https://doi.org/10.4322/rbcv.2023.0015

Standardization and performance evaluation of a virus isolation technique using murine neuroblastoma cell line (N2A) for the rabies virus (RABV) surveillance in Pará State, Brazil*

Padronização e avaliação de desempenho da técnica de Isolamento do vírus da raiva (VRAB) em células de neuroblastoma murino (N2A) no Pará, Brasil

Francisco Amilton dos Santos Paiva,** Alexandre do Rosário Casseb,*** Eliana Vieira Pinto da Silva,** Luanda Bispo Santos do Nascimento Maués,**** Luciana Taborda Corrêae, Thito Yan Bezerra da Paz,** Taciana Fernandes Souza Barbosa Coelho**

Abstract

Rabies is a zoonotic disease caused by the rabies virus (RABV) from the *Lyssavirus rabies* species. In humans, around 60,000 deaths are registered annually in the world due to the disease, while accurate estimation of rabies incidence among other animals, mainly in wildlife, is unfeasible. In recent decades, considerable improvements in diagnostic techniques for RABV were achieved as new methods were developed and standardized. The Brazilian Ministry of Health (MH) recommends the Direct Fluorescent Antibody test (DFA), Viral Isolation in Mice (VIM) and Viral Isolation in Cell Culture (VICC) techniques as diagnostic routine tests. However, even following animal welfare guidelines and rational use of animals, the substitution of VIM by alternative methods, as VICC, has been encouraged. Here, we evaluate the performance of VICC in our laboratory routine, which revealed to be a highly sensitive and specific technique for detecting RABV enabling its implementation as an alternative method to VIM, according to requirements of the MH. Besides the benefits involving the absence of animal use, a shorter laboratory execution time for diagnosis was required, which is a very important factor for the success of the control measures promoted by surveillance agencies. The Evandro Chagas Institute is the Macroregional Reference for diagnosing rabies in the North of Brazil, and the first laboratory in the region to perform the implementation the VICC technique, as described in the present study.

Keywords: Rabies, diagnosis, cell culture, mice, cells.

1. **Introduction**

Rabies is an acute infectious disease caused by *Rabies lyssavirus* (RABV), which is taxonomically classified into the *Mononegavirales* order, *Rhabdoviridae* family, and *Lyssavirus* genus. RABV is harbored and transmitted by mammalian hosts. Its replication occurs in the neurons, compromising the central nervous system (CNS) and leading to encephalomyelitis (STEELE; FERNANDEZ, 1991; BARBOSA *et al*., 2007).

The viral genome is a non-segmented, single stranded, negative-sense RNA molecule ranging from 11.9 to 12.3 kb. Structurally, it comprises two main elements: a lipidic bilayer envelope containing glycoprotein spikes (G), a second membrane formed by the matrix protein (M), the nucleocapsid, which is formed by the genomic RNA attached to the nucleoproteins (N), RNA polymerase (L), and the phosphoprotein (P) (WUNNER *et al*., 1988; CARNIELI, 2009).

Rabies is a neglected zoonotic disease with about 60,000 human deaths reported annually worldwide. However, these numbers may be underestimated due to the impacts of the disease in the different endemic regions. Since accurate estimation of animal deaths by rabies is unfeasible, new diagnostic methods have been implemented, including cell culture and next-generation sequencing (FOOKS *et al*., 2017; HORTON, D. *et al* 2015).

Significant changes occurred in the epidemiological scenario of rabies due to surveillance and control efforts, as the transmission dynamics of the disease, which is being maintained mainly through hematophagous bats. Outbreaks of human rabies transmitted by bats in the North region resulted in 36 deaths in Pará state between the years 2004 – 2005, and 24 deaths in Maranhão state in 2005. In 2018, a new outbreak was recorded in Pará, with 10 deaths reported. All cases were associated to bat spoliation with no post-exposure prophylaxis for rabies (BRASIL, 2019).

