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Short communication

Development of an improved reverse genetics system for Akabane bunyavirus

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A B S T R A C T

Akabane disease, caused by the insect-transmitted Akabane virus (AKAV), affects livestock by causing life-threatening deformities or mortality of fetuses. Therefore, Akabane disease has led to notable economic losses in numerous countries, including Japan. In this short communication, a new T7 RNA polymerase-based AKAV reverse genetics system was developed. Using this system, in which three plasmids transcribing antigenomic RNAs were transfected into cells stably expressing T7 polymerase, we successfully reconstituted the live attenuated vaccine TS-C2 strain (named rTTT), and also generated a mutant AKAV (rTTTΔNSs) that lacked the gene encoding the nonstructural NSs protein, which is regarded as a virulence factor. Analysis of growth kinetics revealed that rTTTΔNSs grew at a much slower rate than the rTTT and TS-C2 virus. These results suggest that our established reverse genetics system is a powerful tool that can be used for AKAV vaccine studies with gene-manipulated viruses.

Akabane virus (AKAV) was first isolated in Japan in 1959 (Oya et al., 1961) and was classified in the genus *Orthobunyavirus*, family Bunyaviridae. Transmission of AKAV occurs in cows, ewes, and goats, primarily through biting midges, in Australia, Southeast Asia, East Asia, the Middle East, and Africa. During pregnancy, AKAV is able to cross the placenta, causing congenital deformities such as arthrogryposis-hydranencephaly syndrome, abortion, premature birth, and stillbirth (Kurogi et al., 1976). These virus-induced complications have been the cause of great economic losses in the livestock industry (Inaba et al., 1975). Although vaccination has reduced the prevalence of Akabane disease, frequent cases still occur in Japan and Korea (Inaba and Matumoto, 1990; Kim et al., 2011). Moreover, antigenic and pathogenic variants of AKAV have been isolated (Akashi and Inaba, 1997; Kamata et al., 2009; Lee et al., 2002; Liao et al., 1996; Miyazato et al., 1989; Ogawa et al., 2007a; Yamakawa et al., 2006). For example, a variant Iriki strain was isolated from a calf presenting non-suppurative encephalitis and neurological symptoms in Japan (Miyazato et al., 1989). The Iriki strain showed low cross-reactivity with antiserum against the current vaccine strain of AKAV in the neutralization tests, suggesting that this and other variants may be the cause of virus persistence even in areas where vaccines are administered. Therefore, it is necessary to reconsider the vaccine strategy to control the disease effectively.

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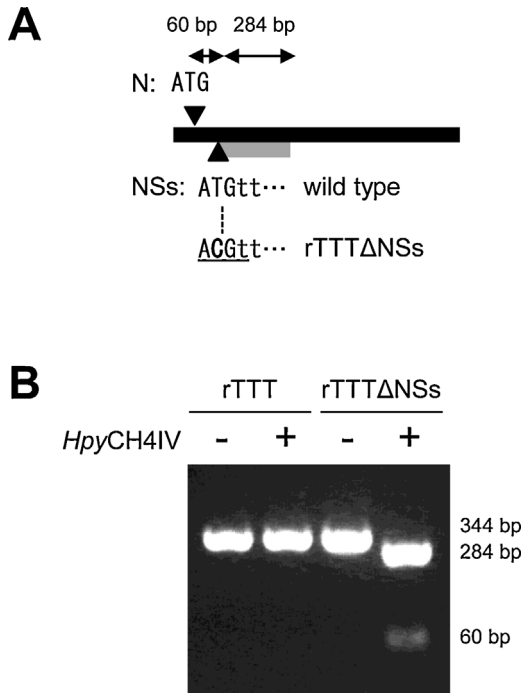

Fig. 1. Construction of recombinant AKAVs. (A) Schematic representation of the nonstructural NSs protein translational start site in the S RNA segment. The NSs start codon was mutated to ablate NSs translation without affecting the amino acid sequence of the nucleoprotein (N). (B) Genetic marker for recombinant AKAV lacking NSs. The mutation in rTTTΔNSs generates a new *Hpy*CH4IV restriction site, which is underlined in (A). After RNA was extracted from the infected cells, RT-PCR was performed to amplify the 344-bp fragment of the NSs open reading frame. No band was observed in the control sample without an RT reaction. *Hpy*CH4IV digestion of the RT-PCR product from rTTTΔNSs yielded two fragments (60 bp and 284 bp).

