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Rabies lyssavirus Isolates from Brazilian Different Reservoirs Species Present Distinct Pattern of Propagation in N2a Cell

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Abstract

Background: Rabies cell culture infection test was developed for the isolation of *Rabies lyssavirus* and as an alternative for the mouse inoculation test. However, tissue culture for street rabies strains produces low viral titer. Here, we assessed the quantity of brain tissue for successful viral isolation toward increased virus titer in effective way.

Methods: Brain tissue isolates from different reservoirs species of Brazil were harvested in different concentration and inoculated in mouse neuroblastoma cells (N2a). These isolates were measured infectious viral titer and cell viability followed by consecutive passages in N2a cells.

Results: Inoculum containing were prominent *Rabies lyssavirus* due to higher viral titer and not significantly dead cell. After consecutive passages in N2a cells *Rabies lyssavirus* variant maintained by vampire bat had remarkable adaptation to the culture system, while isolates from marmoset presents distinct pattern of propagation in N2a cell when compared with other groups.

Conclusion: Based on these results, the isolation followed by viral replication assay may be used in isolates from different reservoirs which enable an effective amplification of the wild type virus strains.

Keywords: Wild type *Rabies lyssavirus*; Viral replication; Dog virus; Bat virus; Marmoset virus

Introduction

Rabies is one of the most important zoonosis that affects the central nervous system of mammals, including the orders Carnivora, Chiroptera and Primate. Due to total number of canine rabies cases has decreased in both North and South America is extremely important over the years to study rabies in wild species, especially bats, since these wild species are nowadays the main perpetuators of rabies [1,2]. A panel of eight monoclonal antibodies (MAbs) against *Rabies lyssavirus* nucleoprotein was developed by the Centro Panamericano de Zoonosis (CEPANZO) and the Organización Panamericana de la Salud (OPS) for antigenic variants (V) identification in both North and South America [3]. This panel established 11 V found in Latin America isolates, including two major reservoir hosts - the dog (V-2) and the vampire bat (V-3), but has not identified the marmoset antigenic profile [2,4,5]. Antigenic variant of vampire bat was also identified in others hosts as cattle and horses, and V of marmoset (V-M) in man [2,4]. Until recently, our knowledge of *Rabies lyssavirus* pathogenesis was limited and largely based on experimental infection with laboratory-adapted strains [6-8]. Moreover, street *Rabies lyssavirus* have distinct biological characteristics (e.g. virus ability to spread from cell to cell, virus internalization and virus replication in a tissue culture system) when compared with tissue culture-adapted strains [6,9,10]. Biochemical and biology investigation frequently require a large quantity of virus, such as viral replication, viral entry and viral spread techniques into host cells. For virus stocks preparation, virus cell isolation is the first procedure, following virus replication in consecutive passages in cells for the preparation of large amount of virus. Unlike street virus, adapted virus in cell culture is easier to obtain a large amount of virus lead to *in vivo* experiments [11-16]. Obstacles such slow development of virus and low efficiency of cell infection have been reported with street virus isolation. In this way, studies have shown that N2a cells are more sensitive for the isolation of street rabies virus than are BHK-21 cells [12,15]. Additionally, techniques for the isolation of street *Rabies lyssavirus* from suspect material in cell cultures have been developed using a 10^{-2} dilution of the original brain tissue [11,12]. As reported previously a 10^{-1} dilution decreased numbers of infected cells when compared with a 10^{-2} dilution due to a possible problem associated with bacterial and/ or toxic contaminants [11,12]. However, another study has shown that 10-fold dilution was necessary to remove or suitably reduce the problem [12,15,17]. Given the fact that virus isolation in culture system is required to produce large quantities of viable infectious virus. In this study we describe the optimal conditions for the isolation of street *Rabies lyssavirus* isolates from different reservoir species by using a rabies tissue culture infection test (RTCT) and replication *in vitro* system. We assess that V-3 group had a higher viral titer when compared with other groups and *Rabies lyssavirus* isolates from different reservoir of Brazil showing a distinct pattern of propagation in N2a cell.