RABV transmission occurs through bites and consequent contact with the saliva of the infected animal, along with scratches and licking on injured skin, or contact with their mucosa. Transmission by aerosols, inadequate manipulation of samples

*Recebido em 22 de maio de 2023 e aceito em 30 de outubro de 2023.
**Evandro Chagas Institute – IEC/SVS/MS, Department of Arbovirology and Hemorrhagic Fevers, Ananindeua, 67030-000, Brazil.
***Federal Rural University of Pará - UFRA, Institute of Health and Animal Production, Belém, 66077-830, Brazil.
****Federal Agriculture Defense Laboratory – LFDA, Av. Almirante Barroso, 1234 Marco Belém, Brazil.

in laboratory, zoophilia, organ transplantation, carcass handling, and undercooked raw meat consumption were also described, but are rare infection sources (MARSTON *et al*. 2018; KOTAIT; CARRIERI; TAKAOKA, 2009; KOPROWSKY, 1996; DUARTE; DRAGO, 2005; FOOKS *et al*. 2009).

The gold standard diagnostic test for RABV is Direct Fluorescent Antibody test (DFA), although it is prone to generating false negative results. For this reason, confirmation of this first diagnosis is mandatory, and must be done by virus isolation techniques, whether in animals or cells. However, due to important bioethical issues, the reduction of the animal use is prioritized whenever possible. In this scenario, the VICC technique is the alternative choice, which can be performed with celerity while presenting high sensitivity and specificity for RABV (BRASIL, 2014; WHO, 2018; CASTILHO *et al.*, 2007; CORONA *et al.,* 2017; DEAN; BELSETH; ATANASU, 1996).

2. **Material and methods**

2.1. *Legal aspects of bioethics and biosafety*

The use of the selected samples was authorized for scientific investigation by the Evandro Chagas Institute (IEC) (Supplementary Material 1), and the Ethics Commission on Animal Use (CEUA) approval under no 03/2020, in accordance with the Ethic Principles on Animal Experimentation (CONCEA) of IEC (Supplementary Material 2).

The handling of samples from suspected rabies cases was performed in a Class II B2 Biosafety cabinet in an Animal Biosafety Level 2 (ABSL-2) or Animal Biosafety Level 3 (ABSL-3) according to the complexity and risks of the procedures, which strictly followed the biosafety standards established by the institution. Also, as a requirement, professionals working in this area must be previously immunized with regular evaluation of their immunological conditions (BRASIL, 2008).

2.2. *Samples*

This study included 215 samples from multiple animal species. The specimens were sent for investigation by the health secretariats and agricultural defense agencies of the Brazilian states in the Amazon region.

Nervous tissue samples from 21 bovines, 42 canines, 5 horses, 6 felines, 10 non-human primates (NHP), and 66 bats, for which 65 tissue samples from salivary glands were also included, were isolated simultaneously, and the sensitivity and specificity of VICC were evaluated in comparison to VIM and DFA. It is important to remember that, depending on the species, the viral load varies according to the anatomy of the brain. For this reason, we request the shipment of the complete material so that the best choice of the examined area can be made in the laboratory. As for bats, the whole animal is always sent, so that, in addition to the tests, the morphological identification of these animals can be carried out with the aid of a dichotomous key.

2.3. *Direct Fluorescenct Antibody Test (DFA)*

The 150 tissue samples were printed on properly identified slides following the technique described by Dean et al. (1996), applying an anti-rabies antibody conjugated to the developer (fluorescein isothiocyanate), diluted at a concentration of 20%. The material was fixed with acetone at -20ºC for at least 30 minutes, followed by incubation in a humid chamber at 37ºC for 30 minutes and washing with phosphate-buffered saline – PBS (Gibco, MA, USA) at pH 7.4 – 7.8, at rest immersed for 10 minutes. The slides were dried at room temperature and finished with buffered glycerin at pH 8.5, and cover slips were placed (DEAN; BELSETH; ATANASU, 1996; HARUÊ, 2017).

The examination was conducted under a fluorescence microscope (Zeiss, Oberkochen, Germany). During the processing, positive and negative controls were added for each set of samples to ensure the reliability of the results (HARUÊ, 2017).

2.4. *Virus Isolation in Mice (vim) or Biological Proof (BP)*

A 20% (weight/volume) suspension was prepared with a minimum of 0.75 g to 1 g of nervous tissue, depending on the species examined, mainly bats, where there are very small species. Nerve tissue samples were macerated in a ceramic degrees and pestle, and 3 mL of PBS were added, followed by centrifugation at 3,000 rpm for 30 minutes, and the supernatant was recovered and used as inoculum.

