Published in final edited form as:

Vet Microbiol. 2016 January 15; 182: 102–107. doi:10.1016/j.vetmic.2015.11.011.

Ebola Virus Mediated Infectivity is Restricted in Canine and Feline Cells

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Abstract

Ebolaviruses and marburgviruses belong to the *Filoviridae* family and often cause severe, fatal hemorrhagic fever in humans and non-human primates. The magnitude of the 2014 outbreak in West Africa and the unprecedented emergence of Ebola virus disease (EVD) in the United States underscore the urgency to better understand the dynamics of Ebola virus infection, transmission and spread. To date, the susceptibility and possible role of domestic animals and pets in the transmission cycle and spread of EVD remains unclear. We utilized infectious VSV recombinants and lentivirus pseudotypes expressing the EBOV surface glycoprotein (GP) to assess the permissiveness of canine and feline cells to EBOV GP-mediated entry. We observed a general restriction in EBOV-mediated infection of primary canine and feline cells. To address the entry mechanism, we used cells deficient in NPC1, a host protein implicated in EBOV entry, and a pharmacological blockade of cholesterol transport, to show that an NPC1-dependent mechanism of EBOV entry is conserved in canine and feline cells. These data demonstrate that cells of canine and feline origin are susceptible to EBOV GP mediated infection; however, infectivity of these cells is reduced significantly compared to controls. Moreover, these data provide new insights into the mechanism of EBOV GP mediated entry into cells of canine and feline origin.

Keywords

Ebola virus; filovirus; canine; feline; VSV recombinant; pseudotypes; entry; infectivity; susceptibility

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Introduction

Ebolaviruses and marburgviruses viruses belong to the family *Filoviridae* and can cause lethal hemorrhagic fever in non-human primates and humans with case fatality rates of 50–90%. There are currently no approved vaccines or antivirals to combat ebolavirus or marburgvirus infection in animals or humans. The catastrophic 2014 outbreak of Ebola virus (EBOV) in West Africa and the unprecedented emergence of this global public health threat in the United States and elsewhere underscores the severity and global importance of this virus and disease. During this outbreak, many questions were raised regarding the unknown potential for domestic pets (*e.g.* dogs and cats) to serve as either a reservoir or vector for the virus, of to be involved in transmission of EBOV to humans and other animals. Indeed, only a limited number of studies have been published on the ability of ebolaviruses to infect cells of canine origin, and no known reports to assess the ability of ebolavirus to infect cells of feline origin (Allela et al., 2005; Chan et al., 2000; Olson et al., 2012; Yang et al., 1998). This lack of knowledge prompted us to undertake this study to investigate the susceptibility of primary canine and feline cells to EBOV GP-mediated infection, and to address, in part, the mechanism of virus entry into these cells.

EBOV GP is required for virus attachment to cells and subsequent entry into endosomes, and the host endo/lysosomal cholesterol transporter protein Niemann-Pick C1 (NPC1) is critical for efficient escape of EBOV from endosomes of primate and human cells leading to a productive infection (Carette et al., 2011). Here, we utilized infectious VSV recombinants and lentivirus pseudotypes expressing the surface GP of EBOV to show that although primary canine and feline cells are susceptible to EBOV-mediated infection, infectivity was restricted significantly compared to that of control primate and human cells. Despite the reduced efficiency in EBOV-mediated entry, we show that the NPC1-dependent mechanism of EBOV entry appears to be conserved in primary canine and feline fibroblasts by using both genetic (NPC1^{−/−} cells) and pharmacological (compound U18666A) approaches. In sum, these findings provide new mechanistic insights into EBOV-mediated entry into canine and feline cells; however, a defined role for domestic pets as a reservoir for the virus, or in the efficient transmission of EBOV from domestic pets to humans, remains to be determined.

