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Assessment of a novel recombinant vesicular stomatitis virus with triple mutations in its matrix protein as a vaccine for pigs

Xinkui Fang[a,b,1], Bing Qi[a,1], Yufang Ma[a,b], Xinchu Zhou[a], Shikuan Zhang[a,b], Tao Sun[a,b,*]

[a] School of Agriculture and Biology, Shanghai JiaoTong University, 800 Dongchuan Rd., Shanghai 200240, China
[b] Shanghai Municipal Veterinary Key laboratory, 800 Dongchuan Rd., Shanghai 200240, China

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ABSTRACT

Vesicular stomatitis virus (VSV) causes a serious vesicular disease responsible for economic losses in the livestock industry. Currently, there are no suitable vaccines to prevent VSV infection. Although the structural matrix (M) protein of VSV has been shown to be a virulence factor in rodent models, its role in the pathogenicity of VSV infection in livestock species is unknown. We hypothesized that VSV with mutations in the M protein represents a novel live attenuated vaccine candidate. To test this, we introduced mutations into VSV M protein using reverse genetics and assessed their attenuation both *in vitro* and in pigs, an important natural host of VSV. A recombinant VSV with a triple amino acid mutation in M protein (VSV_{MT}) demonstrated a significantly reduced ability to inhibit the type I interferon (IFN) signaling pathway and to shutoff host gene expression compared to WT-VSV and a mutant virus with a single amino acid deletion ($VSV_{\Delta M51}$). Inoculation of pigs with VSV_{MT} induced no apparent vesicular lesions but stimulated virus-neutralizing antibodies and animals were protected against virulent VSV challenge infection. These data demonstrate that the M protein is an important virulence factor for VSV in swine and VSV_{MT} represents a novel vaccine candidate for VSV infections in pigs.

1. Introduction

Vesicular stomatitis virus (VSV) possesses a single-stranded negative-sense RNA genome. Livestock species including pigs, cattle, and horses are natural hosts of VSV, which can cause disease involving vesiculation and ulceration of tongue and oral epithelia, with occasional lesions on feet and teats [1]. Two VSV serotypes, New Jersey (VSV_{NJ}) and Indiana (VSV_{IND}), are enzootic from southern Mexico to northern South America [1,2], which can lead to serious economic losses in livestock farming due to the lowered animal productivity and trade barriers. So far, there is no commercial vaccine available to prevent VSV infection. Replication-competent attenuated viruses have been proposed as the basis for developing vaccines due to their capability in stimulating potent immunity *in vivo* [3–5]. With recent developments in molecular technology, construction of recombinant viruses based on reverse genetics has become common for developing novel viral vaccines.

Five structural proteins are encoded by VSV genome, including the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA replicase (L) [1]. In swine, the G protein has been shown to be a determinant of pathogenicity [6]. The M protein is a multifunctional protein involved in virus assembly, budding, and pathogenicity, which is capable of inhibiting the transport of host mRNAs from the nucleus as well as type I interferon (IFN) signaling [7–9]. However, almost all existing data regarding M has been focused on rodent models, which are not natural hosts of VSV. When single point mutations were introduced at the methionine 51 residue of M protein (M51), such as a deletion ($VSV_{\Delta M51}$) or substitution with arginine (VSV_{M51R}), VSV were shown to be attenuated in mice [9,10]. The single amino acid mutation in M51 was shown to affect the ability of VSV to shutoff host gene expression by impairing the binding of M protein to nuclear export factor Rae1 [11]. However, in our study, $VSV_{\Delta M51}$ indicated partial attenuation in pigs. Stojdl et al. demonstrated that another VSV-M mutant strain with two amino acid substitutions (V221F and S226R) could also attenuate virulence in mice, whereas the 50% lethal dose (LD_{50}) of this mutant was lower than that of $VSV_{\Delta M51}$ [9].