Newborn Swiss albino mice were used for inoculation via intracerebral route (KOPROWSKY, 1996; CASTILHO et al., 2007). Animals were maintained in identified micro-isolator cages with daily provided food and water. The proceeding was executed in animal experimentation bioterium level 2 (NBA2).

The mice were observed daily, and all changes were recorded on the inoculation cards for each sample for 21 days. Samples of herbivores and wild animals (e.g., bats) were observed for 30 days. Mice that showed signs of disease were euthanized in a CO2 chamber, and death was certified by a veterinarian through confirmation of cardiorespiratory arrest and observation of cyanosis. Subsequently, the animals were taken to the laboratory, where the nervous tissue was excised in a biosafety level 2 cabin (BSL-2) to proceed with the DFA test. This stage is fundamental since the biological test is a sensitive technique, but it is not specific for rabies, and it is possible to isolate other agents. Since the samples used in this work are from the laboratory routine. For this reason, DFA is performed to confirm the diagnosis of rabies (TAKAOKA, 2009; ZAMUDIO, 2020; BRASIL, 2008).

2.5. *Implantation of Virus Isolation in Cell Culture (VICC)*

The implantation of VICC using the murine neuroblastoma cell line (N2A) followed technical criteria established by the Pasteur Institute of São Paulo, National Reference Laboratory for the diagnostic of rabies.

2.5.1. Cell Culture preparation

N2A cells were slowly thawed at room temperature to avoid excessive celular death. After thawing, 1 mL of the cell suspension was transferred to 25 cm culture bottles with addition of 9 mL of Eagle's Minimum Essential Medium (EMEM) and 10µL of amino acids, after which they were maintained in an incubator (Thermo Scientific, MA, USA) at 37 **°C** and 5% CO2 for 24 h. The cells were then visualized under the microscope to verify their confluence. The EMEM was changed, and the bottles were returned to in the incubator for additional 96 hours.

2.5.2. Cell culture mantainance

This step aims to maintain cells for use or stocking. Initially, 2.5 mL of trypsin were added twice, along with 4 mL of EMEM. From

this material, 1 mL was distributed in 4 bottles, and 9 mL EMEM enriched with 10% fetal bovine serum (FBS) (Gibco, MA, USA) was added. Bottles were stored in the incubator at 37 °C and 5% CO2 for 72 h, after which cells were ready to use.

2.5.3. Conjugated antibody titration

This step consists in the identification of the most suited conjugate titer to be used in the routine required for each reagent batch acquired. The selected dillutions were from 1:5 to 1:320. The choice was based on the intensity of the green and red fluorescence in infected and non-infected cells, respectively.

2.5.4. Inoculation

The applied protocol was described by Webster and Casey (1996). The same suspension used for the VIM was diluted in EMEM and supplemented with 10% FBS. The N2A cell suspension was diluted in EMEM and supplemented with 0,3 mM non-essential amino acids and gentamicin. The cellular concentration was 5 x 105 cells/mL, and the final concentration of nervous tissue was 4%.

Each suspension was inoculated in triplicate in 96-well plates, and positive and negative controls were used for all tests. The plates were maintained in the incubator at 37°C and 5% CO2 for 96 hours. The medium was removed, and the cells adhered to the plates were fixed in 80% acetone for 15 minutes, and DFA was performed as described in item 2.3. After drying, the plates were prepared adding 50 µL of 10% glycerin to each well and analyzed under an inverted fluorescence microscope (Olympus IZ71, Tokyo, Japan) (WHO, 2018; HARUÊ, 2017).

It is important to highlight that the demand for diagnosis is spontaneous and very high. Therefore, the viral titer of each routine sample is impossible to calculate before performing isolation. However, the cell concentration is already standardized for isolation, given that each viral species needs a specific cell concentration. This is a factor that can influence the success of the technique used.

