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J Infect Dis. Author manuscript; available in PMC 2018 December 12.

Published in final edited form as:

J Infect Dis. 2017 December 12; 216(11): 1380–1385. doi:10.1093/infdis/jix486.

Rapid Determination of Ebolavirus Infectivity in Clinical Samples Using a Novel Reporter Cell Line

Markus H. Kainulainen, **Stuart T. Nichol**, **César G. Albariño**, and **Christina F. Spiropoulou**

Viral Special Pathogens Branch, Division of High-Consequence Pathogens and Pathology, Centers for Disease Control and Prevention, Atlanta, Georgia

Abstract

Modern ebolavirus diagnostics rely primarily on quantitative reverse transcription-polymerase chain reaction (qRT-PCR), a sensitive method to detect viral genetic material in the acute phase of the disease. However, qRT-PCR does not confirm presence of infectious virus, presenting limitations in patient and outbreak management. Attempts to isolate infectious virus rely on in vivo or basic cell culture approaches, which prohibit rapid results and screening. In this study, we present a novel reporter cell line capable of detecting live ebolaviruses. These cells permit sensitive, large-scale screening and titration of infectious virus in experimental and clinical samples, independent of ebolavirus species and variant.

Keywords

Ebola; infectivity; reporter cell line; virus isolation

Detecting ebolavirus ribonucleic acid (RNA) in patient samples by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) is a sensitive, first-line method readily adapted to the outbreak setting. However, a positive qRT-PCR result does not consistently equate to presence of infectious virus [1]. Moreover, minor differences in nucleotide sequence may render existing qRT-PCR assays unable to detect an emerging virus. Technically, qRT-PCR is easily scaled up and increasingly automated, whereas infectivity determination by virus isolation remains a more laborious task confined to biosafety level 4 (BSL-4) laboratories. Our standard ebolavirus isolation protocol includes inoculation of Vero-E6 cells, with fixation and immunostaining of the cell monolayer on days 7 and 14 postinfection; this procedure prohibits rapid results. In this work, we establish a reporter cell

Correspondence: C. F. Spiropoulou, PhD, Centers for Disease Control and Prevention, 1600 Clifton Road, G14/SB-153, Atlanta, GA 30333 (ccs8@cdc.gov).

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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line that produces a robust fluorescence signal in response to ebolavirus infection. We have validated this system using stocks of all 5 known virus species of the *Ebolavirus* genus, as well as virus-spiked human blood and serum samples and clinical samples from past outbreaks. Practical and scalable quantification of infectious Ebola virus and related viruses leaves us better prepared to conduct virus persistence studies and to detect emerging viruses in this important genus.

METHODS

Viruses and Biosafety

The following stock viruses were used in the study: Ebola virus/H. sapiens-tc/COD/1976/Yambuku-Mayinga, Sudan virus/H.sapiens-tc/UGA/2000/Gulu-808892, Reston virus/M.fascicularis-tc/USA/1989/Philippines89-Pennsylvania, Bundibugyo virus/H. sapiens-tc/UGA/2007/Butalya-811250, Taï Forest virus/H.sapiens-tc/CIV/1994/Pauléoula-CI, and Marburg virus/R.aegyptiacus-tc/UGA/2007/Kitaka-371Bat-811277. All work with infectious virus was performed in a BSL-4 facility at the Centers for Disease Control and Prevention (CDC).