To characterize the role of M as a virulence determinant in pigs, we constructed two recombinant VSV containing the previously reported attenuating mutations in M; $VSV_{\Delta M51}$ and VSV_{MT}, which

* Corresponding author at: Shanghai Jiaotong University, Animal Science, 800 Dongchuan Road, Minhang District, Shanghai 200240, China.
Tel.: +86-21-34207240.
E-mail address: taosun99@hotmail.com (T. Sun).
[1] These authors contributed equally to the study.

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carried the three mutations ΔM51, V221F, and S226R. Attenuation of phenotype between wild-type (WT) VSV and recombinant M-mutant viruses with mutant M were examined in both *in vitro* and *in vivo* studies.

2. Materials and methods

2.1. Cell lines and porcine embryo fibroblast cell preparation.

The baby hamster kidney cell line (BHK-21) was used for the growth of VSV and for virus neutralization assays. Pig kidney cell line IBRS2 was kindly provided by Prof. Shaobo Xiao (Huazhong Agricultural University, Wuhan, China) and were cultured with MEM medium (GIBCO) with 10% fetal bovine serum (FBS).

The preparation of porcine embryo fibroblast cells (PEF) was based on the method described by Zhang et al. [12]. Briefly, a Landrace pig at 30 days of gestation was euthanized, and the uterus transported to the laboratory for tissue extraction. Fetuses were harvested aseptically and washed twice in Ca^{2+} and Mg^{2+} free PBS (DPBS, GIBCO). After removal of heads and internal organs with fine scissors and forceps, tissues were chopped into small pieces were seeded into a T75 cell culture flask containing Dulbecco's Modified Essential Medium (DMEM, GIBCO) plus 20% FBS (GIBCO) and 1% nonessential amino acids. Tissue explants were cultured at 39 °C with humidified 5% CO_2 atmosphere and attached to the flask 4–6 h later. The fibroblast cells could grow from the tissue pieces. After the adherent cells reached 70–80% confluence, the used medium was discarded, the monolayer washed three times with DPBS followed by digestion using 0.25% trypsin and 0.02% EDTA to harvest the cells. The cells were passaged twice and then frozen using DMEM with 10% DMSO and 20% FBS until use.

2.2. Recovery of recombinant VSV

The VSV_{IND}- based recombinant virus VSV_{XN2} and mutant VSV with methionine 51 in matrix protein deletion ($VSV_{\Delta M51}$) were prepared as described previously [10]. VSV-GFP was the recombinant virus with green fluorescence protein gene (*GFP*) inserted into the additional transcriptional unit between the G–L intergenic junction of VSV [13]. VSV_{XN2} was used as the WT VSV control [10]. A novel recombinant VSV with triple amino acid mutations occurring in matrix protein (M51 deletion, V221F, and S226R) was constructed, and termed VSV_{MT}. The genomic structure of VSV_{MT} was shown in Fig. 1. Plasmid $pVSV_{MT}$ was constructed by substituting *M* gene of $pVSV_{\Delta M51}$ with M_T gene through XbaI and MluI restriction enzyme digestion [14]. Using plasmid $pVSV_{\Delta M51}$ as the template, the M_T gene with V221F, S226R, and M51-deletion was amplified by overlapping PCR. Primers used were shown in Table 1. Briefly, fragment 1 (F1) was amplified with primer P1 and P2, whereas fragment 2 (F2) was amplified with primer P3 and P4. After the first round of PCR, the F1 and F2 fragments were gel purified and mixed in the same tube to set up second round PCR with primers P1 and P4 to construct the M_T gene. The *Pfu* DNA polymerase (Thermo Scientific, USA) was used in all PCR reactions.

Table 1
Overlapping PCR primers for constructing triple mutations in VSV *M* gene (M_T). With plasmid $pVSV_{\Delta M51}$ as the template, mutant *M* gene with V221F, S226R, and M51 deleted (M_T) was amplified by overlapping PCR and cloned with XbaI and MluI sites between *M* gene of $pVSV_{\Delta M51}$ [14]. The bold italics in primer P1 is the XbaI restriction site and italics in P4 is the MluI restriction site. As underlined in primer P3, for V221F, GTC was substituted with TTC, for S226R, AGC was substituted with CGC. Primer P2 and P3 were reverse-complement. Underlined in P2, is the reverse-complement for V221F or S226R respectively.