2.6. *Statistical analysis*

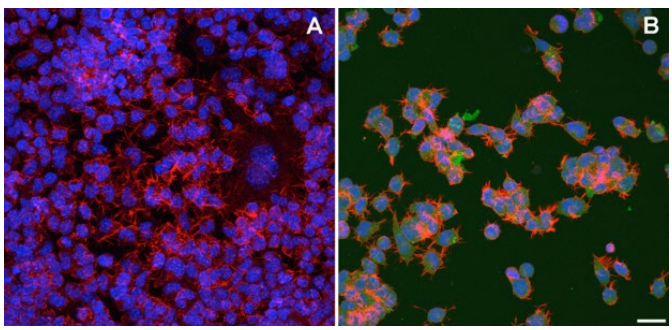
VICC sensitivity and specificity were statistically compared to the routine techniques (VIM and DFA). The statistical analysis was performed using Bioestat 5.3 applying the Kappa test adopting 5% significance level. Along with the statistical analyses, VICC was also evaluated based on practical aspects of the laboratory routine, which allowed a better interpretation and assessment of the data generated from this research.

3. **Results**

From the 65 samples examined, 3 were positive in both VIM and VICC techniques. Figure 1 shows a confocal photomicrograph of a positive chiropteran sample in the VICC technique. The standard operating procedure for the VICC can be consulted in the Supplementary File 3.

The results were concordant in the three techniques across all sample types, bovine, canine, feline, chiropteran (brain) and simian samples. However, from the 5 samples from horses 2 were positive in DFA, while just one was positive in the isolation techniques as shown in Table 1.

Figure 1: Confocal photomicrograph (Leica Camera, Wetzlar, Germany – SP-8 model) of N2A cells used in VICC. (A) Negative control after 96 h of seeding, showing the nuclei of cells stained in blue with DAPI dye. (B) Rabies virus infected cells from a positive sample after 24 hours of inoculation. Actin is stained in red with phalloidin and viral particles labeled with anti-rabies antibody in green. Scale bar: 25 µm.



The concordance observed for VICC and VIM was of 100% sensitivity and specificity, while between the DFA and VICC these values were of 93.3% sensitivity and 100% specificity. The same was observed when comparing DFA and VIM. In these terms, statistical test evaluation through Kappa test (kappa index=0.96, with p<0.0001), demonstrated great concordance between the techniques, and evidenced greater sensitivity for the DFA technique.

Table 1: DFA, VIM and VICC results across specimens and tissue types tested.

Specimen	Tissue	Total	DIAGNOSTIC TECHNIQUE					
			DFA		VIM		VICC	
			+	-	+	-	+	-
Bovines	Brain	21	10	11	10	11	10	11
Canines	Brain	42	0	42	0	42	0	42
Equines	Brain	5	2	3	1	4	1	4
Felids	Brain	6	0	6	0	6	0	6
Chiropterans	Brain	66	3	63	3	63	3	63
	Salivary glands	65	*	*	3	62	3	62
Primates	Brain	10	0	10	0	10	0	10
TOTAL		**215**	**15**	**135**	**17**	**198**	**17**	**198**

Legend: * not applied for the sample **DFA–** Direct Fluorescent Antibody test; **VICC** – Virus Isolation in Cell Culture. **VIM** – Virus Isolation in Mice; (+): reagent/positive; (-): non-reagent/negative.

4. **Discussion**

Clinical diagnosis of rabies is hampered by the non-specificity of the symptoms. For this reason, the implementation of control and prevention measures for rabies notoriously relies on laboratorial diagnosis, which enables better view on its epidemiological scenario. According to the World Organization for Animal Health (WOAH) and the World Health Organization (WHO), DFA is the most recommended technique for RABV diagnosis worldwide, considering its short execution time and high sensitivity. However, the realization of a confirmatory test through a different method, as virus isolation techniques (VIM and VICC), is also recommended to avoid false negative results (WHO, 2018).

Although VIM presents high sensitivity to RABV, the disadvantages related its implementation must be considered, as its extended execution time. Additionally, the technique, must be conducted in institutions in compliance with guidelines for animal welfare, and possess structural capacity for the mantainance of a bioterium facility to accomodate a great amount of animals necessary for routine diagnostics, which considerably raises costs.

In this scenario, cell culture has shown to be very promising when it comes to safety and speed. This technique consists on maintaing living cells in vitro in an adequate and controled environment, while conserving their physiological, biochemical and genetic properties as much as possible. Due to the successful isolation of RABV in cell culture, the replacement of VIM by VICC has been recommended (WHO, 2018; AVMA, 2013).