Materials and Methods

Cells, Plasmids, and Reagents

Primary canine fibroblasts (CFB) and primary feline fibroblasts (FFB-WT and FFB-NPC1^{−/−}) were maintained in DMEM/F12 medium (Gibco) supplemented with 10% fetal calf serum (FCS) and penicillin (100 U/ml)/streptomycin (100µg/ml). The primary FFB cells were collected from the skin of either normal cats (WT) or untreated NPC1^{−/−} cats. Vero-E6 cells, COS-1, BHK21 cells, and human HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM). Madin Darby Canine Kidney (MDCK) cells were maintained in MEM (Gibco) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml)/streptomycin (100µg/ml). All cells were maintained at 37°C in a humidified 5% CO₂ incubator. Compound U18666A was purchased from EMD Millipore (662015-10mg) and a 100mM stock solution was prepared in dH₂O and stored at −20°C. Working concentrations

of 5 and 10 mM of U18666A were prepared fresh in dH₂O immediately before use. Anti-VSV M monoclonal antibody 23H12 (kindly provided by D. Lyles) was used to detect VSV M in Western blotting and immunofluorescence assays.

VSV infection and quantitation

VSV-WT and recombinant VSV-EboGP (Zaire) (expressing mCherry) viruses (Fig. 1) have been described previously (Haines et al., 2012) and were propagated in BHK-21 and Vero-E6 cells, respectively (Haines et al., 2012). Approximately 1×10^6 cells were plated on collagen-coated 6-well plates and infected with VSV-WT or VSV-EboGP at the MOI of 1 for 1 hour. The inoculum was removed, and the cells were washed 3X with PBS, and then incubated for the indicated times in DMEM medium supplemented with 5% fetal calf serum (FCS). Cells were stained with Nucblue, and VSV-EboGP infected cells expressing mCherry were visualized by fluorescence microscopy at 18 hrs post-infection. Virus containing supernatants were harvested at the indicated times post-infection, and titers were determined by standard plaque assay in BHK-21 cells as described previously (Irie et al., 2005). Total and infected cell numbers used to derive percent infection were counted using the “multi-wavelength scoring” segmentation module within MetaMorph image analysis software (Molecular Devices, Downingtown, PA). The total number of cells was determined as the number of Nucblue-stained nuclei and the number of infected cells as nuclei surrounded by mCherry fluorescence. Infected cell extracts were prepared using RIPA buffer (50mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS containing protease inhibitors).

Transduction of lentivirus-GFP pseudotypes

Lentiviral pseudotypes were generated by polyethylenimine transfection of 5×10^6 HEK293T cells with the plasmids pSPAX2 (10.5 μ g, Addgene plasmid #12260), pLenti CMV GFP Hygro (15 μ g, Addgene plasmid #17446) and either pCB6-VSV-G (3.75 μ g) or pCAGGS-Ebola (Zaire) GP- Δ muc (11.25 μ g) (Simmons et al., 2002). EboGP- Δ muc, which has had the mucin domain of the glycoprotein deleted, was used to minimize cytopathic effects on the producer cells. At 24h (VSV-G) or 48h (EboGP- Δ muc) the supernatants were collected and clarified by low-speed centrifugation. 1×10^5 of each cell type were plated into collagen-coated 24-well plates. Unconcentrated supernatants as described above were added to cells before spinoculation at 1200g for 30 min (O'Doherty et al., 2000). The cells were incubated at 37°C for 72h after transduction, then harvested, fixed in 1% paraformaldehyde, and analyzed by flow cytometry for percentage expressing GFP.

Pharmacological inhibition of virus entry

WT feline and canine fibroblasts were mock- or pre-treated with U18666A for 1 hour at the indicated concentrations, and cells were then infected with VSV-WT or VSV-EboGP at an MOI=1 in the absence or presence of U18666A at the indicated concentrations and times. Supernatants were harvested at 10 and 20 hours post-infection, and virions were quantified by standard plaque assay as described previously (Irie et al., 2005).