Reporter Cell Line

Gene synthesis (Integrated DNA Technologies, Coraville, IA) was used to generate a negative-sense minigenome that was initially assembled in the pcDNA5/FRT vector (Invitrogen, Waltham, MA) using InFusion HD cloning (Clontech, Mountain View, CA). The minigenome is driven by the constitutive cytomegalovirus (CMV) promoter and transcribed by host RNA polymerase II. The transcript contains a hammerhead ribozyme at the 5′ end to produce an exact RNA end mimicking that of Ebola virus Makona variant genome (GenBank sequence KP178538.1). The hammerhead ribozyme used here was modeled on the optimization work presented by Yun et al [2] and corresponds to the ribozyme A sequence presented there with the following modifications: stem I (which provides specificity) includes a 7-nucleotide complementarity with Ebola Makona trailer end (GGACACA), and the sequence upstream of this stem is from the vector. The minigenome transcript begins with the 176 base minimal promoter region as identified in defective-interfering Ebola virus particles [3], followed by L messenger RNA (mRNA) untranslated region (UTR) between the polyadenylation signal and stop codon. The minimal promoter region ends in a U base, and the adjacent polyadenylation signal contains a 6 U stretch. The total number of U bases at the junction was left at 6. A codon-optimized sequence for zsGreen ([ZSG] Clontech) in negative sense orientation is followed by the Ebola Makona sequences preceding the nucleoprotein open reading frame, and the transcript 3′ end is trimmed by the “supercut” hepatitis delta virus ribozyme [4]. After initial optimization work (data not shown), the minigenome was transferred into the piggyBac transposon vector PB-CMV-MCS-EF1-Puro (System Biosciences, Palo Alto, CA) using NheI and NotI restriction sites.

To generate stable cell lines, the resulting piggyBac construct was transfected into Vero-E6 cells together with the Super piggy-Bac Transposase construct (System Biosciences) using TransIT LT1 reagent (Mirus Bio, Madison, WI) according to manufacturers’ instructions.

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Three days after transfection, cells were trypsinized and seeded on cell culture dishes as a dilution series. Clones were selected and expanded under 30 μg/mL puromycin. Once enough cells were obtained, clones were ranked by minigenome activation after infection with Ebola virus, and the best-performing clones were expanded further. One such clone was chosen after additional experiments, and it was used here as the Vero-Ebola-reporter cell line. Characterization of the minigenome expression is presented in Supplementary Figure 1.

Sensitivity Assays

Aliquots of diluted virus stocks were repeatedly titered by 50% tissue culture infectious dose ($TCID_{50}$) assays using 6 replicate wells of Vero-E6 cells and Vero-Ebola-reporter cells in parallel. Dilution series were prepared in medium containing 2% fetal calf serum, and 40 μL inoculum were used in each well of a 96-well plate. After 1-hour incubation at 37°C, the inocula were removed and medium without phenol red was added. The apparent titers on Vero-Ebola-reporter cells, as indicated by presence of ZSG-positive cells, were recorded on specified days postinfection until day 7, when the monolayers were fixed and immunostained using rabbit polyclonal antibodies detecting all known species of the genus *Ebolavirus* (in-house reagent 703371). Marburg virus was detected using rabbit polyclonal antibodies (reagent 703358). Quantification data of the ZSG signals upon infection are presented in Supplementary Figure 2.

To simulate low-titer clinical samples, healthy donor serum and ethylenediaminetetraacetic acid (EDTA) blood samples were spiked with stock viruses so that 95% of the mix was blood/serum. The spiked samples were aliquoted and frozen; a new vial was used for each repeat of the experiment. Spiked samples were inoculated neat and as dilution series in phosphate-buffered saline (PBS). The $TCID_{50}$ assays were performed as described above, except that for blood samples, the cells were washed once with PBS after removing the inocula.

Clinical samples obtained during historical outbreak investigations were selected based on their known infectivity status. These samples were tested the same way as the spiked blood samples; however, due to limited sample volumes, 1:10 dilution in PBS was the lowest dilution inoculated, and the experiment was done only once. All storage conditions and number of freeze-thaw cycles from the place of collection to the laboratory could not be ascertained for the clinical samples, and the absolute infectivity values determined did not necessarily reflect the values of the same samples at time of collection. Centers for Disease Control and Prevention Institutional Review Board (IRB) approval was obtained for the use of historical diagnostic material; the IRB waived the requirement to obtain informed consent for this secondary use of archived samples. Healthy donor blood was obtained with informed consent under a separate IRB-approved research protocol.