Internal and external factors interfere on the quality of laboratorial diagnosis of RABV. The first mainly refers to the adequate execution of the laboratory standard operating procedures, which also relies on the experience of the performer. As for the latter, conservation status of the material seems to play a central role, along with the selection of the ideal brain region to be sent for analysis, since the distribution of RABV does not occur homogeneously in the organism and varies for different animal species (GENEVA, 2018).

In this study, the three techniques provided satisfactory and almost completely concordant results, except for a single reagent sample from a horse in the DFA that was negative in the isolation techniques. This event can be explained by the decrease in the load of viable viral particles in the sample. According to Barrosa et al. (2018), the brain stem and spinal cord show the highest concentrations of antigens in horses, which explains the paralytic symptoms that sometimes lead to death before the virus reaches the brain, making them suitable clinical specimens to meet the criteria safe diagnosis. This particularity may cause underestimation of cases of rabies in horses, when only the cerebral cortex is most often available for testing. In addition, the precarious safety conditions for collecting samples in the field lead technicians to remove superficial fragments of nervous tissue, where there is a lower concentration of antigen. These peculiarities are actually observed in laboratory experience. Faced with this type of situation, the best strategy is found in molecular tools.

Kanitz *et al.* (2015) described a similar situation for a bovine sample for which, under optimal replication conditions, RABV could be isolated in VIM and VICC using N2A and BHK-21 (Baby Hamster Kidney) cell lines, but presented a false negative result in DFA, probably due to low viral amounts in the sample.

Besides the previously mentioned techniques, Yang *et al*. (2012) evaluated the application of RT-PCR and RIDA (Rapid Immunodiagnostic Test) as alternative tests for rapid and reliable diagnosis. A panel comprising 110 samples from cattle, domestic dogs and raccoon dogs from Korea was tested in comparison to DFA and VICC, also using N2A cells. The authors observed sensitivity and specificity rates of 100% between the VICC and RT-PCR techniques, while RIDA technique showed sensitivity of 95% and specificity of 98.9%.

Although N2A cells are preferentially chosen for the isolation of RABV, other cell lines may be used for RABV isolation, including BHK-21, CER (Chicken Embryo Related cells), HEK-293 (Human Embryonic Kidney cells) and McCoy cells. Nogueira (2004) observed, in a study including only chiropteran samples, sensitivity and specificity of 90% for N2A cells, against 95% for McCoy cells in both parameters, demonstrating superior accuracy. The study increased the portfolio of cell lines that exhibit effective reproducibility regarding to RABV isolation representing an effective alternative method. The less effort necessary for McCoy cells cultivation was also referred, along with much lower maintenance cost, due to lower concentration of reagents required in comparison to N2A, including FBS for supplementation.

The celerity of VICC technique (4 days), in contrast to VIM (up to 30 days), demonstrated its unquestionable relevance for the epidemiological surveillance of rabies, enabling fast implementation of control measures following the identification of animal rabies and imminent risk to humans. Indeed, this characteristic is key to the superior performance VICC technique in comparison to VIM. Moreover, from 215 samples tested by VIM technique, 15 required retesting, which delayed even more the final result, an event also reported by other authors (AVMA, 2013).

In this study it was observed that, the execution time of DFA ranges between 2-4 hours, depending on the number of slides examined, without the need for repetition. A total of 153 samples were tested by DFA. In the VICC technique, from 215 samples tested, 214 took 96 hours (4 days) to produce definitive results. A single horse sample required retesting. this intercurrence increased the time for the definitive result to 192 hours (8 days).

And of the 215 samples, 48 were from carnivores (canine and feline) and required a period of 21 days of observation, while the remaining 167 samples were observed for 30 days until the confirmation of a negative result. The 17 positive samples varied from 7-14 days from day “zero” until the appearance of the first clinical signs. Reinoculation of 15 samples was necessary, increasing the execution time from 30 to up to 94 days.

Some indirect benefits about the maintainance of N2A cell lines in stock at the laboratory are the support for the diagnosis of other diseases in the Department of Arbovirology and Hemorrhagic Fevers, as well as turning cultured antigens available for subsequent antigenic analyses, and supporting the execution of RABV serum neutralization technique.