Sample	Year	Host	Antigenic Variant*
IP 2476	2014	*Canis familiaris*	V-2
IP 2478	2014	*Canis familiaris*	V-2
IP 1729	2014	*Canis familiaris*	V-2
IP 2484	2014	*Canis familiaris*	V-2
IP 2480	2014	*Canis familiaris*	V-2
IP 2477	2014	*Canis familiaris*	V-2
IP 2489	2014	*Canis familiaris*	V-2
IP 2479	2014	*Canis familiaris*	V-2
IP 1410	2014	*Desmodus rotundus*	V-3
IP 3444	2014	*Artibeus lituratus*	V-3
IP 4292	2013	*Desmodus rotundus*	V-3
IP 4660	2013	*Diphylla ecaudata*	V-3
IP 2303	2014	*Artibeus lituratus*	V-3
IP 2258	2014	*Artibeus lituratus*	V-3
IP 3664	2015	Bat host species undetermined	V-3
IP 3667	2015	Bat host species undetermined	V-3
IP 1304	2014	*Callithrix jacchus*	V-M (Marmoset**)
IP 1301	2014	*Callithrix jacchus*	V-M (Marmoset**)
IP 3109	2011	*Callithrix jacchus*	V-M (Marmoset**)
IP 2884	2014	*Callithrix jacchus*	V-M (Marmoset**)
IP 6294	2014	*Callithrix jacchus*	V-M (Marmoset**)
IP 1770	2012	*Callithrix jacchus*	V-M (Marmoset**)
IP 3113	2011	*Callithrix jacchus*	V-M (Marmoset**)

IP: Samples registered in the Pasteur Institute, São Paulo, Brazil. V-2: Antigenic variant maintained in domestic dog. V-3: Antigenic variant maintained in vampire dog. V-M: Antigenic variant maintained in marmoset.* antigenic variants were found, as defined in the monoclonal antibody panel produced by the Centers for Disease Control and Prevention (CDC) Atlanta, USA. ** antigenic profile previously reported in isolates from marmoset [4].

Table 1: RABV isolates from different reservoirs, year in which the samples were collected, species from which the samples were collected and antigenic characterization of rabies virus isolates.

Material and Methods

Cells

Neuroblastoma cell line (N2a) (N2a, ATCC, CCL-131) grew in Eagle's minimum essential medium, supplemented with 10% fetal bovine serum, 3% of gentamicin and 3% nonessential amino acids (complete medium) at 37°C and 5% CO_2 atmosphere.

Virus strains

Twenty-three *Rabies lyssavirus* isolates from different reservoirs were obtained from first passage isolates in Swiss-Webster mice inoculated with 20% suspensions of central nervous systems (CNS) (Table 1) and propagated seventh-passage in N2a cells.

All *Rabies lyssavirus* isolates were characterized antigenically using eight monoclonal antibody panel from the Centers for Disease Control and Prevention (CDC) in Instituto Pasteur from São Paulo. The samples isolated from domestic dogs, bats and marmoset were characterized as variant 2 (V-2), variant 3 (V-3) and marmoset variant (V-M), respectively [3,4]. Challenge Virus Standard (CVS-31), brain tissue uninfected and complete medium were used as controls [18].

Inoculum preparation

Brain tissue of different street *Rabies lyssavirus* strain (Table 1) were macerated, homogenized manually on ice and diluted with complete medium at 20%, 10% and 5% (w/v) concentrations and then centrifuged at 3,000 xg for 30 minutes.

Virus titration

For virus titration by direct fluorescent antibody assay, N2a cells in 96-well plates (Corning/Costar) were infected using different dilutions of a brain homogenate (20%, 10% and 5%) with serial 10-fold virus dilutions in minimum essential medium (MEM) and incubated at 37°C for 3 days [19]. Foci were counted using a fluorescence microscope, and virus titers calculated in focus-forming units (FFU) per milliliter. The expression of the virus titer was presented on a logarithmic scale. All titrations were determined in quadruplicate.

Cell viability

For each well, 0.1 mL of N2a cell (5×10^5 cells/mL) suspension, 0.04 mL of the brain suspension (20%, 10% and 5%) and 0.1 mL of medium complete were added in a 96-well plate (Corning/Costar). After 72 hours, trypsinized cells were suspended and viable cells were identified and enumerated in a hemocytometer by 0.2% trypan blue exclusion.

Virus replication in consecutive passages

Nine *Rabies lyssavirus* strains were obtained from first passage isolates in mice inoculated with 20% suspensions of CNS, and propagated seventh-passage in N2a cells in the presence of complete medium. For each well, 1 mL of N2a cell (1×10^6 cells/mL) suspension, 0.5 mL of the tissue culture suspension and 2.5 mL of medium complete were added in a 6-well plate (Corning/Costar). After 72 hours, each passage was titrated as described above.

