PRESENCE OF RABIES VIRUS IN SALIVARY GLANDS: INSIGHTS FROM THE SARAWAK OUTBREAK FROM 2017 TO 2018

NAIM, M.S.N.[1*], AHMAD FIKRI, A.Y.[1], WAN NORMAZIAH, W.O.B.[1], NORAZURA, A.H.[1], NURSHUHADA, A.H.[1], NURHAFIZA, H.[1], ASNIZA, S.[1], EZDIANI, A.[1], AND SOHAYATI, A.R.[2]

1 Veterinary Research Institute, Perak, Malaysia

2 Veterinary Public Health Division, Department of Veterinary Services Malaysia, Putrajaya, Malaysia

**Corresponding author*: msnaim@dvs.gov.my

ABSTRACT. Rabies is a fatal zoonotic disease that poses a significant public health concern in Malaysia, particularly during outbreaks in domestic dog populations. While the detection of rabies virus in brain tissue is well established, data on viral presence in the salivary glands of suspected rabid dogs in Malaysia remain limited. This study aimed to investigate the presence of rabies virus in the salivary glands of dogs confirmed positive for rabies by brain tissue analysis during the Sarawak outbreak from 2017 to 2018. A total of 39 salivary gland samples from dogs confirmed brain-rabies-positive by the Fluorescent Antibody Test (FAT) in a previous study were analyzed for viral detection using the Rapid Tissue Culture Infection Test (RTCIT). Out of 39 samples, 17 (43.59%) were positive for rabies virus by RTCIT, while 22 (56.41%) were negative. All RTCIT-positive samples were subjected to further confirmation by nested Reverse Transcription Polymerase Chain Reaction (RT-PCR), resulting in 16 positive and one negative sample. These findings demonstrate that rabies virus can be detected in salivary glands of suspected rabid dogs, highlighting the role of these tissues in viral shedding and transmission. To the best of our knowledge, this represents the first documented detection of rabies virus in the salivary glands of suspected rabid dogs in Malaysia. These results underscore the importance of including salivary gland analysis in rabies surveillance and diagnostic protocols to enhance understanding of disease transmission dynamics.

Keywords: rabies, RTCIT, virus isolation, rapid test, nested RT-PCR

INTRODUCTION

Rabies is a highly contagious viral disease that affects the central nervous system of mammals, including humans (Moges, 2015; Otolorin *et al*., 2015). The virus primarily replicates in muscle tissue at the site of infection before entering peripheral nerves and traveling to the central nervous system (Hemachudha *et al*., 2013). Following central nervous system infection, the virus disseminates to several organs including the salivary glands, which is crucial for viral transmission (Mahadevan *et al*., 2016).

The prior study conducted by Jimenez *et al*. (2018) demonstrated the successful isolation of rabies virus from salivary glands tissues of confirmed rabid carnivores, including striped skunks and various wild and domestic carnivores, during the period of 2013 to 2015 in northern Colorado. Another study has indicated that rabies virus can be detected in salivary glands even before the onset of clinical symptoms, making these tissues particularly important for early diagnosis and understanding disease progression (Fisher *et al*., 2018). In another study, Hoary foxes (*Pseudoalopex vetulus*) were found to exhibit rabies-positive salivary glands when analyzed using Fluorescent Antibody Test (FAT) and Mouse Inoculation Test (MIT) (Silva *et al*., 2009). Additionally, recent findings conducted by Guo *et al*. (2024) revealed that viral loads in salivary glands can reach significant levels during the preclinical phase, highlighting their importance in viral shedding and transmission.

In Malaysia, rabies has a history of sporadic outbreaks dating back to 1924, with major incidents occurring in 1945 (Ganesan *et al*., 1993), often linked to canine movement (Lim, 1998). Through extensive control programs, the country successfully eradicated the disease, being declared officially rabies-free by 1999 (Ganesan *et al*., 1993; Lim, 1998). However, this status was lost in 2015 with a significant re-emergence involving canine cases in the northern Peninsular Malaysia states of Perlis, Kedah and Penang (Navanithakumar *et al*., 2019; WAHIS Interface, 2015). A more sustained and severe outbreak began in East Malaysia, Sarawak in July 2017, which continues to report both animal and human cases (Wada *et al*., 2024). Between 2017 and 2020, Sarawak recorded over 3,000 positive animal samples, predominantly in dogs (Sim *et al*., 2021).