Ultimately, it is important to point out that other tools are in the process of being implemented, including RT-qPCR, a further step towards the total suspension of the animal use for the RABV diagnosis at Evandro Chagas Institute. However, we emphasize the importance of the VICC technique, which is currently incorporated into the laboratory routine, and greatly contributes to a safer and more rapid diagnosis, in accordance with the guidelines related to animal welfare.

Acknowledgements

To the Federal University of Pará

To Evandro Chagas Institute and the Department of Arbovirology and Hemorrhagic Fevers for the support and realization of this study.

To the Federal Agricultural Defense Laboratory (LFDA) for the kind donation of N2A cells from the ATCC/USA.

To Dr. José Antônio Picanço Diniz for the microscopy images

To technician Armando de Sousa Pereira, for his contribution to the laboratory routine.

Referências

AVMA, AVMA. Guidelines for the Euthanasia of Animals: 2013 Edition. **American Veterinary Medical Association, Schaumburg (IL)**, 2013.

BARBOSA, Taciana Fernandes Souza *et al*. Epidemiologia molecular do vírus da raiva no estado do Pará no período de 2000 a 2005: emergência e transmissão por morcegos hematófagos (desmodus rotundus). **Caderno de Saúde Coletiva**, Rio de Janeiro, v. 3, n. 15, p.329-348, 2007.

BARROSA, Noelle de Castro; LANGONI, Hélio; VICTÓRIA, Cassiano; MENOZZI, Benedito Donizete; MANTOVAN, Karine Bott; LATOSINSKI, Giulia Soares; JOAQUIM, Sâmea Fernandes. Results of laboratory diagnosis of rabies in herbivores (cattle and horses): a retrospective study. **Brazilian Journal of Veterinary Medicine**, [S.L.], v. 40, p. 1-5, 2018.

BRASIL. Ministério da Saúde Secretaria de Vigilância em Saúde Departamento de Vigilância Epidemiológica. Ministério da Saúde. **Manual de Diagnóstico Laboratorial da Raiva:** Normas e Manuais Técnicos. Brasília: Editora Ms, 2008. 110 p.

BRASIL. Ministério da Saúde. Secretaria de Vigilância em Saúde (Ed.). **Raiva: o que é, causas, sintomas, tratamento, diagnóstico e prevenção.** 2019. Disponível em: <http://portalms.saude.gov.br/saude-de-a-z/raiva>. Acesso em: 05 abr. 2019.

BRASIL. Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de Vigilância Epidemiológica. **Normas técnicas de profilaxia da raiva humana**. Brasília, 2014. 60 p.

CARNIELI JUNIOR, Pedro. **Caracterização Genética de Vírus da Raiva Isolados de Bovinos e Equinos no Período de 1997-2002 Em Área Epidêmica do Estado de São Paulo, Brasil.** 2009. 77 f. Tese (Doutorado) - Curso de Ciências, Universidade de São Paulo, São Paulo, 2009.

CASTILHO, Juliana Galera et al. Padronização e aplicação da técnica de isolamento do vírus da raiva em células de neuroblastoma de camundongo (N2A). **Boletim Epidemiológico Paulista**, São Paulo, v. 4, n. 47, p.12-18, nov. 2007.

CORONA, Thaila Francini; BÖGER, Beatriz; ROCHA, Tatiana Carneiro da; SVOBODA, Walfrido Külh; GOMES, Eliane Carneiro. Comparative analysis of Mouse Inoculation Test and Virus Isolation in Cell Culture for rabies diagnosis in animals of Parana, Brazil. **Revista da Sociedade Brasileira de Medicina Tropical**, [S.L.], v. 51, n. 1, p. 39-43, fev. 2017. FATpUNIFESP (SciELO). http://dx.doi.org/10.1590/0037-8682-0303-2017

DEAN, D. J.; ABELSETH, M. K.; ATANASU, P. The fluorescence antibody test. In: **Laboratories Techniques in Rabies**. MESLIN, F. X.; KAPLAN, M. M.; KOPROWSKI, H.(eds.). Geneva,World Health Organization, 1996. p.88-95.