Statistical analyses

Isolates of each variant were evaluated by statistical analysis. Viral titer and cell viability data were calculated and checked for normality. Each group were compared statistically by using a two-way ANOVA with Tukey's multiple comparisons test for multiple groups. Student's t test was used to compare two groups. A P-value of less than 0.05 was considered statistically significant. All analyses were conducted by using Prism software (GraphPad Software®, San Diego, CA, USA).

Results

The amount of the brain tissue may be a determining factor for *Rabies lyssavirus* isolation due to quality and quantity of sample. To establish more stable condition for *Rabies lyssavirus* isolation, firstly, we assessed the viral titer and the death cell with virus inoculum containing different amounts of brain tissue (20%, 10% and 5%).

We evaluated samples from different Brazilian reservoirs species, of whom 8 were V-2, including viruses maintained in domestic dog, 8 were V-3, including viruses maintained in hematophagous bat and 7 were V-M including viruses maintained in marmoset (Table 1).

The mean titration results obtained for each brain tissue concentration of isolates of each variant are shown in Figure 1. No significant differences between samples sharing the same antigenic profile each group were observed. We verified that virus isolation in N2a cell culture occurred in all samples and dilutions of brain tissue, however using inoculum containing 20% of brain's tissue the virus titer was significantly higher in V-3 and V-2 groups ($p<0.001$) compared with other brain tissue concentration (Figure 1a). No significant differences of virus titer were identified in V-M group between inoculum containing brain tissue diluted in different concentration (Figure 1a).

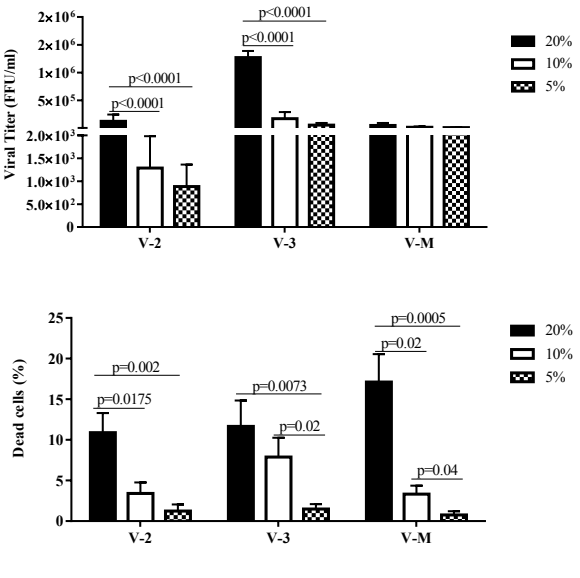

Figure 1: Viral titer (a) and percentage of dead cells (b) in different RABV variant. V-2: Antigenic variant maintained in domestic dog. V-3: Antigenic variant maintained in vampire dog. V-M: Antigenic variant maintained in marmoset. 20%: inoculum containing 20% of brain's tissue. 10%: inoculum containing 10% of brain's tissue. 5%: inoculum containing 5% of brain's tissue. Data are mean ± standard deviation. P value: calculated between inoculum containing brain tissue diluted in different concentration. Statistical analyses were conducted using the two-way ANOVA with Tukey's multiple comparisons test.

When examining relationship between brain inoculum and viral titers we observed that virus titer is greater in cell culture with larger amount of tissue, V-M group's exception (Figure 1a).

Poor-quality sample could interfere with viral isolation as a result of the increased number of dead cells. This study observed that the inoculum containing 20% of CNS had a higher percentage of dead cells when compared with the other dilutions (Figure 1b).

We also investigated the relationship between virus titer and dead cell percentage in each group. Virus titer and death cell in culture system was higher when a larger amount of brain tissue were inoculated, showing a positive relation among all concerned parameters (Figure 2). Interestingly, V-3 group had a higher viral titer when compared with other groups independent of tissue concentration used (Figures 1 and 2).

