Published in final edited form as:

J Virol Methods. 2017 September ; 247: 28–31. doi:10.1016/j.jviromet.2017.05.005.

Susceptibility of neuroblastoma cells to rabies virus may be affected by passage number

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Abstract

Maintaining a healthy, continuous immortalized cell line is essential for rabies laboratories that perform virus isolation assays and test for the presence of viral neutralizing antibodies. Individuals who routinely work with rabies virus, such as rabies laboratory employees, or those who may have a high potential for exposure to rabies virus, including veterinarians, should be tested for the presence of anti-rabies viral neutralizing antibodies (VNA) every 6 to 24 months, depending on potential exposure level. The gold standard for serum neutralization assays require the use of live rabies virus and cells that are sensitive to rabies virus infection. Additionally, virus isolation assays are routinely performed in rabies laboratories as a back-up for the direct fluorescent antibody test (dFAT). Currently there are no guidelines or publications recommending the use of low, intermediate, or high passage cell lines in rabies assays. In this study, we compared the sensitivity of intermediate, high, and extremely high passaged neuroblastomas to rabies virus using virus isolation, serum neutralization, and real time RT-PCR techniques. Additionally, cells were examined microscopically to determine changes in morphology and dissemination of rabies virus antigen between intermediate, high, and extremely high passage cells. No significant difference was found between cell passage numbers and viral susceptibility between intermediate and high passaged cells. However, extremely high passaged cells (1200 passages) were less susceptible to viral infection and/or produced less virus following inoculation. As a result, rabies laboratories that use viral isolation and serum neutralization assays should regularly assess cell susceptibility to ensure the integrity and repeatability of the test.

Introduction

Rabies virus (RV) is endemic throughout most areas of the world. With a fatality rate of nearly 100%, rabies laboratories have a responsibility to accurately and promptly diagnose rabies submissions. Although the gold standard technique for rabies diagnosis is the direct fluorescent antibody test (DFA), inclusion of a backup test for any submission that includes a history of a bite is highly recommended (World Organization for Animal Health, 2011).

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Several rabies laboratories in the United States and Canada use the Tissue Culture Infection Test (TCIT), a virus isolation technique, to confirm the absence of rabies antigen in diagnostic samples from bite cases that are negative for rabies antigen on the DFA.

Additional techniques, such as serology and virus isolation, are common methods employed in many rabies laboratories. The New York State Department of Health (NYSDOH) Rabies Laboratory receives approximately 1000 human and between 3,000 and 7,000 animal serum samples annually to assess the presence of anti-rabies viral neutralizing antibodies (VNA). The NYSDOH also receives serum and cerebral Spinal Fluid (CSF) samples for ante-mortem human rabies testing.

Because of their susceptibility to RV, murine neuroblastomas C-1300 (NA) are the most common continuous cell line used for virus isolation and serum neutralization assays (Rudd, 1987). Some laboratories prefer not to maintain a continuous culture of cells and contract with in-house tissue culture facilities or private laboratories to supply fresh cells on a daily or biweekly basis. Due to the Clinical Laboratory Evaluation Program (CLEP) certification, the NYSDOH Rabies Laboratory is required to maintain records of media, cell passage technique, passage number, and instrument certification. Additionally, because the submission of ante-mortem human cases requires emergency testing, the NYSDOH rabies laboratory maintains a continuous culture of NA cells.

Currently, there is no standard recommendation for the passage age of cells used to perform RV assays in laboratories. The ability of continuous cell lines to exist almost indefinitely has presented numerous concerns of subculturing practices that lead to cellular cross-contamination when multiple cell lines are employed in individual laboratories (American Type Culture Collection, ATCC). Long-term subculturing places selective pressure on cell line traits leading to, for example, faster growing cells that eventually overrun slower proliferators in the population. In addition, cell lines maintained in culture over a long period of time may experience mutations that alter the original functional characteristics of the cell lines identified at earlier passage levels (Hughes et al., 2007).

