NOTE
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Establishment of a reverse genetics system for rabies virus strain Komatsugawa

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ABSTRACT. The rabies virus strain Komatsugawa isolated from a dog in Tokyo in the 1940s retains biological properties as a field strain, providing an effective model for studying rabies pathogenesis. To facilitate molecular studies on the pathogenesis, this study aimed to establish a reverse genetics system for the Komatsugawa strain. By transfecting the full-length genome plasmid of this strain, infectious virus with artificially introduced genetic markers in its genome was rescued. The recombinant strain had biological properties similar to those of the original strain. These findings indicate that a reverse genetics system for the Komatsugawa strain has successfully been established.

KEYWORDS: Japan, Komatsugawa, rabies virus, reverse genetics, street strain

Rabies is a lethal neurological disease affecting all mammal species including humans that is caused by infection with rabies virus (RABV), a member of the genus Lyssavirus of the family Rhabdoviridae. No definite therapy for this disease has yet been established, resulting in approximately 59,000 human deaths every year, mainly in developing countries in Asia and Africa [5]. Therefore, the development of a therapeutic approach is an important and urgent task for minimizing and eventually eliminating human deaths by rabies. In nature, field RABVs (so-called “street strains”) with distinct genetic backgrounds are maintained among various animal host species and all of them have the potential to be transmitted to humans. Therefore, to establish a therapeutic approach that is universally effective in all rabies patients, it is necessary to exhaustively characterize street RABV strains with various genetic backgrounds and then to identify a key phenomenon in the pathogenic mechanisms that is conserved among all street strains.

To understand the rabies pathogenesis at the molecular level, a reverse genetics (RG) system for RABV is undoubtedly useful, as it enables rescue of an infectious virus from cloned cDNA and thus discreetional manipulation of the viral genome. For example, the RG system permits generation and the following phenotypic examination of a series of RABV variants with a mutation(s) in a viral protein, leading to the identification of an important functional domain on the protein that is involved in viral pathogenesis. Since the first report of the RG system for RABV [22], systems for several laboratory and vaccine strains (so-called “fixed strains”) have been established [6, 12, 28, 29] and utilized in various research aspects [2, 4, 8, 10, 24]. The findings obtained in those studies include useful information for understanding RABV pathogenesis. However, the pathogenic mechanisms revealed by using fixed RABV strains do not always reflect those of street strains that are the actual cause of rabies in clinical patients. Generally, fixed strains have been established after serial passages of a street strain in animal brains and, in many cases, cultured cell lines and therefore can acquire biological phenotypes that are different from those of street strains. This indicates the limitation of the pathogenesis studies using only fixed strains.

Since the first establishment of the RG system for a street RABV strain with a silver-haired bat-derived strain, SHBV-18 [3], RG systems for several street strains including RABV-Dog, RABV-Fox, 1088, 8743THA, GD-SH-01, and QS-05 have been reported [1, 7, 21, 25, 27], facilitating studies on the pathogenesis by street strains. However, there is a genetic bias on the street strains that were chosen for the establishment of RG systems: the street strains stated above belong to the phylogenetic clade Bats, Cosmopolitan or Asian, only three of the eight major clades that were previously defined by a whole genome-based phylogenetic analysis [26]. This highlights the necessity for the establishment of RG systems for street strains that belong to the remaining five clades (Africa-2 and -3, Indian subcontinent, RAC-SK, and Arctic-related clades) for understanding the intrinsic pathogenic mechanisms conserved among

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street strains with different genetic backgrounds, which includes the mechanisms involved in viral neurovirulence, neuroinvasiveness and immune evasion.