Despite this critical re-emergence and the global understanding of salivary glands as the key site of viral shedding for transmission, robust data regarding viral isolation from the salivary glands of suspected rabid animals in Malaysia remains a crucial and unaddressed gap in epidemiological surveillance. Understanding the presence of infectious virus in these tissues is vital for both diagnostic confirmation and epidemiological studies related to transmission risk. Therefore, this study aimed to investigate the presence of rabies virus in salivary glands samples collected from suspected rabid dogs during the Sarawak outbreak. Viral detection was performed using the RTCIT to confirm the presence of infectious virus, with subsequent molecular confirmation of positive results conducted via nested RT-PCR analysis.

MATERIALS AND METHOD

Samples processing

Veterinary Research Institute received 39 samples of salivary gland and brain of suspected rabid dogs during Sarawak Rabies Outbreak 2017-2018. All the samples were from suspected infected areas in Kuching (26 samples), Serian (10 samples), and Samarahan (3 samples). The brain samples were analyzed and found to be all positive during the previous study conducted by Naim *et al*. (2022). Specifically, the brain tissues were confirmed positive for rabies antigen using the Fluorescent Antibody Test (FAT) and RTCIT. In contrast, this study will focus exclusively on testing samples from salivary glands. The RTCIT, a rapid virus isolation method, was employed for the salivary glands as it is the direct indicator of infectious viral shedding and thus transmission potential, which is the primary focus of salivary glands analysis.

The methods for sample preparation and RTCIT were based on standard rabies diagnostic techniques described by Rupprecht *et al*. (2018). Briefly, the salivary glands were ground in a mortar and pestle. The tissues were suspended in Viral Transport media (VTM) containing 5% fetal bovine serum, 200 IU/mL penicillin, 200 µg/mL streptomycin, 0.50 µg/mL amphotericin B, 200 µg/mL kanamycin sulphate (all obtained from Gibco, USA), and 7.5% of sodium bicarbonate. The mixture was centrifuged at 3000 rpm for 10 minutes at 4°C. The resulting supernatant was collected as the tissue culture infection inoculum. The samples were stored at -80°C until further study.

Rapid Tissue Culture Infection Test (RTCIT)

The RTCIT was conducted using four-well Lab-Tek tissue culture chamber slides (Nalgene Nunc International, Lab-Tek® II). Each well of chamber slides was seeded with 5x105 viable BHK-21 (ATCC®) cells per milliliter. A 1:10 diluted salivary gland suspension was introduced into the wells and incubated for 1 hour at 37°C with 5% CO_2. Following

incubation, the suspension was aspirated and replaced with a fresh growth medium. The chamber slides were then incubated for 5 days at 37°C with 5% CO_2. After the incubation period, the medium was removed, and the slides were air dried and fixed with 80% cold acetone for 30 minutes. The FAT was subsequently performed, and the chamber slides were examined under 20X and 40X magnification using a fluorescence microscope to identify the presence of apple green fluorescence foci indicative of rabies virus. The assays were repeated using supernatant from the first passage for a second passage and subsequently a third passage, to maximize the sensitivity of the assay.