To determine if passage number effects the outcome of rabies serology and TCIT assays, we compared the viral load of NA cells over 43 passages, NA cells over 800 passages, and NA cells over 1200 passages using the TCIT, serum neutralization (SN), quantitative real time RT-PCR, and fluorescent microscopy. Our results demonstrate that cultured cells used in RV assays should be evaluated for specificity and sensitivity at various passage levels.

Methods and Materials

Cells passaged 43 to 106 times were considered intermediate passage (IP) cells, high passage (HP) cells from 851 to 925, and extremely high passaged (EHP) cells from 1200 to 1273. The TCIT was performed as described previously (Rudd et al., 1987), CVS-11 RV and NA cells. The titer of CVS-11 was 10^5 TCID₅₀/ml with a Ct value of 30. Comparisons between cell passage numbers occurred simultaneously. To perform the TCIT, 90 μ l of Eagles Growth Media was added to the wells of a 96 well tissue culture plate for virus dilution. Next, 10 μ l of CVS was added to the first well for a 1:10 dilution. Ten microliters

was removed from the first well and added to the second well for a 1:100 dilution. This was repeated through the 10^{-4} dilution. Using a cell concentration of 6×10^5 per ml, 50 microliters of the cell suspension was added to the wells and allowed to grow for approximately 3 days. Following this incubation, the plates were removed from the incubator and the supernatant was removed from each well retained for real time RT-PCR. Supernatant recovered from cells was processed for real-time RT-PCR as described elsewhere (Dupuis et al., 2015).

Serum neutralization test was performed as described previously in Trimarchi et al., 1996. Fifty microliters of growth media was added to test wells of a 96 well plate. Next, 50 ul of Rabies immune globulin (RIG, Federal Drug Administration) was added to the first well and eight -2 fold dilutions were done out to the dilution of 1:512. Fifty microliters of 10^5 TCID₅₀/ml CVS-11 was added to all wells containing RIG. Cells were allowed to grow for approximately three days and the plates were processed as described above. Real time RT PCR was not performed on wells containing RIG.

Once the supernatant was removed for molecular analysis, the cells were fixed overnight with 1:1 methanol/ 10% formalin. Following fixation, the plates were washed twice for 30 minutes with 0.01 M phosphate buffered saline (PBS) (pH 7.6) to remove fixative. The plates were stained with a rabies conjugate (DFA 3, Millipore, USA) for 30 minutes. Following the staining procedure, the plates were washed with PBS twice for 5 minutes. Each well was viewed under a florescent microscope to evaluate infectivity, antigen dissemination, and antigen morphology.

Results

Using the student's T test, the average Ct values from IP, HP, and EHP rabies infected NA cells were not significantly different ($p>0.05$). The difference in Ct values are compared graphically in Figure 1. Despite the higher Ct values at passage 91 and 95 in figure 1, there was no visible difference in the TCIT assay. These results did not repeat on the subsequent tests, passages 92 and 96 respectively, and may be the result of a technical error on the part of the individual performing the collection or test, the robot performing the RNA extraction and real-time PCR assay. The Ct value of the EHP cells was approximately a log greater but the difference was not statistically significant. Microscopic examination comparing the percent of infected cells per well revealed no obvious difference in rabies virus antigen morphology or percent of cells infected between IP and HP cells. Figure 2. Although, EHP cells were less susceptible to rabies virus infection, antigen morphology remained similar to IP and HP cells. Figure 2 A-D. Using the TCIT test, CVS antigen was present through the 10^{-5} dilution in all cell passage types. However, the number of infected cells was greater in the IP and HP cells as opposed to the EHP cells.

When RIG was included, rabies virus was neutralized until RIG was diluted 10^{-3} in IP and HP cells and at a 10^{-5} dilution in old cells. Figure 2 E-F. The amount of virus produced in IP and HP cells overwhelmed RIG at a lower dilution thereby preventing neutralization at a lower dilution as compared to EHP cells. The difference in viral neutralization demonstrates

the susceptibility and/or viral production in IP and HP cells is increased when compared to EHP cells.