The RABV strain Komatsugawa is the only existent Japanese street strain, which was isolated from a dog in Tokyo in the 1940s before eradication of rabies in Japan in 1957. Phylogenetic analyses previously revealed that this strain belongs to the Arctic-like clade [11, 13, 23], of which street strains have barely been characterized phenotypically. We recently demonstrated by comprehensive phenotypic analyses that, even though the Komatsugawa strain has experienced 19 brain-to-periphery passages in guinea pigs and more than 20 intracerebral passages in mice after the original isolation from a dog [19], this strain retains biological phenotypes including virulence that are similar to those of the representative street strains RABV-Dog and 1088 [23]. These findings indicate that the Komatsugawa strain provides an effective model for studying rabies pathogenesis. In this study, to facilitate studies on the pathogenic mechanisms of street RABVs, we aimed to establish an RG system for the Komatsugawa strain. We recovered the recombinant Komatsugawa strain from cloned cDNA (rKoma) and compared the biological phenotypes, including virulence in mice, of this strain with those of wild-type Komatsugawa strain (wtKoma).

To construct a full-length genome plasmid of the Komatsugawa strain, a total of five cDNA fragments (1.5–3.8 kb in length) that collectively cover the whole viral genome (11,927 nt) were amplified by reverse transcription (RT)-polymerase chain reaction (PCR) from genomic RNA extracted from a wtKoma stock prepared in previous study [23]. These cDNA fragments were assembled on a pUC19 plasmid vector by stepwise cloning. The complete genomic cDNA assembled on the pUC19 vector was located downstream of a T7 promoter and upstream of a hepatitis delta virus antigenomic ribozyme cDNA so that the positive-sense genomic (antigenomic) RNA can be synthesized under the control of T7 RNA polymerase. During the RT-PCR and cloning steps described above, two genetic markers that distinguish rKoma from wtKoma were introduced into the cDNA fragments. Specifically, a silent mutation (A-to-C nucleotide substitution, shown as a positive-sense cDNA) was introduced into the G gene at position 3962 (based on the genome nucleotide sequence of wtKoma [GenBank accession No. LC553558.1]). In addition, a T-to-A substitution was introduced into a nucleotide at position 5083 to generate an SpeI recognition site (5083 ACTAGT 5088, substitution site underlined) in the G-L noncoding region of rKoma. The former and latter genetic markers were designated G-Mk#1 and G-Mk#2, respectively. The resulting plasmid was subject to sequencing by the conventional Sanger method to confirm the integrity of the nucleotide sequence of the cloned cDNA.

Details of the construction of the full-length genome plasmid are available from the authors on request.

According to the protocols for the RG system for the street RABV strain 1088 [7], we tried to rescue infectious rKoma from cloned cDNA. Briefly, BHK/T7-9 cells that constitutively express T7 RNA polymerase [12] were grown in a 24-well tissue culture plate and were transfected by using TransIT-LT1 (Mirus, Madison, WI, USA) with the full-length genome plasmid (2.0 μg/well) together with helper plasmids expressing N, P, G, and L proteins, according to the protocol for the rescue of the street 1088 strain [7, 12]. The culture supernatants were collected at 4 days post-transfection and were inoculated into mouse neuroblastoma clone C1300 cells (NA cells) [16]. At 6 days post-inoculation, the NA cells were fixed and immunostained for viral N protein by using a monoclonal antibody 13–27 [17]. The immunostaining visualized viral N protein-positive foci in NA cells inoculated with supernatants from the transfected BHK/T7-9 cells (Fig. 1, left). In contrast, when BHK/T7-9 cells were transfected without the helper plasmid expressing viral RNA polymerase L protein, foci were not formed in the NA cells (Fig. 1, right). These findings indicated that infectious rKoma was generated by transfection with the full-length genome plasmid and the helper plasmids.