Results

Infection of primary canine and feline fibroblasts with VSV-EboGP

We first sought to determine whether primary cells or cell lines of canine or feline origin were susceptible to infection with VSV-EboGP. Vero-E6 (standard primate cell line for EBOV propagation), COS-1 (primate fibroblast cell line), and HEK293T (human epithelial cell line susceptible to EBOV infection) cells were used as epithelial and fibroblast control cells (Fig. 2). The indicated cells were infected with VSV-EboGP at an MOI=1.0, and virions were quantified from supernatant samples harvested at either 10 or 20 hours post-infection (Fig. 2). Importantly, we found a significant restriction in infectivity in both FFB and CFB cells at 10 and 20 hours post-infection compared to control Vero-E6 cells (Fig. 2). Indeed, virion production from FFB and CFB cells was reduced between 2–3 orders of magnitude compared to that from Vero-E6 cells (Fig. 2). It should be noted that infectivity of VSV-EboGP was also found to be restricted in additional established and primary canine cells, including Madin Darby Canine Kidney (MDCK) and canine mesenchymal stem cells (CMSC) in multiple experiments (Han and Harty, data not shown). These results suggest that primary canine and feline fibroblasts are significantly less permissive to infection with VSV-EboGP than are multiple control cells.

In addition to virus titration, we used confocal microscopy to visualize and quantify VSV-EboGP infected Vero-E6, CFB, FFB-WT, and FFB-NPC1^{-/-} cells expressing mCherry (Fig. 3). The indicated cells were either mock-infected, or infected with VSV-EboGP at an MOI=1.0, and mCherry expression was visualized at 18 hrs post-infection, which allowed for multiple rounds of virus replication (Fig. 3). Representative confocal images revealed robust expression of mCherry fluorescence in 94% of infected Vero-E6 cells. In contrast, 1% of CFB were productively infected with VSV-EboGP, whereas 61% and 5% of FFB-WT and FFB-NPC1^{-/-} cells were productively infected with VSV-EboGP, respectively (Fig. 3). Together, results described above indicate that infectivity of VSV-EboGP is constrained in primary canine and feline fibroblasts compared to control cells, and suggest that expression of host NPC1 is important for optimal infectivity of VSV-EboGP.

VSV-EboGP infection of WT and NPC1^{-/-} feline cells

To further evaluate whether an NPC1 dependent mechanism of EBOV entry is indeed conserved in feline cells as it is in primate and human cells, we performed a timecourse infection of Vero-E6, WT, and NPC1^{-/-} feline fibroblasts with either VSV-EboGP or VSV-WT as a control (Fig. 4). Cells were infected with the indicated virus at an MOI of 1.0, and cells and virion containing supernatants were harvested at 10 and 20 hours post-infection. Supernatant samples were titrated, and viral growth curves were graphed (Figs. 4A and 4B). Robust titers of both VSV-EboGP and VSV-WT were quantified from Vero-E6 cells at 10 and 20 hrs post-infection (Figs. 4A and 4B). In contrast, the titers of VSV-EboGP from FFB-WT cells were reduced at 10 and 20 hrs post-infection by approximately 24- and 20-fold respectively, and an even greater reduction in VSV-EboGP titer was observed from FFBNPC1^{-/-} cells at both 10 and 20 hrs post-infection compared to that from FFB-WT and Vero-E6 cells (Fig. 4A). The reduction in titers of VSV-WT from feline cells compared to that from Vero-E6 cells was less pronounced (approximately 5- and 10-fold at 10 and 20 hrs

post-infection, respectively), and importantly, titers of VSV-WT from FFB-WT and FFB-NPC1^{-/-} cells at both 10 and 20 hrs post-infection were essentially identical (Fig. 4B). Western blotting was used to detect and quantify the levels of VSV M protein synthesized in infected cell extracts, and these findings revealed a close correlation between the levels of VSV M proteins synthesized in infected cells and corresponding viral titers (Fig. 4C). Indeed, a marked difference in expression of VSV M was observed in FFB-WT and FFB-NPC1^{-/-} cells infected with VSV-EboGP (Fig. 4C, compare lanes 2 and 3), whereas essentially no difference in expression of VSV M was observed in FFB-WT and FFB-NPC1^{-/-} cells infected with VSV-WT (Fig. 4C, compare lanes 5 and 6).