Primers	Primer sequences
P1	5′CCGG***TCTAGA***GGAGAATTCATCTCTGTC3′
P2	5′CTAGCTCATTTGAAGTGGCGGATAGAATCCAGGAACCACGCTCCAGATGC 3′
P3	5′GCATCTGGAGCGTGGTTCCTGGATTCTATCCGCCACTTCAAATGAGCTAG 3′
P4	5′ CCGG***ACGCGT***AAACAGATCGATCTCTGTT 3′

Rescue of VSV_{MT} was performed as previously described [15]. Briefly, co-transfection of $pVSV_{MT}$ with helper plasmids, pBS-N, P, and L, was performed in BHK21 cells infected with a recombinant vaccinia virus (vTF7-3) expressing T7 RNA polymerase. At 48 h posttransfection, culture supernatants were collected and filtered through a 0.2 μm filter into fresh BHK21 cells. Cells were checked daily for infection. If typical cytopathic effect (CPE) was observed 2–3 days afterwards, supernatants were collected and viruses were plaque purified in Vero cells. Individual plaques were isolated and seed stocks amplified in BHK21 cells. To identify rescued recombinant VSV, typical bands for L, G, N/P, and M proteins were detected through Western blotting using specific convalescent sera from VSV_{XN2}-infected mice [13]. VSV_{MT} was also confirmed with RT-PCR before use. Primers covering *M* gene were as follows, forward primer: 5′ATGAGTTCCTTAAAGAAGATT3′; reverse primer: 5′TTTGAAGTGGCTGATAGA3′.

Viral stocks were amplified by passage at low multiplicity of infection (MOI) of 0.01 in BHK21 cells. Recombinant viruses were concentrated by ultracentrifugation at 30,000 rpm/min for 2 h and frozen at −70 °C. Virus was titrated by plaque assay on Vero cells. Briefly, 90% confluent Vero cells in 12-well plates were infected with optimally diluted VSV and then covered with low melting temperature agar (Invitrogen, Carlsbad, CA, USA) after rinsing with phosphate buffered saline (PBS). At 24 h postinfection (p.i.), 1% crystal violet was used to stain Vero cells and viral titers were quantified.

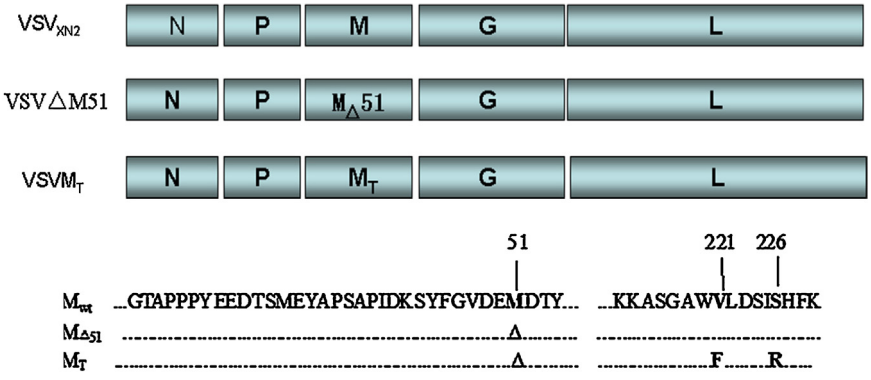

Fig. 1. Construction of recombinant VSVs with different mutant M proteins. The parental VSV genome encodes the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the large RNA polymerase (L). M_{wt}: wild-type M protein, $M_{\Delta 51}$: M protein with M51 deleted, M_T: M protein harboring triple-sited mutations (M51deleted, V221F and S226R).