To check whether rKoma has the artificially introduced genetic markers in the genome, a cDNA fragment (nucleotide positions from 3134 to 6699) containing both G-Mk#1 and G-Mk#2 sites was amplified by RT-PCR by using RNA extracted from a viral stock

![Fig. 1. Detection of infectious rKoma rescued from cDNA by inoculation to mouse neuroblastoma (NA) cells. To rescue the rKoma from cDNA, BHK/T7-9 cells were transfected with the full-length genome plasmid together with four helper plasmids expressing N, P, G, and L proteins, according to the protocol for the rescue of the street 1088 strain [7]. At 4 days after transfection, the supernatant was collected and inoculated into NA cells. The NA cells at 6 days post-inoculation were fixed and immunostained with an anti-RABV N protein mouse monoclonal antibody 13–27 [17]. A negative control was prepared by inoculating with the supernatant from the recovery experiments without transfection with the L protein-expressing helper plasmid. The scale bars correspond to 200 μm.](image-url)
as a template (Fig. 2A). RT-PCR, but not PCR without the RT step, resulted in amplification of a cDNA fragment with the expected size (3,566 bp) (Fig. 2B). These results indicated that the cDNA fragment was amplified from the viral genomic RNA, not from the transfected plasmid DNA. Direct sequencing confirmed the presence of an A-to-C nucleotide substitution at the position that corresponds to genome nucleotide position 3962 on the amplified cDNA fragment (Fig. 2C). After SpeI digestion, the amplified cDNA fragment was subjected to sequencing by the conventional Sanger method after agarose gel purification. The genetic marker (G-Mk#1) in the rKoma gene is indicated on the nucleotide sequences. Nucleotide numbers are based on the genome nucleotide sequence of wtKoma [GenBank accession No. LC553558.1] (Fig. 2D). The amplified cDNA fragments from rKoma and wtKoma were digested with SpeI before electrophoresis. M: molecular weight marker, +: cDNA fragments treated with SpeI, -: without SpeI.

**Fig. 2.** Detection of genetic markers in the rKoma genome. (a) Schematic diagram of the cDNA fragment amplified by RT-PCR for checking the presence of the genetic markers (G-Mk#1 and 2) introduced into the rKoma genome. The sizes of the rKoma and wtKoma cDNA fragments after SpeI digestion are also indicated. (b) A cDNA fragment of 3,566 bp including the genetic marker sites was amplified by RT-PCR by using an RNA extract from an rKoma stock as a template. An RNA extract from a wtKoma stock was used for a positive control. M: molecular weight marker, +: with the RT step, -: without the RT step (c). The cDNA fragments from rKoma and wtKoma were subjected to sequencing by the conventional Sanger method after agarose gel purification. The genetic marker (G-Mk#1) in the rKoma gene is indicated on the nucleotide sequences. Nucleotide numbers are based on the genome nucleotide sequence of wtKoma [GenBank accession No. LC553558.1]. (d) The amplified cDNA fragments from rKoma and wtKoma were digested with SpeI before electrophoresis. M: molecular weight marker, +: cDNA fragments treated with SpeI, -: without SpeI.
fragment from rKoma was cleaved into two fragments of 1,954 bp and 1,612 bp, whereas the fragment from wtKoma was not cleaved (Fig. 2D). These findings demonstrated that rKoma has both G-Mk#1 and G-Mk#2 in the genome, proving that the Komatsugawa strain can be genetically manipulated by modifying viral cDNA on the full-length genome plasmid.

Next, to examine the growth ability of rKoma in cultured cells, a multiple-step growth curve of this strain in NA cells was compared with that of wtKoma (Fig. 3A). On the first day after viral inoculation of NA cells at a multiplicity of infection of 0.001, the titers of both rKoma and wtKoma in the culture supernatants were under the detection limit (<1.0 × 10^2 focus-forming units [FFU]/mL). From

![Graph showing viral titers over days post infection](image)

**Fig. 3.** Growth curves and focus sizes of rKoma and wtKoma in mouse neuroblastoma (NA) cells. (a) Growth curves of rKoma and wtKoma in neuroblastoma (NA) cells. Each virus was inoculated at a multiplicity of infection (MOI) of 0.001 into NA cells. Viral titers in culture supernatants collected at 1, 3, and 5 dpi were determined by a focus assay as reported previously [20]. This assay was carried out in triplicate.