Lastly, we used indirect immunofluorescence to identify the intracellular localization of VSV M in FFB-WT or FFB-NPC1^{-/-} cells infected with either VSV-WT or VSV-EboGP at 5 hrs post-infection (Fig. 4D). Based on previously published results in human cells (Carette et al., 2011; Cote et al., 2011; Krishnan et al., 2012; Miller et al., 2012), we expected to observe a more punctate staining pattern for VSV M in VSV-EboGP infected FFB-NPC1^{-/-} cells compared to FFB-WT cells, if VSV-EboGP is indeed dependent on expression of feline NPC1 to escape from the endosome. Indeed, we found that the pattern of VSV M expression was predominantly cytoplasmic in VSV-EboGP infected FFB-WT cells, whereas the pattern of M expression was less abundant and predominantly punctate in VSV-EboGP infected FFB-NPC1^{-/-} cells (Fig. 4D). In contrast, FFB-WT and FFB-NPC1^{-/-} cells infected with VSV-WT displayed a similar cytoplasmic staining pattern for VSV M (Fig. 4D).

Pharmacological inhibition of NPC1 in feline and canine cells restricts EBOV-mediated entry

We used a pharmacological approach to help confirm results from our genetic approach above that suggest that feline NPC1 expression is important for efficient entry of VSV-EboGP. Briefly, FFBWT or CFB-WT cells were infected with VSV-EboGP or VSV-WT at an MOI of 1.0 in the absence or presence of compound U18666A; an inhibitor of intracellular cholesterol transport that yields a phenotype similar to that of NPC1 mutant cells (Cote et al., 2011). Cells and supernatants were harvested at 10 and 20 hrs post-infection, and infectious virions were quantified by standard plaque assay (Fig. 5, panels A, B, D, and E). We observed a 1–2 log reduction in titers of VSV-EboGP from FFB-WT cells at 10 and 20 hrs post-infection in the presence of 5 or 10 μ M U18666A (Fig. 5A). In contrast, no significant reduction in titers of VSV-WT from FFB-WT cells was observed in the presence of 5 or 10 μ M U18666A (Fig. 5B). Similarly, we observed a 2–3 log reduction in titers of VSV-EboGP from CFB-WT cells at 10 and 20 hrs post-infection in the presence of 5 or 10 μ M U18666A (Fig. 5D). In contrast, no significant reduction of titers of VSV-WT from CFB-WT cells was observed in the presence of 5 or 10 μ M U18666A (Fig. 5E). Reduced expression of VSV M was evident in both FFB-WT (Fig. 5C) and CFB-WT (Fig. 5F) cells infected with VSV-EboGP in the presence of U18666A (Figs. 5C and 5F, compare lane 1 with lanes 2 and 3), whereas levels of VSV M in FFB-WT or CFB-WT cells infected with VSV-WT in the presence of U18666A were comparable to those in VSV-WT infected cells in the absence of U18666A (Figs. 5C and 5F, compare lane 4 with lanes 5 and 6). Together, these findings suggest that the NPC1-dependent mechanism for efficient and

productive entry of EBOV appears to be conserved in primary feline and canine fibroblasts (Cote et al., 2011; Haines et al., 2012; Krishnan et al., 2012; Lee et al., 2013).

Transduction of canine and feline cells with EBOV GP lentiviral pseudotypes

In addition to using infectious VSV recombinants to investigate EBOV-mediated entry into canine and feline cells, we utilized lentiviral vectors encoding a GFP reporter and pseudotyped with either VSV-G or EboGP as a second confirmatory approach (Medina et al., 2003; Simmons et al., 2003a; Simmons et al., 2003b). Vero-E6, CFB, FFB-WT, and FFB-NPC1^{-/-} cells were spinoculated with the indicated lentiviral pseudotypes and analyzed for expression of GFP by flow cytometry after 72 hrs (Fig. 6). We observed no significant difference in the percentage of Vero-E6 cells expressing GFP in the populations transduced with the VSV-G versus EboGP pseudotyped lentiviruses ($p = 0.85$); however, CFB, FFB-WT, and FFB-NPC1^{-/-} cells all showed significant decreases in transduction by the EboGP lentivirus compared to that by the VSV-G lentivirus (Fig. 6). Indeed, CFB cells transduced with EboGP showed a 6.4-fold decrease in transduction relative to the VSV-G vector ($p = 0.0065$), and the FFB-WT cells showed a 4.1-fold decrease ($p = 9.6 \times 10^{-5}$) (Fig. 6). In correlation with results from the recombinant VSV experiments, transduction of the FFB-NPC1^{-/-} cells by the EboGP lentivirus was least efficient, while the FFB-NPC1^{-/-} cells remained susceptible to transduction by the VSV-G lentivirus (Fig. 6). Taken together, these findings correlate well with those obtained above using VSV recombinants.