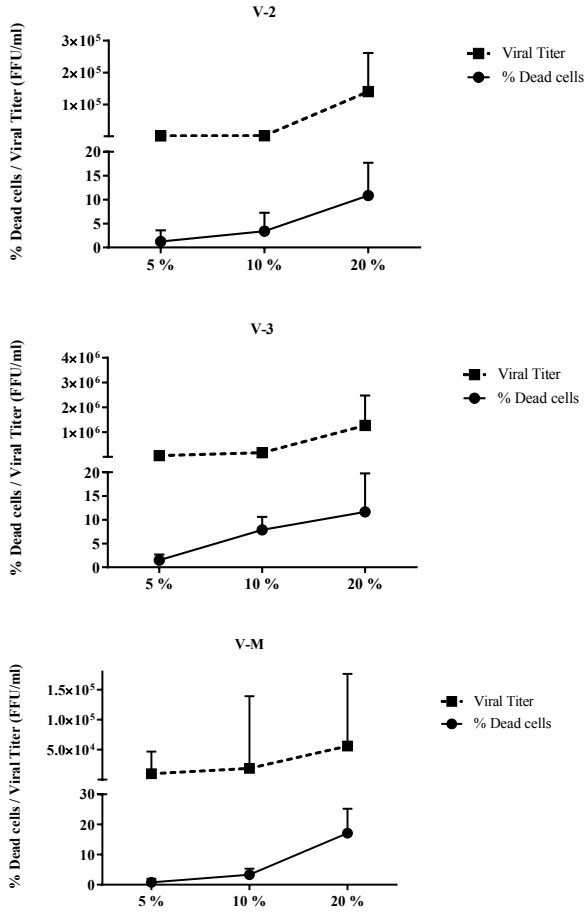

Figure 2: Relationship between viral titer and percentage of dead cells in different RABV variants. V-2: Antigenic variant maintained in domestic dog. V-3: Antigenic variant maintained in vampire dog. V-M: Antigenic variant maintained in marmoset. 20%: inoculum containing 20% of brain's tissue. 10%: inoculum containing 10% of brain's tissue. 5%: inoculum containing 5% of brain's tissue. Data are mean ± standard deviation. P value: calculated between inoculum containing brain tissue diluted in different concentration. Statistical analyses were conducted using the two-way ANOVA with Tukey's multiple comparisons test.

For *Rabies lyssavirus* propagation, its isolation in cell culture system is a basic prerequisite for continuous cultivations and enables an easy

transfer of supernatant in serial passage. Thus, supernatant containing viral particle were inoculated at 7 serial passages in N2a cells to propagate the viruses. Here, we used the viral inoculum containing 20% of brain tissue due to the higher titer obtained in isolation cell culture rabies and reasonable cell death. Virus propagation in 6-well microplate showed more efficient than in 96-well microplate, reached a minimum titer of 6×10^4 TCID 50/mL in a single passage (Figure 3). As shown in Figure 3, infective virus titers of samples increased exponentially during supernatant passages in N2a cells. Extracellular virus reached the maximum titer (p=0.026) at seventh passages in V3 group (Figure 3). However, in V-2 and V-M group virus reached the maximum titer at 5th passage (p=0.002) and 3th (p=0.005) passage, respectively (Figure 3).