RESULTS

Our reporter cell system uses an Ebola virus minigenome expression cassette (Figure 1A and Supplementary Figure 1) that was introduced into Vero-E6, our standard cell line for ebolavirus isolation, by a transposon system. Upon infection, the viral replication complex recognizes promoters on the minigenome RNA and uses it as template to transcribe mRNA

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for the fluorescent protein, ZSG. Infectious virus can therefore be detected by monitoring ZSG fluorescence in live cells without additional reagents. As seen in Figure 1B, Ebola virus infection results in strong ZSG signals in a subset of infected Vero-Ebola-reporter cells, distinguishing infected from non-infected cultures. Importantly, infections with the other members of the *Ebolavirus* genus (Sudan, Bundibugyo, Reston, and Taï Forest viruses) also resulted in strong ZSG fluorescence, demonstrating that our system can detect multiple species of ebolaviruses with only minor differences in the activation potential (quantified in Supplementary Figure 2). The more distantly related filovirus, Marburg virus, could not activate the reporter.

Having established that infected Vero-Ebola-reporter cultures could be identified by the ZSG signal, we next evaluated the sensitivity of the system in comparison with virus detection by the standard immunofluorescence assay. We reasoned that titer determinations by the $TCID_{50}$ method would serve this purpose. In this assay, replicate wells are infected with 10-fold serial dilutions of the sample until no infectivity remains. Therefore, reduced sensitivity would manifest as failure to detect ZSG in the highest dilutions where live virus actually still exists, leading to reduced titer values. Apparent titers were determined daily by the reporter assay to assess the time required for reliable results. As shown in Figure 2A, all 5 ebolaviruses could be quantified using the reporter cells. By day 7, titer values determined by ZSG reporter signal closely resembled the values obtained by immunofluorescence on Vero-E6 cells, indicating that the sensitivity of the Vero-Ebola-reporter system is similar to that of the standard assay.

Next, the system was tested with material relevant for diagnostics and virus discovery. In contrast to stock titration, detecting low levels of infectivity in clinical material may be hindered by exposure of cell monolayers to high concentrations of viscous original sample. For a stringent test, human EDTA blood and serum were spiked with Ebola or Sudan virus stocks to obtain 2 samples with infectivity values close to the $TCID_{50}$ detection limit. As shown in Figure 2B, live Ebola or Sudan viruses in the 3–4 log/mL range could be reproducibly detected in blood by day 3, and <2-log/mL concentrations could be detected by day 4. Similar results were obtained with spiked serum samples. Mock-spiked control blood and serum samples tested negative in both $TCID_{50}$ and Vero-Ebola-reporter cell line assays (data not shown).

Finally, actual clinical material was tested. Original samples known to contain live virus at unknown titers were selected; Ebola, Sudan, and Bundibugyo viruses, the 3 viruses known to cause major outbreaks in humans, were represented. All 3 viruses could be detected directly from clinical material (Figure 2C). Two samples (Ebola and Bundibugyo) at relatively high 5-log and 6-log/mL titers, respectively, could be judged positive by as early as 24 hours postinfection. One Sudan sample with 3-log/mL infectivity, as well as 2 samples (Ebola and Bundibugyo) with infectivity below the quantification limit, produced positive results in the Vero-Ebola-reporter system by day 3 postinfection. One Sudan sample was negative on Vero-E6 cells but positive on Vero-Ebola-reporter cells (1 of 6 wells on day 7).

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DISCUSSION

Molecular methods, such as qRT-PCR, have rightly become the first-line method to diagnose cases of Ebola virus disease. However, there is no substitute for culturing clinical samples in vitro or in vivo to determine presence of live virus and transmission risk. The rapid advances in the field of molecular diagnostics have not reached virus isolation, which remains largely manual work. Reporter cell systems have emerged to bridge the gap for some viruses, yet expanding the approach is not trivial; the technical principle used necessarily depends on the biology of the virus in question. Viruses whose genomes exist as deoxyribonucleic acid (DNA) in host nuclei, including herpesviruses and lentiviruses, have been detected after stable integration of plasmids with reporter genes under viral promoters [5, 6]. Infection activates transcription of the reporter gene from the DNA template. Reporter cell lines for RNA viruses must rely on alternative principles. Flaviviruses [7] and enteroviruses [8] have been detected by linking cleavage of specific peptide sequences by viral proteases with reporter activation. Influenza, a negative-strand RNA virus with a nuclear replication cycle, has been detected using the minigenome approach [9]. A stable minigenome cell line has also been developed for a positive-strand RNA virus with a cytoplasmic replication cycle [10].

