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Modifying the *NSs* gene to improve live-attenuated vaccine for Rift Valley fever

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Vaccines for exotic emerging diseases are considered for populations concerned about economic loss or social panic by natural or intentional introduction of those pathogens into their countries. In particular, zoonotic diseases transmitted by native mosquitoes may cause devastating economic loss and public health impact in the absence of effective countermeasures. A number of countries are at risk of potential spread of Rift Valley fever virus (RVFV; family *Bunyaviridae*, genus *Phlebovirus*) from endemic areas in Africa. RVFV establishes vertical transmission in floodwater *Aedes* species, a primary vector of RVFV, and is also maintained by horizontal transmission between mosquito vectors and animals [1]. RVFV infection results in more than 90% death in newborn lambs, 40–100% of abortion or fetal malformation in pregnant ewes, and causes a similar abortion in cattle, goat or other ruminants [2]. Most of the human patients of RVF typically show a biphasic febrile illness and less than 1% of patients suffer from lethal hemorrhagic fever, thrombosis or neurological disorder, while 1–10% of patients develop partial or complete blindness [3]. After 82 years since the first recognized RVF outbreak in Kenya in 1930, RVF is now endemic to most of sub-Saharan Africa and has spread into Egypt, Madagascar and Yemen [4]. In the USA, RVFV is classified as a Category A Priority Pathogen by the NIH/the

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RVFV is a negative-stranded RNA virus with a tripartite genome named small (S)-, medium (M)- and large (L)-segments; S-segment encodes N and nonstructural NSs proteins in an ambisense manner, M-segment encodes envelope Gn and Gc proteins, nonstructural NSm proteins and the less-characterized 78-kD protein, and L-segment encodes L protein (RNA-dependant RNA polymerase) [4].

Effective vaccines will minimize the impact of RVFV introduction into nonendemic countries. Past studies demonstrated that neutralizing antibodies play an important role in protection from lethal RVFV challenge [5]. Considering that a large number of ruminants are infected during RVF outbreaks, RVF vaccines should rapidly induce high levels of protective neutralizing antibodies in both ruminant and humans with a single dose. Currently, only a formalin-inactivated The Salk Institute-Government Service Division (TSI-GSD)-200 vaccine is available with an Investigational New Drug status in the USA. TSI-GSD-200 is produced from the Entebbe strain (a wild-type [wt] RVFV isolate from mosquitoes in the 1940s) at high containment facilities, and there is a limitation in available doses, while at least three doses are required for inducing a protective level of neutralizing antibody [5,6]. A live-attenuated vaccine strain, Smithburn vaccine, generated by numerous intracerebral passages in suckling mice, has been used in endemic countries since the 1950s. Smithburn vaccine causes abortion in vaccinated pregnant ruminants, and recent sequencing analysis showed a potential reassortment with the wt RVFV strain [7]. A live-attenuated candidate vaccine, MP-12, was generated in the 1980s from an Egyptian isolate, ZH548 strain, by 12 serial passages in human diploid lung MRC-5 cells in the presence of a chemical mutagen, 5-fluorouracil [8]. Ruminants vaccinated with a single dose of MP-12 elicit high levels of neutralizing antibodies [5]. MP-12 has 23 mutations compared with the parental ZH548 strain, among which some of the 19 mutations in M- and L-segments are considered to be responsible for its attenuation, while the S-segment still has a virulent phenotype [5,9]. A study suggested that MP-12 retains residual virulence in pregnant ewes during early pregnancy [10], whereas the safety in pregnant ruminants at later stages and newborn lambs has been demonstrated [5]. Furthermore, more than 100 human volunteers were successfully vaccinated with MP-12 vaccine without notable adverse effects [5]. Thus, MP-12 is much safer than the Smithburn strain, and is considered one of the most promising candidate vaccines for RVF in humans and ruminants in the USA [5]. In addition, MP-12 is the RVFV strain exempted from select agent rule, and can be handled in a biosafety level 2 laboratory.