To check statistical significance, two-way analysis of variance (ANOVA) with Sidak’s multiple-comparison test was conducted by using GraphPad Prism ver. 8.3 (GraphPad Software, San Diego, CA, USA). Error bars: means ± SEM, ns: not significant (P ≥0.05). (b) Focus formation by rKoma and wtKoma in NA cells. NA cells were inoculated with each virus at an MOI of 0.0002 and then overlaid with eagle’s minimal essential medium (E-MEM) containing 1% methylcellulose. At 3 days after inoculation, the cells were fixed and immunostained with an anti-RABV N protein antibody 13–27. The scale bars correspond to 200 μm. (c) Quantification of the focus area. A total of 50 foci of each strain were randomly selected and photographed to quantify their areas by Image J software. Each column represents the average area. Student’s t-test was conducted by using GraphPad Prism to check statistical significance. Error bars: means ± SEM, ns: not significant (P ≥0.05).
3 days post-inoculation (dpi), the titers of both strains increased exponentially and reached over $1.0 \times 10^5$ FFU/mL at 5 dpi. There was no statistically significant difference between titers of rKoma and wrKoma at 3 and 5 dpi ($P \geq 0.05$). These findings indicated that the growth abilities of rKoma and wrKoma in NA cells are comparable.

To compare cell-to-cell spread of rKoma in NA cells with that of wrKoma, focus sizes of the respective strains in NA cells were examined. Immunostaining for viral N protein revealed that focus size of rKoma was similar to that of wrKoma (Fig. 3B). A quantitative analysis supported this observation: there was no statistically significant differences between focus areas of rKoma and wrKoma (Fig. 3C). These findings indicated that the efficiency of cell-to-cell spread of rKoma is almost identical to that of wrKoma.

Finally, to compare the virulence of rKoma and that of wrKoma in mice, 6-week-old male ddY mice (Japan SLC, Shizuoka, Japan) (5 mice/group) were inoculated with $1.0 \times 10^2$ FFU or $1.0 \times 10^3$ FFU of the respective strains into the left thigh muscle and were observed daily for 30 days for clinical signs. The mice that showed severe neurological signs were humanely euthanized according to the protocols approved by the Committee for Animal Research and Welfare of Gifu University (approval no. H30-202). After intramuscular inoculation, both rKoma and wrKoma caused lethal infection in mice in a dose-dependent manner (Table 1). Importantly, the lethal dose 50 of rKoma ($4.8 \times 10^2$ FFU) was comparable to that of wrKoma ($6.8 \times 10^2$ FFU), indicating that the virulence of rKoma in mice is almost identical to that of wrKoma.

In conclusion, all of the findings described above indicated that rKoma, which has biological characteristics similar to those of wrKoma, was recovered from cloned cDNA. Thus, we have successfully established an RG system for an RABV strain that belongs to the Arctic-like phylogenetic lineage for the first time.

The RG system for the Komatsugawa strain established in this study will be a strong tool to study molecular mechanisms for RABV pathogenesis in the future. We previously demonstrated by using the fixed strain Nishigahara that the N, P and M proteins play important roles in the pathogenesis by exerting their evasive functions against the host type I interferon system and apoptotic cell death [8, 9, 14, 15, 18]. These findings indicate that the N, P and M proteins have the potential to be a therapeutic molecular target. However, these protein functions have not exhaustively been examined with street strains with distinct genetic backgrounds, thus questioning whether these functions are universally conserved among street RABVs. To resolve this question, it is necessary to generate viral variants with defects in the functions by using RG systems for various street strains including the Komatsugawa strain and then to investigate their phenotypes involved in the pathogenesis. In addition, the RG system for the Komatsugawa strain will facilitate molecular studies on replication mechanisms that are conserved among street RABV strains, which will also provide useful information about therapeutic targets for rabies.

CONFLICT OF INTEREST. The authors declare no conflicts of interest associated with this manuscript.

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