Discussion

The recent emergence of EVD in individuals living with domestic pets highlighted unanswered questions and deficiencies in our understanding not only of the ability of domestic animals to be infected with EBOV, but also the potential role of domestic animals, particularly pets such as dogs and cats, in transmission and spread of EBOV to humans. Canines are known to be highly prevalent in and around areas of EVD outbreaks in Africa. Other than a single, unconfirmed serology study of dogs at the epicenter of a 2002 EVD outbreak in Gabon (Allela et al., 2005), little to no information is available on the ability of dogs or cats to be infected, or serve as vectors or reservoirs for ebolavirus transmission. Without a critical assessment of this potential, we cannot formulate appropriate measures to control transmission and spread of this global public health threat. Thus, a more comprehensive understanding of the susceptibility and risk of domestic animals becoming infected with EBOV, and their potential role in the dynamics of EBOV transmission via casual contact or contact/ingestion of infected meat, is warranted.

Although a complete and comprehensive understanding of the possible role of domestic animals in EBOV transmission using live virus and *in vivo* models is beyond the scope of this manuscript, we began to address this topic by first assessing the permissiveness of various canine and feline cells to EBOV GP-mediated infection. In addition, we took advantage of our unique NPC1^{-/-} feline cells to investigate, in part, the mechanism of EBOV-mediated entry into feline and canine cells using genetic and/or pharmacological approaches. Results from two independent yet complementary approaches (VSV recombinants and lentivirus pseudotypes) revealed that feline fibroblasts were more

susceptible to EBOV GP-mediated entry than both primary and established canine cells; however, both canine and feline cells were generally refractive to EBOV GP-mediated entry compared to control primate and human cells.

Using primary feline fibroblasts expressing or lacking NPC1 as well as pharmacological approaches, we found that the dependence on host NPC1 expression for optimal EBOV-mediated entry and infection appears to be conserved in both feline and canine fibroblasts. Indeed, results from indirect immunofluorescence studies revealed an altered punctate pattern of M protein localization during early entry stages of VSV-EboGP infection of FFB-NPC1^{-/-} cells, compared to VSV-EboGP infection of FFB-WT cells. This altered pattern of M protein localization was not observed in either FFB-WT or FFB-NPC1^{-/-} cells infected with VSV-WT. The observed punctate staining pattern of M protein in VSV-EboGP infected FFB-NPC1^{-/-} cells suggests that the virus is unable to escape the endosome efficiently due to the lack of the NPC1 receptor, and thus aggregates in the endosome. In contrast, VSV-EboGP did efficiently escape the endosome and enter/replicate in the cytoplasm of FFB-WT cells expressing NPC1. This mechanistic finding is important as it both confirms the conservation of this entry mechanism in domestic animal species, and implies that future antiviral therapeutics targeting NPC1 function may be effective in preventing EBOV entry and infection in multiple species.

Our findings provide new insights into both the permissiveness of cells from domestic pets to be infected with EBOV, and the conservation of the NPC1-dependent mechanism of entry in these cells that is utilized by EBOV. These results suggest that cells of canine and feline origin are less susceptible to EBOV-mediated infection than cells of primate and human origin; however, the mechanistic basis of this restriction remains to be determined. Based on our findings, we can only speculate that domestic pets may not serve as major reservoirs or vehicles for casual transmission of EBOV to other animals or humans. If dogs and cats can indeed be infected with EBOV at low levels, then one cannot rule out the possibility of humans or animals becoming infected with EBOV following ingestion of contaminated meat from these animals. However, additional experiments will be required to prove conclusively whether domestic pets can be productively infected with EBOV and play a role in the dynamics of EBOV transmission.