Discussion

Alterations in cell susceptibility to infectious agents based on passage number has not been well studied. Searching scientific blogs on the internet, one can find anecdotal reports of decreased susceptibility to viruses in high passaged cells. However, peer reviewed publications investigating differences between low and high passage cells and viral susceptibility are limited. McClurkin et al., 1974, evaluated a continuous line of low passage (8-19) and intermediate (83-89) passaged bovine turbinate cells for susceptibility to multiple bovine viral diarrheal (BVD) isolates. Despite a significant increase in the number of chromosomes in the intermediate passaged cells, viral susceptibility was not altered. Additionally, morphological and growth characteristics remained the same between low and intermediate passaged cells. Conversely, Jensen and Norrild, 2000, reported decreased susceptibility to herpes simplex virus I (HSV-1) infection in intermediate passaged human embryonic lung fibroblasts. They compared cells following 27, 32, 40, and 48 passages and found significantly fewer infectious virus particles in cells following 48 passages. The plaque size decreased as the passage number increased, albeit the difference was not significant. The authors hypothesized the smaller plaque size was the result of decreased virulence. In addition to the decrease in susceptibility, the authors noted alterations in cell structure increased in intermediate passage number cells.

Additional publications have reported changes in cellular response to various external stimuli such as toxins and androgens. Esquenet et al., 1997, reported significant differences in cellular response between low and high passaged cells when exposed to androgens and retinoic acid. Chin-Mei et al., 1997, described decreased doubling time in high passage cells yet no change in sensitivity to ionizing radiation. Boukamp et al., 1997 also reported decreased population doubling time and significantly increased cloning efficiency in HaCaT cells. Maurer et al., 2016, reported that high passage cells may be at a disadvantage when exposed to toxic agents. According to their study, high passage cells were less likely to recover from exposure to a mitochondrial neurotoxicant as compared to low passage cells. Low passage cells were capable of buffering K⁺ induced depolarization where as high passaged cells were not.

There is no established definition of low, intermediate, or high passage cells. The cells we defined as intermediate cells (passage 43-106) could be considered by some publications to be high passage cells, others as intermediate passage cells. Some publications report genetic changes can occur in continuous cells lines prior to passage 43, therefore it is possible that changes affecting susceptibility in our cell lines may had occurred prior to the first passage we tested (Maurer et al., 2016, Kato et al., 2008, Jensen and Norrild, 2000, Boukamp et al., 1997).

The goal of this study was to determine what, if any, impact the cell passage number had on susceptibility to rabies virus. Cellular changes that alter cell susceptibility to rabies could have a profound outcome on viral assays used in rabies laboratories. Despite the lack of

statistical significance, the susceptibility to rabies virus infection between IP/HP and EHP cells was sufficiently different to affect the outcome of our neutralization and real-time RT-PCR assays. This study demonstrates the assay variation that can occur when extremely high passaged cells are used. If the use of HP or EHP is necessary due to limited cell quantity, type of cell, and so forth, developing an SOP to regularly assess cell susceptibility is necessary to ensure the integrity of the test.

Acknowledgments

We thank the New York State Department of Health, Wadsworth Center Tissue Culture and Media core for their media services. We are grateful to the Wadsworth Center Illustration Department for their assistance with Figure 2. This work was supported by NIH grant 5K08AI085031 to April D. Davis.

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Highlights

- Cell susceptibility to rabies virus remained consistent among intermediate and high passage neuroblastoma cells but extremely high passaged cells were less susceptible to rabies virus.
- Rabies virus antigen load, morphology, and dissemination were consistent between intermediate and high passage neuroblastoma cells. Antigen morphology in EHP was similar to IP and HP cells
- Viral growth between intermediate and high passaged cells was similar but there was a notable difference when using extremely high passaged cells.

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Figure 2.

Micrographs illustrating rabies virus antigen in IP and EHP cells. A. IP and B. EHP cells inoculated with 1×10^5 TCID₅₀ CVS at a magnification of 100 $\times$. C. Increased magnification (200 $\times$) of same cells as in micrographs A and B. E. IP and F. EHP cells inoculated with 1×10^5 TCID₅₀ CVS and challenged with a constant dose of Rabies Immunoglobulin (RIG) at a magnification of 100 $\times$. In both E and F, RIG is at a 10^{-5} dilution.