RVFV lacking the *NSs* gene as novel live-attenuated vaccine candidates

Researchers sought to improve live-attenuated vaccines for RVF. Bouloy *et al*. demonstrated that NSs is the major virulence factor of RVFV by using clone 13 strain (C13), which is a natural plaque isolate from the 74HB59 strain, encoding a 69% in-frame truncation in the *NSs* gene [11,12]. C13 was evaluated as a live-attenuated veterinary vaccine candidate in ruminants, and its efficacy and safety was demonstrated in pregnant ewes at early pregnancy [13]. Bird *et al*. generated the ΔNSsΔNSm-rRVFV using reverse genetics for Egyptian

ZH501 strain [14]. The ΔNSsΔNSm-rRVFV lacks both *NSs* and *NSm* in ZH501 genome, which resulted in attenuation of both S- and M-segments. The safety and efficacy of ΔNSsΔNSm-rRVFV were demonstrated in ewes at early pregnancy. A difference of the wt RVFV strain lacking *NSs*/*NSm* from the MP-12 strain is that attenuation is derived from a lack of functional *NSs* gene. A large truncation in the *NSs* gene renders RVFV incapable of causing a reversion to virulence, and it also works as a marker of differentiation of infected from vaccinated animals (DIVA) by measuring anti-NSs IgG. On the other hand, C13 encodes wt RVFV M- and L-segments, while ΔNSsΔNSm-rRVFV encodes wt RVFV L-segment. Those vaccines should not cause viremia as long as vaccinated animals are immune competent, while C13 causes viremia in immune-compromised host animals [12,15]. In such a case, mosquitoes may be infected with those vaccine strains. C13 can replicate in two major mosquito vectors: *Aedes vexans* and *Culex pipiens* [16]. Surprisingly, RVFV lacking *NSm* was shown to have a decreased infection rate in *Culex quinquefasciatus* [17]. It will be important to understand the mechanism of mosquito transmission using NSm, and to apply the knowledge to the development of new-generation vaccines.

New-generation vaccines for RVF in nonendemic countries

RVF vaccines for humans should demonstrate efficacy and safety through clinical trials. Nonreplicating vaccines such as subunit vaccine, DNA vaccine, virus-like particle or replicon vaccine that allow a single cycle replication in infected cells may be safer than live-attenuated vaccines [5,18,19], while the efficacy and production strategy are important limiting factors for their development. Assuming that only workers who handle infected animals require vaccination, live-attenuated vaccine has a merit in inducing a high level of protective neutralizing antibody in healthy immune-competent persons within a week.

Our group aims to develop a modified MP-12 vaccine for veterinary and human use by using reverse genetics. Although the safety of MP-12 has been shown in ruminants and humans [5], residual virulence in pregnant ewes and a lack of DIVA marker may be of a concern in the veterinary field. As described earlier, MP-12 S-segment encodes a virulent phenotype, and the major virulence factor NSs is functional. NSs induces the inhibition of host general transcription by sequestering transcription factor IIH p44 subunit proteins and by promoting post-translational degradation of transcription factor IIH p62 subunits, the inhibition of IFN-β gene promoter by specifically interacting with SAP30, and the post-translational degradation of dsRNA-dependent protein kinase [4]. We generated rMP12-C13type that encodes a C13-like 69% in-frame truncation of *NSs*. The rMP12-C13type induced a high level of neutralizing antibodies in outbred CD1 mice, and showed a similar efficacy with parental MP-12 [20]. The rMP12-C13type, but not parental MP-12, accumulated viral proteins in dendritic cells at the local draining lymph node after subcutaneous inoculation, while viremia was induced in mice vaccinated with MP-12 but not with rMP12-C13type [20]. Thus, removal of *NSs* from MP-12 does not affect the efficacy of MP-12 vaccine in a mouse model. The rMP12-C13type is attenuated in the S-, M- and L-segments, and encodes a DIVA marker. In fact, none of the mice vaccinated with rMP12-C13type induced anti-NSs IgG [20]. Further study is required to know whether the rMP12-C13type is immunogenic in ruminant and humans.

Introduction of foreign genes into MP-12 in place of *NSs* may increase its immunogenicity in ruminants or humans without affecting the safety and the DIVA marker. We generated rMP12-mPKRN167, which encodes a dominant-negative form of mouse protein kinase in place of *NSs*, and tested its efficacy in mice [20]. The rMP12-mPKRN167 significantly increased the abundance of viral proteins at draining lymph nodes without inhibiting type-I interferon responses, and conferred 100% protection from wt RVFV challenge by a single-dose vaccination [20]. We also generated MP-12 encoding other phlebovirus *NSs*; for example, Sandfly fever virus, Toscana virus and Punta Toro virus, and are currently testing their immunogenicity and safety in the mouse model.

Live-attenuated RVF vaccines have a promising performance in safety and efficacy for RVF only, and thus cost–benefit analysis will be essential to develop such vaccines. Novel technology platforms to stimulate protective immune responses for several pathogens will be of future interest, replacing traditional ones, which can be supported by industry partners with minimum investment. We should keep developing both types of technologies for unexpected emergence of RVFV in the future.

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