoculated in 5 mL of 2x YT (Yeast Extract Tryptone) (TEKNOVA) containing 50 µg/mL of Kanamycin (LC-Kan-VSNJV plasmid), or Carbenicillin (pTIT plasmids) and incubated at 37 °C overnight while shaking. Plasmids were purified using the QIAprep kit (QIAGEN) following the manufacturer instructions. Plasmids containing correct size inserts were identified by restriction endonuclease analysis and sequenced to confirm fidelity to the wild type sequence. Selected cultures were used to inoculate 250 mL in 2x YT medium as described above. Plasmids were purified using the QIAGEN Plasmid Maxi Kit and re-sequenced. Sequencing reactions were conducted using multiple sets of primers as previously described (Pauszek and Rodriguez, 2012).

As a result of the recombination reactions, four stable plasmids were

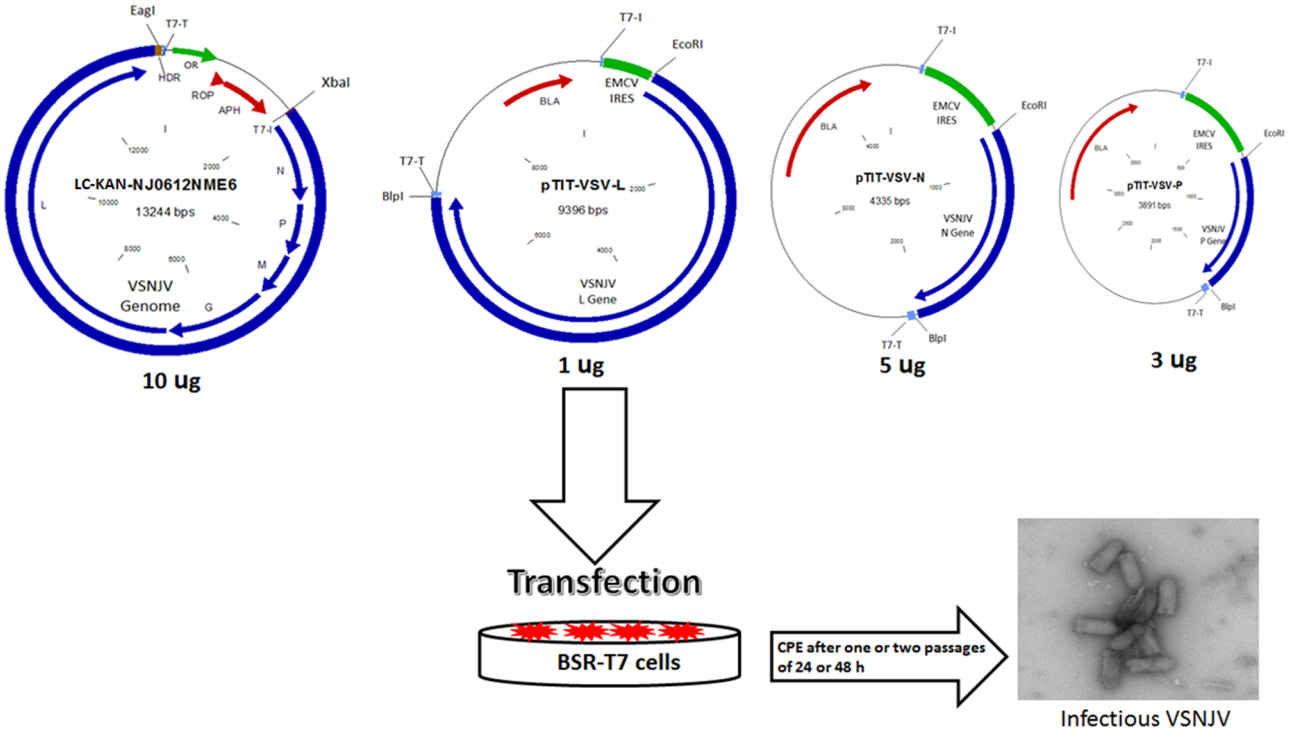


**Fig. 2.** Recovery of rNJ0612NME6. Specific concentrations of developed plasmids containing the full-length genome of VSV (LC-Kan-NJ0612NME6), and individual genes N (pTIT-VSNJV-N), P (pTIT-VSNJV-P), and L (pTIT- VSNJV-L) were used to transfect BSR-T7/5 cells at 75–80% confluency using a calcium phosphate transfection kit (ThermoFisher). After an overnight incubation, cells were split into T25 flasks, and typical CPE produced by VSV was observed after 24–48 hrs. Plasmid features include: restriction sites (EagI, XbaI, EcoR1, and BlpI), T7 promoter (T7-I), T7 terminator (T7-T), replication origin (OR), gen ROP, E.coli regulatory protein (ROP), kanamycin resistance gene (APH), ampicillin resitance gene (BLA), hepatitis delta ribozyme (HDR), and encephalomyocarditis virus internal ribosome entry site (EMCV IRES).