In this study, we use the minigenome principle to cover viruses of genus *Ebolavirus*, which are negative-strand RNA viruses with a cytoplasmic replication cycle. This work was inspired by other groups that have previously reported on successful swapping of Ebola and Reston virus replication complexes and minigenomes, providing evidence that viruses within the genus *Ebolavirus* have conserved transcription and replication signals [11–13]. Some experimental systems have even indicated that this cross-activation extends to Marburg virus [12, 13], but in other systems [11, 14], as well as ours, such cross-activation was not observed. Indeed, our system detects all 5 known ebolaviruses in stock samples and all the tested ones in patient serum and blood. We consider cross-activation by multiple ebolaviruses advantageous, because detecting any type of ebolavirus, especially in clinical material, is arguably a situation of public health concern. Although the assay involves growing live ebolaviruses and is therefore confined to BSL-4 laboratories, it is easily scalable and simple to perform, considerably increasing the capacity to detect infectious virus. We intend to use the cell line in projects involving ecological and clinical samples potentially containing live Ebola virus and its relatives, those known to date, and those that may yet be discovered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Tatyana Klimova for critical editing of the manuscript, Shannon Bonds (Emory University) for help in obtaining healthy donor blood, and Ketan Patel for assisting in accessing samples. We acknowledge that more interesting work on virus reporter cells exists than could be referenced in this report’s short format. We encourage the interested reader to look into the topic beyond the key publications referenced herein.

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Financial support. This work was done with funds allocated for Viral Special Pathogens Branch by the Centers for Disease Control and Prevention.

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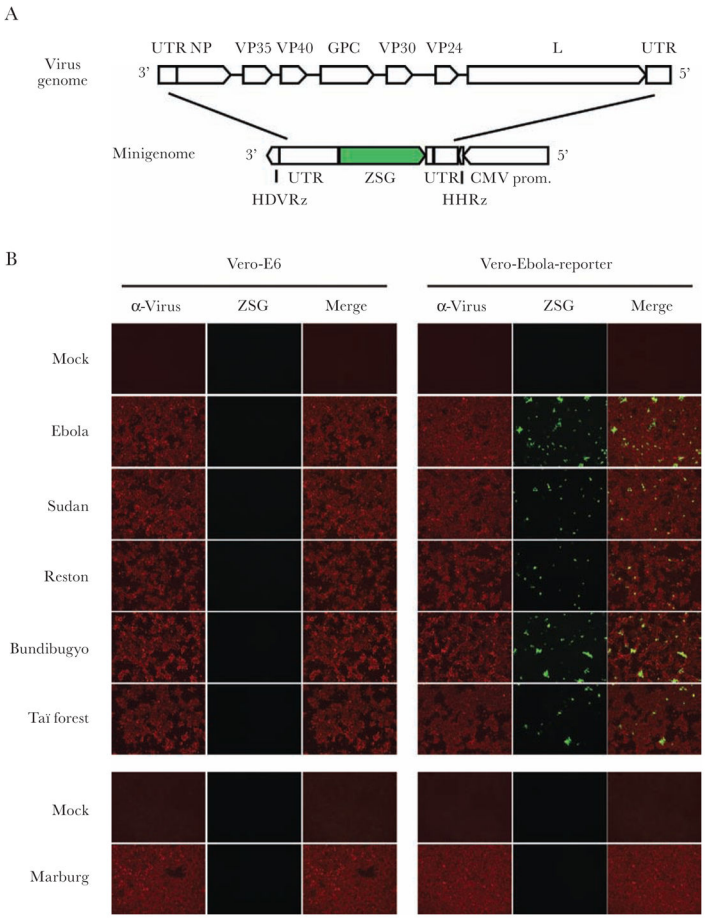

Figure 1.