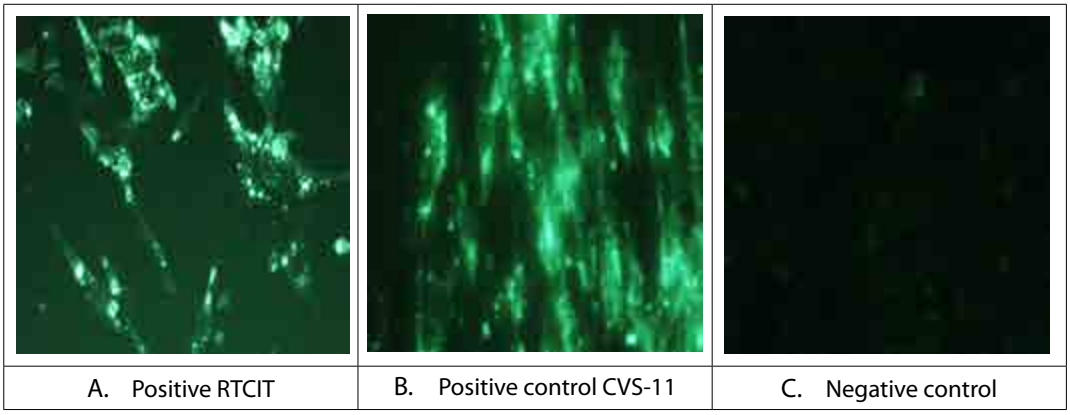
Nested RT-PCR

Only rabies positive samples of salivary gland tested by RTCIT will be subjected to nested RT-PCR. The samples were extracted for viral RNA using TRIzol® (Thermo Fisher Scientific, USA) according to the manufacturer's instructions in accordance with WOAH standard procedure. The RT-PCR (Promega Corporation, Madison USA) amplification was performed using 4 sets of primer (RVD007, RVD008, RVD009 and RVD010) followed by nested RT-PCR amplification using 4 sets of primer (RVD011, RVD012, RVD013 and RVD017) as described by the Australian Animal Health Laboratory (AAHL) method QA/13-80-6 (Wang and Meehan, 2014). The amplicons of the secondary PCR were visualized by 1.5% gel electrophoresis.

RESULTS

Out of 39 salivary gland samples tested by RTCIT, 17 (43.59%) were positive, whereas 22 (56.41%) were negative. The results for both positive and negative samples remained consistent upon observation through the third passage, with no additional samples converting from negative to positive. All positive samples exhibited an apple green fluorescence while negative control showed blank background (Figure 1).

Nested RT-PCR was performed on these 17 RTCIT-positive samples, confirming 16 (94.1%)



A. Positive RTCIT	B. Positive control CVS-11	C. Negative control

Figure 1. Fluorescent microscopic results of RTCIT for rabies virus detection. The figure displays representative images of BHK-21 fixed and stained via the FAT. **A)** A positive RTCIT result from tissue culture inoculated with salivary gland homogenate of a suspected rabid dog, showing characteristic apple-green fluorescence (rabies virus antigen foci). **B)** Positive control using the standard CVS-11 virus. **C)** Negative control (uninfected cells) showing a blank background.

Figure 2. Agarose gel electrophoresis of nested RT-PCR products targeting the rabies virus nucleoprotein gene (498 bp). Lane M: 100 bp DNA ladder; Lanes 1–17: samples tested by RTCIT; Lane 4: RTCIT-positive but nested RT-PCR-negative; Lane +: positive control (CVS-11 strain); Lane– : negative control.

as positive, while one sample was negative by RT-PCR. The nested RT-PCR analysis of the RTCIT-positive samples demonstrated amplification, with the primary RT-PCR yielded 606 bp product and the secondary PCR yielded a 498 bp product. No amplification band was observed in the negative control template (Figure 2).

DISCUSSION

To our knowledge, this is the first report demonstrating the detection of rabies virus in the salivary gland tissues of suspected rabid dogs in Malaysia. The study cohort consisted of 39 dogs whose brain tissues were previously confirmed as rabies positive using FAT test. The test is the gold-standard method for definitive diagnosis, ensuring a highly reliable positive status for the animals included in this study.

Out of 39 of the salivary gland samples, 17 (43.59%) were positive whereas 22 (56.41%) were negative by RTCIT. The finding that 43.59% of dogs confirmed brain positive for rabies also harbored infectious virus in their salivary gland is highly significant, as it directly quantifies the transmission risk via viral shedding during the Sarawak outbreak. The successful isolation of the virus in cell culture (RTCIT) confirms that the virus was present at biological relevant, high concentrations sufficient (high titer) for transmission. This high titer of virus detected by RTCIT provides the significant level of virus detection requested.

The detection of the rabies virus in the first passage indicates that the concentration of the virus was above the threshold, suggesting that a single passage is sufficient to detect infectious virus in salivary gland or brain. This aligns with the finding by Hostnik *et al*. (2014), who reported successful isolation of vaccine virus in the salivary gland of a red fox using a single passage virus isolation technique. Crucially, the positive and negative RTCIT results for all 39 samples remained consistent upon observation through the third passage. This confirms the robustness of the first passage results and supports the methodology chosen for rapid diagnostic implementation.