DUARTE, Leila; DRAGO, Maria Carmo. **A Raiva.** 2005. 28 f. Tese (Doutorado) - Curso de Virologia, Universidade de Évora, Évora, 2005.

FOOKS, Anthony R. *et al*. Emerging technologies for the detection of rabies virus: challenges and hopes in the 21st century. **PLoS neglected tropical diseases**, v. 3, n. 9, p. e530, 2009.

FOOKS, Anthony R. *et al*. Rabies. **Nature Reviews Disease Primers**, Reino Unido, v. 3, p.17091-17109, 30 nov. 2017. Springer Nature. http://dx.doi.org/10.1038/nrdp.2017.91.

GENEVA. WHO. Classification of lyssaviruses. In: GENEVA. WHO. **Expert Consultation on Rabies:** WHO Technical Report Series. 1012. ed. Geneva: Who, 2018. Cap. 3. p. 10-15. (Third report.).

HARUÊ, Carolina Freire Tamura. Diagnóstico da Raiva: Estudo comparativo entre a efetividade dos testes de cultivo celular e inoculação viral em camundongos. Trabalho de Conclusão de Curso. Universidade Estadual Paulista Júlio de Mesquita Filho. Faculdade de Medicina Veterinária, 2017.

HORTON, D. *et al.* Complex epidemiology of a zoonotic disease in a culturally diverse region: phylogeography of rabies virus in the Middle East. PLoS Neglected. Tropical Disease. **9**, e0003569 (2015).

KANITZ, Fabio Adriano **et al**. Virus isolation in cell culture for confirmatory diagnostic of rabies in bovine specimens. **Ciência Rural**, [S.L.], v. 45, n. 12, p. 2193-2196, dez. 2015.

KOPROWSKY, H. The mouse inoculation test. In: **Laboratories Techniques in Rabies.** MESLIN, F. X.; KAPLAN, M. M.; KOPROWSKI, H.(eds). Geneva, World Health Organization, 1996, p. 80-87.

KOTAIT, Ivanete; CARRIERI, Maria Luiza; TAKAOKA, Neide Yumie. **Manual Técnico do Instituto Pasteur:** Raiva – Aspectos gerais e clínica. 8. ed. São Paulo: Instituto Pasteur, 2009. 57 p.

MARSTON, Denise *et al*. The lyssavirus host-specificity conundrum — rabies virus — the exception not the rule. **Current Opinion in Virology**, [s.l.], v. 28, p.68-73, fev. 2018. Elsevier BV. http://dx.doi.org/10.1016/j.coviro.2017.11.007.

NOGUEIRA, Yeda L. Estimativa de validade de um novo método de Isolamento de vírus rábico. **Revista de Saúde Publica**, São Paulo, v. 2, n. 38, p. 315-322, 2004.

STEELE, J.H.; FERNANDEZ, P.J. History of rabies and global aspects. In: BAER, G.M. (Ed.) **The natural history of Rabies**. 2. ed. Boca Raton: CRC Press, 1991, p. 1-24

WORLD HEALTH ORGANIZATION. WHO Expert Consultation on Rabies, Third Report. WHO Technical Report Series, no. 1012, 2018.

WUNNER, William H. *et al*. The Molecular Biology of Rabies Viruses. **Reviews Of Infectious Diseases**, Chicago., v. 10, n. 4, p.771-784, Nov./Dec. 1988.

YANG, Dong-Kun; SHIN, Eun-Kyung; OH, Yoon-I; LEE, Kyung-Woo; LEE, Chung-San; KIM, Seo-Young; LEE, Jeong-A; SONG, Jae-Young. Comparison of four diagnostic methods for detecting rabies viruses circulating in Korea. **Journal Of Veterinary Science**, [S.L.], v. 13, n. 1, p. 43-48, 2012. The Korean Society of Veterinary Science.

ZAMUDIO, Raphaela Mello. CELULAS HEK-293: UMA ALTERNATIVA PARA ISOLAMENTO VIRAL NO DIAGNÓSTICO DA RAIVA. Trabalho de Conclusão de Curso (Especialização). Secretaria de Estado da Saúde de São Paulo. Instituto Pasteur, São Paulo, 2020. Programa de Especialização em Diagnóstico Laboratorial da Raiva.