Fluorescent and bioluminescent reporter mouse-adapted Ebola viruses maintain pathogenicity and can be visualized *in vivo*

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Supplementary Material and Methods

Rescue of recombinant viruses

Viral RNA from the MA-EBOV strain Mayinga was used as a template for RT-PCR amplification to generate 4 overlapping fragments of similar size spanning the full-length genome. These fragments were gel-purified and used to assemble a full-length clone into a T7 transcription vector. The final plasmid contained the full-length anti-genome preceded by the T7 RNA polymerase promoter and followed by the hepatitis delta virus ribozyme and the T7 polymerase terminator. The full-length clone was later modified to introduce two different reporter genes, ZsGreen1 (ZsG, Clontech) and nano-luciferase (nLuc, Promega), independently or together. Reporter genes were inserted through molecular recombination using the InFusion cloning system (Takara Bio).

For single-reporter genomes, ZsG or nLuc were fused to the self-cleaving P2A peptide and to the EBOV VP40 open reading frame (ORF). For double-reporter genomes, two strategies were used. In the first, nLuc was fused to P2A and to EBOV NP ORF, and ZsG was fused to P2A and to EBOV VP40 ORF. In the second strategy, ZsG was directly fused to nLuc, and that cassette was fused to P2A and to EBOV VP40 ORF.

Rescue of all recombinant MA-EBOV strain Mayinga viruses was performed by transfecting a 70% confluent monolayer of BSR-T7/5 cells in 12-well plates with 1.5 μg of each of the full-length clones, together with 4 support plasmids: 0.5 μg pC-L, 0.25 μg pC-NP, 0.05 μg pC-VP35, and 0.05 μg pC-VP30. Construction of these support plasmids is described in [29]. Supernatants from transfected cells were harvested 7 days post transfection, clarified by low-speed centrifugation, and passaged twice in fresh monolayers of Vero-E6 cells. Rescue events were confirmed by immunostaining infected cell monolayers or by monitoring expression of the reporter genes. All recombinant viruses were sequenced by NGS, and the viral genomic sequences were identical to those in the full-length plasmids.

Next generation sequencing

Viral RNA was prepared for sequencing using the KAPA RNA HyperPrep kit with RiboErase (Roche) according to the manufacturer's instructions. NGS was performed using paired-end 2 × 150 bp chemistry on either Illumina MiniSeq or iSeq instrument. Analysis was performed using CLC Genomics Workbench (v21.0.3).

RT-qPCR primers and probe sets

EBOV RNA was quantified using an RT-qPCR assay targeting EBOV NP (forward primer: AGAAATGAACCCTCCGGCTC; reverse primer: ATCATCTGACTCCAAGGGCG; probe: ACCCACTGGACGATGCCGACG). The housekeeping genes Ppia (forward primer: CCCACCGTGTTCTTCGAC; reverse primer: TCCTTTCTCTCCAGTGCTCAG; probe: GAGCCCTTGGGCCGCGTCTC) and Gusb (forward primer: CCCAAGGGTTACTTTGTCCAG; reverse primer: TGGTATAGAGGACCACAGATCG;

probe: TTCTTCAACTATGCGGGACTGCA) were used to standardize RNA harvested from tissues. These assays have been validated in house as appropriate controls. Standard curves were generated by serially diluting 10-fold an RNA oligo specific for EBOV NP (AGAAATGAACCCTCCGGCTCAACCAGCCCTCGCATGCTGACACCAATTAACGAAGA GGCAGACCCACTGGACGATGCCGACGACGAGACGTCTAGCCTTCCGCCCTTGGAGT CAGATGAT).

Immunoblotting

Cell lysates were heated to 99°C for 5 min and 15 μL lysate was separated using 4–12 % Bis-Tris (ThermoFisher) SDS-PAGE and transferred to a nitrocellulose membrane. Western blotting was carried out using the Pierce™ Fast Western Blot Kit according to the manufacturer's instructions. EBOV proteins were identified using rabbit anti-EBOV NP pAb (IBT Bioservices #0301-012) and rabbit anti-EBOV VP40 (IBT Bioservices #0301-010). Reporter proteins were identified using mouse anti-ZsGreen1 monoclonal antibody (ThermoFisher #TA180002) and mouse anti-NanoLuc monoclonal antibody (Promega #N7000). Mouse anti-α-tubulin monoclonal antibody (Sigma) was included as a loading control.

Mouse husbandry

Mice were housed in a climate-controlled laboratory with a 12 h day/night cycle and provided with commercially available, chloroplast-free mouse chow (Teklad Global 18% Protein Rodent Diet) and water *ad libitum*; and group-housed on a mix of corn cob (Bed-o'Cobs® ¼", Anderson Lab Bedding) and paper bedding (Enviro-dri® and Carefresh®) with cotton nestlets in an isolator-caging system (Tecniplast GM500) with a HEPA-filtered inlet and exhaust air supply.

Clinical scoring

Mice were assessed daily for clinical signs and weight change. Clinical signs in mice were scored based on 14 parameters: 2 points each for quiet, dull, responsive (QDR) disposition, hunched back, ruffled coat/piloerection, or hypoactivity; 3 points each for dehydration or abnormal huddling; 5 points each for ataxia/circling/tremors/paresis, abnormal breathing, or anemia; 7 points for weight loss of >20%; 10 points each for inability to bear weight, paralysis, frank hemorrhage or bleeding, moribund state, or weight loss of >25%. Animals were humanely euthanized with isoflurane vapors when end-point criteria were reached (clinical score ≥10) or at study completion (14 dpi).

Fluorescence imaging *in situ*

Fluorescence was visualized and imaged *in situ* using a Canon PowerShot G12 camera in conjunction with a Dark Reader camera filter (#AF580), Dark Reader spot lamp (#SL10S), Dark Reader hand lamp (#HL34T), and Dark Reader glasses (#AG16) from Clare Chemical Research (Dolores, CO).