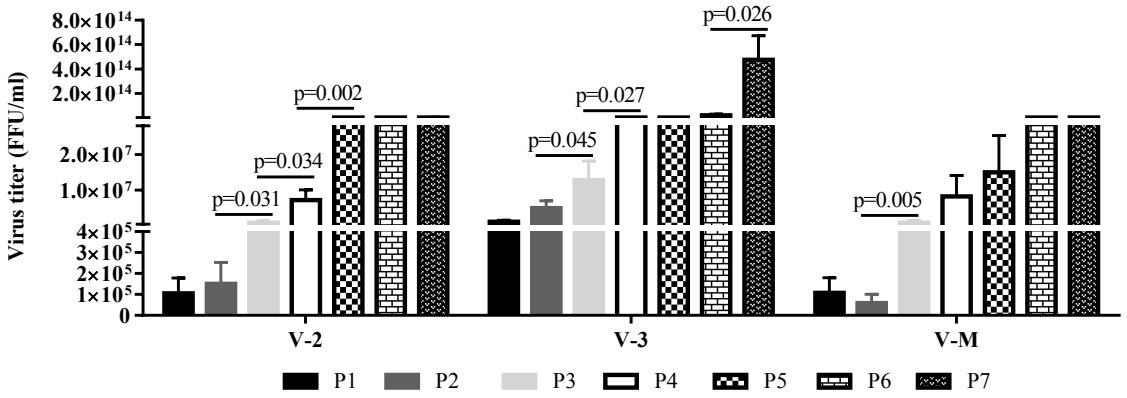

Figure 3: Virus replication in seven consecutive passages in N2a cells. V-2: Antigenic variant maintained in domestic dog. V-3: Antigenic variant maintained in vampire dog. V-M: Antigenic variant maintained in marmoset. 20%: inoculum containing 20% of brain's tissue. 10%: inoculum containing 10% of brain's tissue. 5%: inoculum containing 5% of brain's tissue. P: N2a-cell-passaged street rabies virus. Data are mean ± standard deviation. P value: calculated between inoculum containing brain tissue diluted in different concentration. Statistical analyses were conducted using the two-way ANOVA with Tukey's multiple comparisons test.

Discussion

Isolation of street *Rabies lyssavirus* in tissue cultures have been used to virus isolation [12-20]. It is previously shown that 10% brain suspension is optimum for isolation of *Rabies lyssavirus* in N2a cells [12,15,17]. Another study shows that 20% of the original brain present same results [16]. However, some difficulties such as low efficiency of cell infection and slow replication of street rabies viruses is observed. Here, we showed that *Rabies lyssavirus* isolates from different hosts could have different titer in the virus isolation and virus propagation in N2a cells dependent on brain suspension concentration used.

As shown in Figure 1, viral titer in V-M group was equal independent of brain tissue concentration used. However, in V-2 and V-3 group showed higher viral titer in tissue culture when used inoculum containing 20% of brain tissue (Figure 1). Unlike the other groups, no evidence of a significantly increased was observed in viral titer of V-M group until third passage in N2a cells (Figure 3). We also assessed that regardless amount of brain tissue used in the viral isolation, the V-3 group had a higher viral titer compared with other groups, showing remarkable adaptation of RABV strain from vampire bat to the culture system (Figures 1-3). Taken together, our results provide evidence that isolated from different reservoirs are differently adapted and replicated in N2a cells.

These results support the hypothesis that street viruses may use different cell surface molecules [21]. Accordingly, *Rabies lyssavirus* strains originating from bats and dogs could exhibit differences in glycoproteins, thus using different receptors to infect cells [6,10,21,22]. Moreover, other non-structural proteins, such as P protein interacts with the dynein light chain LC8, which it could involve in regulatory role in viral polymerase activity [23].

It is also known that the spillover of a RABV strain from one species to another may be benefited by virus subpopulations that have a selective advantage in a new host. In this way, it is possible that that isolated from bat will be best adapted to overgrow in N2a cells due the most promiscuous of bat RABV strain [24].

Importantly, the finding that *Rabies lyssavirus* isolated from marmoset seems not propagate efficiently in N2a cells. Our results show the inadequate interaction between *Rabies lyssavirus* of V-M group with N2a cells, suggesting the possibility of a better adaptation of *Rabies lyssavirus* of V-M group in other cell types.

Serial passage of street *Rabies lyssavirus* demonstrated low efficiency in N2a cell infection in 96-well plates (data not shown). Nevertheless, street rabies viruses seeded in 6-well plates showed high virus propagation in N2a cells (Figure 3) as shown in material and methods. Thus, high cell concentration and low viral load could be more effective for street virus replication.

Previous studies have shown that decomposed sample could cause problems associated with dead cell due to bacterial and/ or toxic contaminants [24-26]. The trypan blue method is a very common assay for assessing cytotoxicity in laboratory research, where live cells with intact cell membranes are not colored [27-29]. We observed positive relationship among amount of brain tissue, viral titer and number of dead cells (Figures 1 and 2). Nevertheless, our data

indicated that about 10 percent of dead cell observed in 20% brain tissue's inoculum did not affect viral isolation (Figure 1b).

Together, these data indicate that inoculum containing 20% brain tissue concentration is optimal for isolation and replication of the *Rabies lyssavirus* in N2a cells due to higher viral titer, discreet cytotoxicity, and consequently effective for acquiring of a high viral titer after consecutive passage in N2a cells. However, inoculum containing 5% of brain's tissue would be better for laboratory diagnosis by reason of using a smaller brain tissue's amount and detecting rabies virus.

In conclusion, our data show that this method provides a good option for virus isolation and replication will help in the producing enough virus material that could be used to characterize street stains *in vitro* and *in vivo*. In addition, replication system could be a good alternative in samples with low virus titer or in decomposed tissue situations.

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