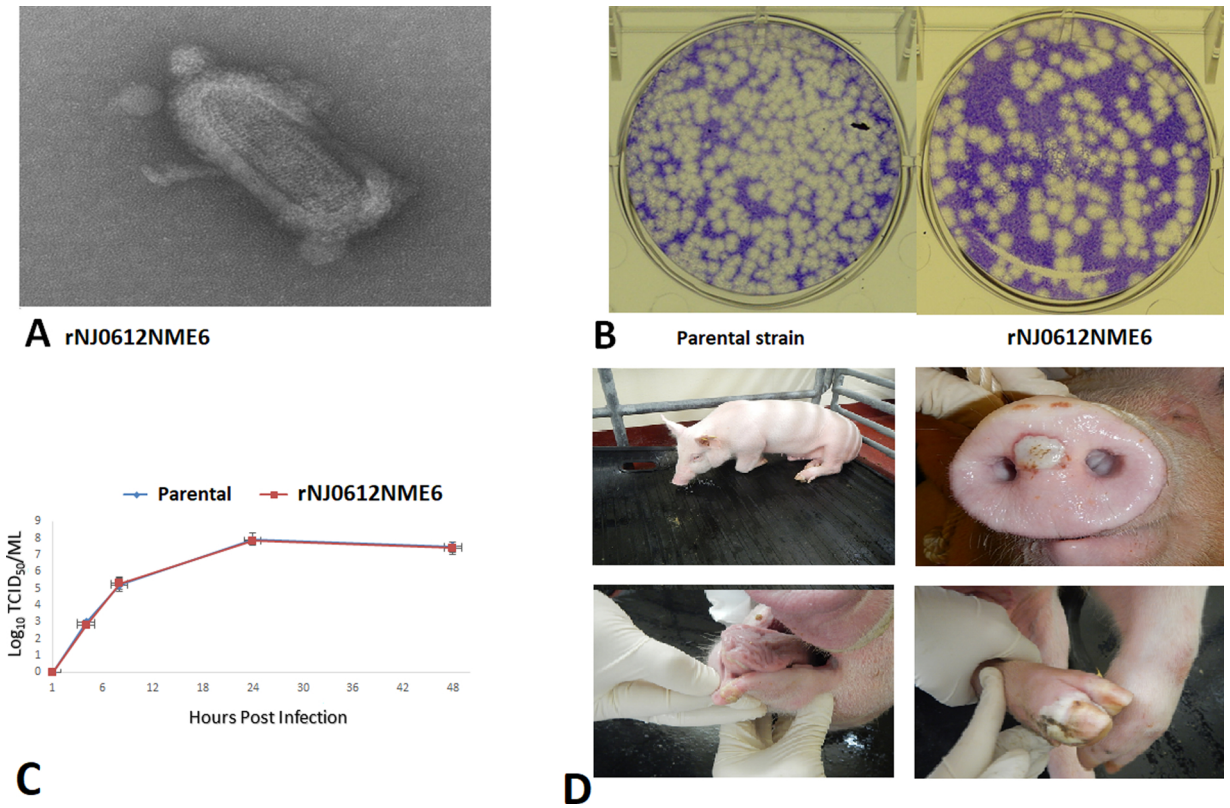


**Fig. 3.** *In vitro* and *in vivo* characterization of rNJ0612NME6. A) rNJ0612NME6 confirmed the typical rhabdovirus bullet shape when purified and visualized by electronic microscopy. B) Infection of BHK-21 cells with rNJ0612NME6 and the parental wild-type virus confirmed similar plaque formation, and growth kinetics (C). D) Intradermal inoculation of the snout skin of four pigs with $1 \times 10^7$ $TCID_{50}$ of rNJ0612NME6 resulted in the classical VSV clinical features including: depression, fever, vesicle development at the inoculation site, and systemic infection evidenced by the presence of epithelial lesions at secondary sites (lips and feet). Nasal and oral swabs analyzed by real time RT-PCR and viral isolation confirmed the presence of rNJ0612NME6.

obtained: LC-Kan- NJ0612NME6, pTIT-VSNJV-N, pTIT-VSNJV-P, and pTIT-VSNJV-L (Fig. 2). The recovery of recombinant VSNJV (rNJ0612NME6) was achieved in a vaccinia virus free system using BSR-T7/5 cells as previously described (Buchholz et al., 1999; Harty et al., 2001). More details about this process are shown in Fig. 2, and supplementary file 2.

The presence of rNJ0612NME6 in the supernatant of transfected BSR-T7/5 cells was confirmed by real time PCR conducted as previously described (Velazquez-Salinas et al., 2018b). The extracted RNA was treated with 2 units of DNAseI (BioLabs) to remove any residual/contaminating LC-Kan-NJ0612NME6. Additionally, the infection of rNJ0612NME6 in Vero cells was neutralized with a pool of polyclonal antisera collected during the experimental infection of pigs with the parental strain (Velazquez-Salinas et al., 2018b). Furthermore, full-length genomic sequence analysis confirmed 100% sequence identity between rNJ0612NME6 and the parental virus.