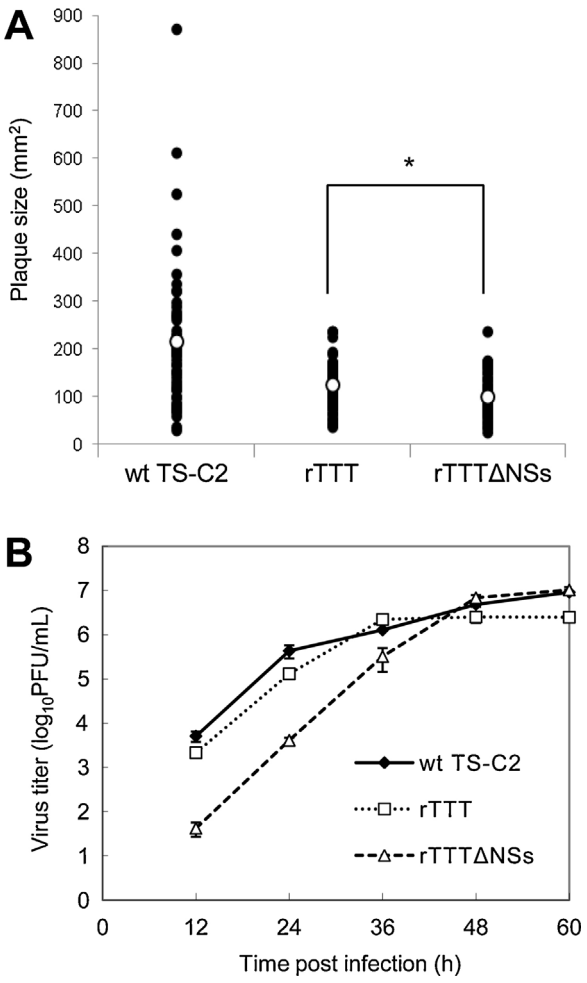

Fig. 2. Characterization of recombinant AKAVs. (A) Plaque sizes of recombinant viruses. The results are shown for 50 randomly selected plaques for each virus. The mean plaque sizes are represented by open circles (*, $P < 0.05$; two-tailed Student's *t*-test). (B) Growth kinetics of wt and recombinant viruses. HmLu-1 cells were infected with each virus at an MOI of 0.01. Supernatants were collected at the time points shown, and the number of PFUs was determined. The results shown are the means ± SD of three independent experiments.

Members of the genus *Orthobunyavirus* have a genome comprising three unique species of single-stranded RNA, designated as S (small), M (medium), and L (large). The L RNA segment of AKAV encodes RNA-dependent RNA polymerase (L protein), while the M segment encodes envelope glycoproteins Gn and Gc and a nonstructural NSm protein. The envelope glycoproteins are responsible for virus neutralization and attachment to mammalian or insect cells (Ludwig et al., 1991). The S segment encodes a nucleoprotein (N), which shares antigenic determinants with the nucleoproteins of some other species in the genus (Akashi et al., 1997), and a nonstructural NSs protein, which acts as a type I interferon antagonist and is involved in the regulation of host protein synthesis, thereby functioning as a virulence factor (Weber et al., 2002).

Reverse genetics systems are useful for studying basic mechanisms as well as practical applications for many viruses, albeit they occasionally pose technical difficulties for negative-sense RNA viruses. We previously developed an RNA polymerase I-based reverse genetics system using the OBE-1 strain for AKAV (Ogawa et al., 2007c). However, its efficiency for virus rescue was substandard, lacking the robustness of the reverse genetics system. To address this flaw, we sought to establish a T7 RNA polymerase-based reverse-genetics system, which has provecd to be effective in rescuing other bunyaviruses (Blakqori and Weber, 2005; Bridgen and Elliott, 1996; Ikegami et al., 2006)

We selected the TS-C2 vaccine strain (Kurogi et al., 1979) (purchased from the National Veterinary Assay Laboratory) as the donor virus for development of the reverse genetics system in this study, since this system may be used to generate potential AKAV vaccine candidates in the future. For example, vaccine candidates such as reassortants having the S and L segments of TS-C2 and M segment of the antigenic variants can be easily constructed by using the reverse genetics system. First, we determined both end sequences of the viral genome, which were required for cloning of viral cDNAs into plasmids for reverse genetics. To this end, viral RNA was extracted from virions in the supernatant of hamster lung (HmLu-1) cells infected with the TS-C2 strain using the Viral RNA mini kit (Qiagen, Hilden, Germany). The RNA was transcribed by SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using specific primers designed based on the OBE-1 sequence (Ogawa et al., 2007b) (Table 1, RT), after which poly C tails were added using the 5′ RACE system (Invitrogen). Next, we performed PCR amplification of the cDNA ends with GoTaq Green Master Mix (Promega, Madison, WI, USA) using the anchor and gene-specific primers (Table 1, first PCR). The abridged universal primer, which is homologous to the adapter region of the anchor primer, and other specific primers (Table 1, nested PCR) were used for the second amplification reactions of both ends. All fragments were cloned into pCR2.1-TOPO (Invitrogen) and sequenced using standard protocols (3130xl Genetic Analyzer; Applied Biosystems, Foster City, CA, USA).

We then amplified full-length cDNAs of the TS-C2 strain by LA *Taq* polymerase (TaKaRa Bio, Shiga, Japan) using gene-specific primer sets (Table 1), TA cloned, and sequenced. The GenBank accession numbers assigned to the TS-C2 strain sequences are