Although 39 samples of brain tissue were confirmed positive by FAT, rabies virus isolation by the RTCIT from the salivary glands was negative in 22 (56.41%) of these cases. The RTCIT repeated through up to three passages yielded no additional positive results, likely due to viral load below the detection threshold of the assay (Panning *et al*., 2010). Additionally, intermittent or absent viral shedding in saliva during the early or late stages of infection may explain the negative findings (Delpietro *et al*., 2001). The absence of infectious virus in salivary glands despite central nervous system infection aligns with evidence

that viral dissemination to peripheral tissue is not guaranteed in every infected individual (Jimenez *et al*., 2019; Fook *et al*., 2017). Given consistent negative RTCIT results, it is concluded that viral loads were minimal or absent in these salivary glands, mitigating the risk of transmission from these samples. This highlights the limitations of virus isolation assays compared to molecular diagnostic methods and the complexity of rabies virus shedding dynamics, which have important implications for diagnostic and epidemiology (Panning *et al*., 2010; Yang *et al*., 2012)

All 17 samples that were positive by RTCIT were further analyzed using nested RT-PCR, with 16 of the samples were in concordance with RTCIT. The results indicated that rabies virus RNA can be detected using this technique, thus proving the test is sensitive and specific in demonstrating the presence of rabies disease from the infected specimens. However, it is noted that one sample was RTCIT positive but RT-PCR negative. This discrepancy may be due to the presence of inhibitors interfering with the amplification process (Cai *et al*., 2018) or conversely, a viral load below the detection threshold of the RT-PCR assay (Nadin-Davis *et al*., 2009). This RT-PCR detection threshold issue for the single discrepant sample is distinct from the 22 RTCIT-negative samples, which failed to show infectious virus replication entirely.

The demonstration of substantial quantities of viral antigen in suspected rabid dogs underscores the significant risks associated with handling suspected animals (Beauregard *et al*., 1969). Furthermore, Carey *et al*. (1983) reported that the rabies infected animals often exhibit high titer in salivary glands compared to their brain tissues. Transmission of rabies typically occurs through the introduction of the virus via the saliva of infected animals through bites, open wounds or unwrapped cuts or contact with mucous membranes (Langley, 2009). Consequently, rabies prevention can be achieved by minimizing direct contact with rabid animals, their mucous membranes, and open wounds. Additionally, providing comprehensive training to wildlife workers, veterinarians, animal handlers and laboratory workers is essential, as preventive measures are more effective than post-exposure interventions (Bano *et al*., 2016).

The laboratory plays a crucial role in rabies control by emphasizing the accuracy and speed of case confirmation. This study highlights the importance of the RTCIT method, which provides a high degree of sensitivity and specificity for confirming infectious virus. The fast and accurate confirmation of infectious virus in salivary glands reinforces the necessity of immediate public health intervention.

CONCLUSION

In conclusion, this study confirmed the presence of infectious rabies antigen in the salivary gland of rabid dogs. This is the first documented detection of rabies virus in the salivary glands of suspected rabid dogs in Malaysia. The successful isolation of infectious virus via RTCIT of the brain-positive dogs demonstrates that the virus was present at a known significant and transmissible level (high titer), underscoring the high risk of transmission. Additionally, it has revealed that suspected rabid dogs may serve as possible sources of exposure to incidents of the rabies outbreak in Sarawak.

While the salivary gland samples tested negative by RTCIT, including one sample that was RTCIT-positive but RT-PCR negative, these results provide critical information on the limits of viral shedding and potential diagnostic discrepancies. The high proportion of RTCIT negative samples suggests that viral shedding in the salivary glands is not guaranteed even in confirmed rabid dogs, or may be below the assay's detection limit.

Further studies should include both RTCIT and RT-PCR testing on all salivary gland samples to better assess diagnostic accuracy. Expanding the sample size and including samples from other regions in Malaysia will strengthen the findings. Additionally, molecular characterization of detected rabies virus strains and investigation of quantitative viral loads at different stages of infection are recommended to precisely estimate the level of infectious virus in the salivary glands and improve understanding of rabies transmission and support more effective control strategies.