Finally, to assess the phenotypic characteristic of rNJ0612NME6, in *vitro* and *in vivo* experiments were conducted, including the infection of pigs, a natural host of VSNJV. Experimental infections of pigs were conducted under an experimental protocol approved by the institutional animal care and use committee (IACUC protocol #245-05-14R) in a biosafety level 3 (BSL-3Ag) facility at the United Sates Department of Agriculture's Plum Island Animal Disease Center (PIADC). The results of these experiments confirmed similar phenotypic characteristics of rNJ0612NME6 and the parental virus both *in-vitro* and *in-vivo* (Fig. 3). Additional information about the methodology used to perform these experiments is summarized in supplementary file 2.

In conclusion, the aim of this study was to validate the efficiency of the site-specific recombination approach based on the Infusion assembly method for the rapid development of a full-length cDNA clone derived from a highly virulent field strain of VSNJV. The results obtained in this study concur with a previous publication utilizing a similar approach with classical swine fever virus (Kamboj et al., 2015), supporting the efficiency of this methodology for the rapid and efficient assembly of vector plasmids. Overall, the cloning process was slightly more efficient for the supporting pTIT vector plasmids than for LC-Kan-NJ0612NME6 which contains multiple fragments. The results of this study will contribute to the improvement of the previously described methodology for developing full-length cDNA clones of VSV (Whelan et al., 1995; Lawson et al., 1995) by excluding multiple sub-cloning steps, thereby allowing for rapid genetic manipulation of new emerging VSV strains using a vaccinia virus-free system.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.01.003.

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