Acknowledgements

We thank D. Lyles for kindly providing VSV M monoclonal antibody 23H12. We also thank D. Argento for assistance in preparing the figures, L. King for critical reading of the manuscript, C. Berry for statistical analysis, and members of the Harty, Freedman, and Bates labs for helpful discussions. This work was supported in part by NIH grant R33 AI102104 and a University Research Foundation (URF) grant to R.N.H and NIH grant R01 AI081913 to P.B. Imaging experiments were performed in the PennVet Imaging Core Facility on instrumentation supported by NIH S10RR027128, the School of Veterinary Medicine, the University of Pennsylvania, and the Commonwealth of Pennsylvania.

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Highlights

- Cells of canine and feline origin are susceptible to Ebola GP-mediated entry.
- However, Ebola GP-mediated infectivity of canine and feline cells is restricted.
- The NPC1-mediated mechanism of Ebola entry is conserved in canine and feline cells.

Fig. 1. Schematic diagram of the genome of VSV-WT compared to that of VSV-EboGP recombinant

VSV-EboGP expresses mCherry from a separate ORF. The VSV G protein on the surface of VSV-WT virions is replaced by EBOV GP (Zaire) on the surface of VSV-EboGP virions.

Fig. 2. Infection of primary canine and feline fibroblasts with VSV-EboGP

The indicated cells were infected with VSV-EboGP at an MOI=1.0, and virions from supernatant samples harvested at 10 (black bar) and 20 (gray bar) hours post-infection were quantified by plaque assay. Results are from three or more independent experiments, and error bars represent SD. Welch's t-test was used to compare mean titer measurements of all cell types with Vero-E6 cells. * = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.0001$.

Fig. 3. Confocal microscopy to assess infectivity of canine and feline cells to VSV-EboGP Infection

Representative confocal images of Vero-E6, CFB, FFB-WT, and FFB-NPC1^{-/-} cells mock-infected, or infected for 18 hrs with VSV-EboGP at an MOI=1.0. Cell nuclei were stained with Nucblue, and VSV-EboGP infected cells express mCherry (red). Scale bar = 200μm in both the lower (10×) and higher (40×) magnifications. Percent infection is shown for VSV-EboGP infected cells, as determined by high content analysis. Quantitation of all mock-infected cells yielded percentages <0.05%.

Fig. 4. Growth Kinetics of VSV-EboGP Infection in Feline Cells

Vero-E6, FFB-WT, and FFB-NPC1^{-/-} cells were infected with VSV-EboGP (**A**) or VSV-WT (**B**) at an MOI of 1.0, and cells and supernatants were harvested at 10 and 20 hrs post-infection. Growth curves for VSV-EboGP (**A**) and VSV-WT (**B**) from the indicated cells at 10 and 20 hrs post-infection are shown. Data are from three independent experiments. **C**) VSV M protein was detected in the indicated infected cell extracts at both 10 and 20 hrs post-infection using anti-VSV M mAb. **D**) The intracellular localization of VSV M (green) in FFB-WT and FFB-NPC1^{-/-} cells infected for 5 hrs with either VSV-WT or VSV-EboGP was determined by indirect immunofluorescence using anti-VSV M mAb. Cell nuclei were stained with Nucblue.

Fig. 5. Compound U18666A inhibits VSV-EboGP infection in canine and feline cells
 FFB-WT or CFB-WT cells were infected with VSV-EboGP (**A and D**) or VSV-WT (**B and E**) at an MOI of 1.0 in the absence (0 μ M) or presence of 5.0 or 10.0 μ M U18666A, and cells and supernatants were harvested at 10 and 20 hrs post-infection. Growth curves for VSV-EboGP (**A and D**) and VSV-WT (**B and E**) in the absence or presence of U18666A at 10 and 20 hrs post-infection are shown. Data are from two independent experiments. VSV M protein was detected in the indicated infected cell extracts at both 10 and 20 hrs post-infection from FFB-WT (**C**) and CFB-WT (**F**) cells using anti-VSV M mAb. Cellular actin was detected as a loading control.

Fig. 6. Transduction of Canine and Feline Cells by EboGP Pseudotype

The indicated cell types (x-axis) were transduced with lentivirus-GFP pseudotypes expressing either VSV G (black bars) or Ebola GP (gray bars), and the percentage of cells expressing GFP after 72 hrs was determined by flow cytometry (y-axis). Data are from three independent experiments. *** = p value <0.01 (unpaired t test).