REFERENCES

1. Bano, I., Sajjad, H., Shah, A.M., Leghari, A., Mirbahar, K. H., Shams, S., and Soomro, M. (2016). A review of rabies disease, its transmission and treatment. *Journal of Animal Health and Production*, *4(4),* 140–144. https://doi.org/10.14737/journal.jahp/2016/4.4.140.144
2. Beauregard, M. and Casey, G.A. (1969). Demonstration of rabies antigen in salivary glands of rabies suspected animals. *Canadian Journal of Comparative Medicine*, *33*(1), 55–58. https://pubmed.ncbi.nlm.nih.gov/4237297
3. Cai, D., Behrmann, O., Hufert, F., Dame, G., and Urban, G. (2018). Direct DNA and RNA detection from large volumes of whole human blood. *Scientific Reports*, *8*(1), 1–11. https://doi.org/10.1038/s41598-018-21224-0
4. Carey, A.B. and McLean, R.G. (1983). The ecology of rabies: Evidence of co-adaptation. *Journal of Applied Ecology*, *20*(3), 777. https://doi.org/10.2307/2403126
5. Delpietro, H. A., Larghi, O.P., and Russo, R.G. (2001). Virus isolation from saliva and salivary glands of cattle naturally infected with paralytic rabies. *Preventive veterinary medicine*, *48*(3), 223–228. https://doi.org/10.1016/s0167-5877(00)00187-2
6. Fisher, C.R., Streicker, D.G., and Schnell, M.J. (2018). The spread and evolution of rabies virus: Conquering new frontiers. *Nature Reviews Microbiology*, *16*(4), 241–255. https://doi.org/10.1038/nrmicro.2018.11
7. Fooks, A.R., Cliquet, F., Finke, S., Freuling, C., Hemachudha, T., Mani, R.S., Müller, T., Nadin-Davis, S., Picard-Meyer, E., Wilde, H., and Banyard, A.C. (2017). Rabies. *Nat Rev Dis Primers*. Nov 30;3:17091. doi: 10.1038/nrdp.2017.91.
8. Ganesan, J. and Sinniah, M. (1993). Occurrence of human rabies in peninsular Malaysia. *The Medical journal of Malaysia*, *48*(2), 194–199.
9. Guo, X., Zhang, M., Feng, Y., Liu, X., Wang, C., Zhang, Y., Wang, Z., Zhang, D., and Guo, Y. (2024). Transcriptome analysis of salivary glands of rabies-virus-infected mice. *Frontiers in Microbiology*, *15*. https://doi.org/10.3389/fmicb.2024.1354936
10. Hemachudha, T., Ugolini, G., Wacharapluesadee, S., Sungkarat, W., Shuangshoti, S., and Laothamatas, J. (2013). Human rabies: Neuropathogenesis, diagnosis, and management. *The Lancet Neurology*, *12*(5), 498–513. https://doi.org/10.1016/s1474-4422(13)70038-3
11. Hostnik, P., Picard-Meyer, E., Rihtarič, D., Toplak, I., and Cliquet, F. (2014). Vaccine-induced rabies in a red fox (*Vulpes vulpes*): Isolation of vaccine virus in brain tissue and salivary glands. *Journal of Wildlife Diseases*, *50*(2), 397–401. https://doi.org/10.7589/2013-07-183
12. Jimenez, I., Spraker, T., Anderson, J., Bowen, R., and Gilbert, A. (2018). Isolation of rabies virus from the salivary glands of wild and domestic carnivores during a skunk rabies epizootic. *Journal of Wildlife Diseases*, *55*(2), 473. https://doi.org/10.7589/2018-05-127
13. Langley, R.L. (2009). Human fatalities resulting from dog attacks in the United States, 1979-2005. *In Wilderness and Environmental Medicine* (Vol. 20). http://www.who.int/classifications/apps/
14. Lim, K.G. (1998). Rabies raises its ugly head once more. *Medical Journal of Malaysia, 53*(1), 4-5.
15. Mahadevan, A., Suja, M.S., Mani, R.S., and Shankar, S.K. (2016). Perspectives in diagnosis and treatment of rabies viral encephalitis: Insights from pathogenesis. *Neurotherapeutics*, *13*(3), 477–492. https://doi.org/10.1007/s13311-016-0452-4
16. Moges, N. (2015). Epidemiology, prevention and control methods of rabies in domestic animals: Review article. *European Journal of*

Biological Sciences, *7*(2), 85–90. https://doi.org/10.5829/idosi.ejbs.2015.7.02.93255