Ebola virus minigenome schematic and reporter cell activation by different filoviruses. (*A*) Schematic presentation of Ebola virus genome and the minigenome cassette. (*B*) Activation of zsGreen (ZSG) reporter upon infection. Vero-E6 cells and Vero-Ebola-reporter cells were infected at multiplicity of infection = 0.5 and immunostained 3 days postinfection. Abbreviations: CMV prom., cytomegalovirus promoter; GPC, glycoprotein precursor; HDVRz, hepatitis delta virus ribozyme; HHRz, hammerhead ribozyme; L, ribonucleic acid (RNA)-dependent-RNA-polymerase; NP, nucleoprotein; UTR, untranslated region; VP, viral protein; ZSG, zsGreen; α-Virus, immunostaining against the virus with specific antibodies.

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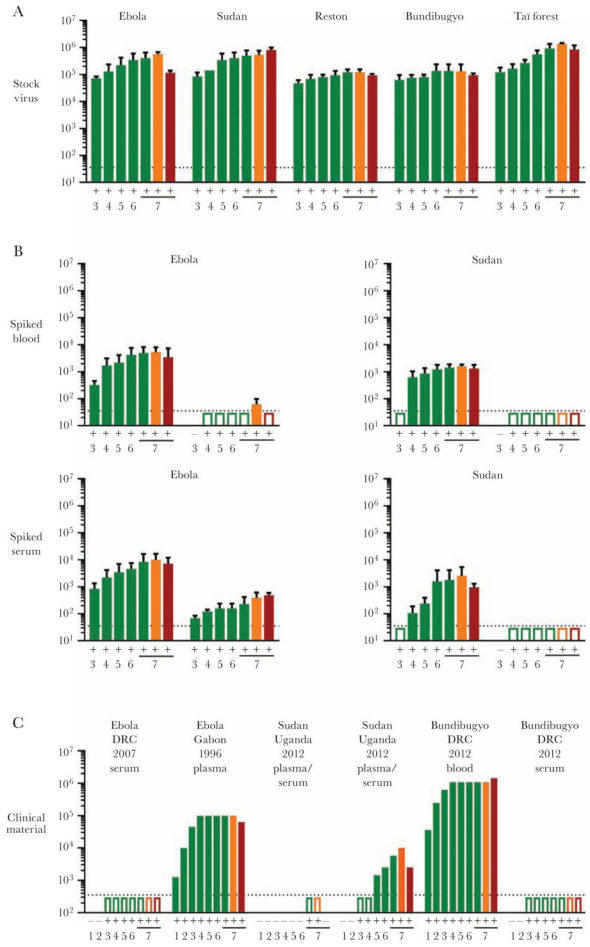

Figure 2.

Quantifying infectious virus with the reporter cell line. All inserts represent virus titer values as 50% tissue culture infectious dose $(TCID)_{50}$/mL. (*A*) Stock virus titration. (*B*) Ethylenediaminetetraacetic acid blood and serum samples spiked with Ebola or Sudan viruses at 2 concentrations. (*C*) Clinical samples. Green bars represent titers determined by zsGreen (ZSG) signal. Orange and red bars represent titers determined by immunostaining on Vero-Ebola-reporter cells and Vero-E6 cells, respectively. Day postinfection is depicted by numbers, and positive (all repeats) or negative results are depicted by +/− symbols. Dashed horizontal lines indicate $TCID_{50}$ quantification limit. Open bars below quantification limit represent time points at which live virus was detected in all repeat experiments, but at least 1 repeat did not produce enough signal for $TCID_{50}$ calculation. *A* and *B* represent averages and standard deviations from 3 independent experiments. *C* presents a single experiment. Abbreviation: DRC, Democratic Republic of the Congo.