17. Nadin-Davis, S.A., Sheen, M., and Wandeler, A.I. (2009). Development of real-time reverse transcriptase polymerase chain reaction methods for human rabies diagnosis. *Journal of Medical Virology*, *81*(8), 1484–1497. https://doi.org/10.1002/jmv.21547
18. Naim, M.S.N., Ahmad Fikri, A.J., Wan Normaziah, W.O.B., Norazura, A.H., Nurshuhada, A.H., Nurhafiza, H., Asniza, S., Ezdiani, A., and Sohayati, A.R. (2022). Development and validation of virus isolation for rabies diagnosis. *Malaysia Journal of Veterinary Malaysia*, *13*(2), 25–31.
19. Navanithakumar, B., Sohayati, A.R., Rohaiza, Y., Sarah Dandang, A., Mariani, H., Leonora, T.M., and Dorothy, K.S. (2019). An overview of rabies outbreak in Malaysia, ordinances and laws. *Malaysia Journal of Veterinary Research, 10*(2), 148-158.
20. Otolorin, G.R., Aiyedun, J.O., Mshelbwala, P.P., Ameh, V.O., Dzikwi, A.A., Dipeolu, M.A., and Danjuma, F.A. (2015). A review on human deaths associated with rabies in Nigeria. *Journal of Vaccines & Vaccination*, *6*(1), 262. https://doi.org/10.4172/2157-7560.1000262
21. Panning, M., Baumgarte, S., Pfefferle, S., Maier, T., Martens, A., and Drosten, C. (2010). Comparative analysis of rabies virus reverse transcription-PCR and virus isolation using samples from a patient infected with rabies virus. *Journal of clinical microbiology*, *48*(8), 2960–2962. https://doi.org/10.1128/JCM.00728-10
22. Rupprecht, C.E., Fooks, A.R., and Abela-Ridder, B. (2018). *Laboratory techniques in rabies* (5th ed., Vol. 1). World Health Organization.
23. Silva, M.L.C.R., Da Silva Lima, F., De Barros Gomes, A.A., De Azevedo, S.S., Alves, C.J., Bernardi, F., and Ito, F.H. (2009). Isolation of rabies virus from the parotid salivary glands of foxes (*Pseudalopex vetulus*) from Paraíba State, Northeastern Brazil. *Brazilian Journal of Microbiology*, *40*(3), 446–449. https://doi.org/10.1590/s1517-83822009000300004
24. Sim, B.N.H., Liang, B.N.W., Ning, W.S., and Viswanathan, S. (2021). A retrospective analysis of emerging rabies: a neglected tropical disease in Sarawak, Malaysia. *The journal of the Royal College of Physicians of Edinburgh*, *51(2)*, 133–139. https://doi.org/10.4997/JRCPE.2021.207
25. Yang, D.K., Shin, E.K., Oh, Y.I., Lee, K.W., Lee, C.S., Kim, S.Y., Lee, J.A., and Song, J.Y. (2012). Comparison of four diagnostic methods for detecting rabies viruses circulating in Korea. *Journal of Veterinary Science*, *13*(1), 43–48. https://doi.org/10.4142/jvs.2012.13.1.43
26. Wada, Y.A., Mazlan, M., Noordin, M.M., Mohd-Lila, M.A., Fong, L.S., Ramanoon, S. Z., and Zahli, N.I.U. (2024). Rabies epidemiology in Malaysia (2015-2023): A cross-sectional insights and strategies for control. *Vaccine*, 42(26), 126371. https://doi.org/10.1016/j.vaccine.2024.126371
27. WAHIS Interface (2015). Follow-up report No. 1 (Final report). World Animal Health Information Database Interface, World Organisation for Animal Health (OIE). Accessed on 3 Jan 2019 at https://www.oie.int/wahis_2/ public/wahid.php/Reviewreport/Review?page_refer=MapEventSummary&reportid=18929
28. Wang, J. and Meehan, B. (2014). Molecular Diagnosis at CSIRO Animal, Food and Health Sciences Australian Animal Health Laboratory: Nucleic Acid Detection for Disease Diagnosis and Emergency Disease Investigation – Rabies Virus PCR (including Lyssavirus). [AAHL Regional Programme]. Australian Centre for Disease Preparedness (ACDP).