COMPARISON OF SENSITIVITY AND SPECIFICITY BETWEEN FLUORESCENT ANTIBODY TEST (FAT), HISTOPATHOLOGY AND NESTED POLYMERASE CHAIN REACTION (NESTED RT-PCR) FOR RABIES DETECTION DURING OUTBREAK 2015

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ABSTRACT. Rabies is a zoonotic disease caused by an RNA virus of genus Lyssavirus affecting all mammals, commonly dogs. Classical rabies virus (RABV) is the most highlighted, causing animal infection and public concern. There are two types of classical RABV, known as furious rabies and dumb rabies. Furious rabies is presented by aggressive behaviour whereas the dumb type normally displays no changes in behaviour and is followed by paralysis. Besides that, hypersalivation, photophobia, and hydrophobia could be other clinical symptoms observed in rabid animals. The objective of this study is to compare the sensitivity and specificity of test methods available in diagnosing rabies. This comparison was made between the Fluorescent Antibody Test (FAT), histopathology, and nested polymerase chain reaction (nested RT-PCR) on the brain samples collected during the rabies outbreak in 2015. Sensitivity and specificity were calculated based on the sensitivity and specificity formula. Neither histopathology nor nested RT-PCR could produce good sensitivity results as compared to FAT. However, both histopathology and nested RT-PCR provide higher specificity results compared to the FAT. Other alternative tests in conjunction with rabies diagnostic tests should be established.

Keywords: FAT, histopathology, nested RT-PCR, sensitivity, specificity

INTRODUCTION

Rabies is the deadliest zoonotic disease caused by an RNA virus of the genus Lyssavirus of the family Rhabdoviridae and order Mononegavirales (ICTV, 2009). Classical rabies virus (RABV) is the most highlighted in causing animal infection and public concern (Kaw *et al*., 2011). Transmission of the virus is through direct contact with infected saliva or nerve tissue via an open wound (Tsiang, 1993). Most human rabies is from dog bites where the victim eventually dies due to poor post-bite treatment (Nyasulu *et al*., 2021).

In Malaysia, rabies cases were last reported in 1995 in Terengganu in a dog on a fishing boat in a coastal village (Sunny *et al*., 2013). After 20 years, one case was reported each in Perlis, Penang, and Kedah (neighbouring states to Thailand) (Baker, 2015). Three years-old male dog bit the owner after being force feed during a state of illness. The dog was euthanized upon the owner's request. Post-mortem was performed without any significant findings. The dog brain was then sent for a Rabies Fluorescent Antibody Test (FAT), and the result came out positive. At the same time, histopathology and nested polymerase chain reaction (nested RT-PCR) were also conducted and the results showed positive for rabies. Thus, passive surveillance was initiated within a 30 km radius from case foci in early August till the end of December 2015.

A few tests are useful for confirmatory diagnosis, or as an alternative test in virus identification and epidemiological studies as well as the ability to detect the presence of RABV. These tests include the FAT, histopathology, and nested RT-PCR. The FAT, known as the gold standard, is performed according to the procedure described by the World Organisation

for Animal Health (WOAH) and the World Health Organization (WHO) (Meslin *et al*., 1996). The FAT is conducted using fluorescein isothiocyanate (FITC) tagged with monoclonal antibodies and appeared as a brilliant apple-green object against a dark background (Prabhu *et al*., 2018). While histopathology is a diagnosis of rabies in recognition of the presence of acute polioenchephalo-myelitis, craniospinal ganglionitis, mononuclear perivascular infiltrates, diffuse glial proliferation, and regressive changes in neuronal cells and glial nodules as well as the presence of Negri bodies (Hananeh *et al*., 2015). Developed Negri bodies contain genome, antigenome, and mRNA (Lahaye *et al*., 2009). In addition, the nested RT-PCR is a rapid and sensitive RNA detection technique and is a supplementary test to detect the RABV (Fazilah *et al.*, 2021). Given the different detection targets, this study aims to evaluate the sensitivity and specificity of the currently available test method against rabies in Malaysia.

MATERIALS AND METHODS

Collection of Samples

Passive surveillance was activated following the first case, and 607 dogs were sampled from 3 northern states of Peninsular Malaysia, including Perlis, Kedah, and Penang. Each dog was then put to sleep humanely using a lethal dose of sodium pentobarbital to obtain its brain. The dog head was packed in a tertiary containment system within a cooler box filled with ice pack which was then transported to the laboratory immediately. All procedures were performed following the Laboratory Biosafety Guidelines for Handling and Processing specimens (updated Sep 20, 2022). The post-mortem was conducted to extract parts of the brain for further testing. A part of the brain was separated according to the selected test methods. Formalin was used to fix the brain parts for histology.

Fluorescent Antibody Test (FAT)

The FAT was performed using the right and left sides of the brain using samples such as the hippocampus, cerebrum, cerebellum, and brain stem. The tissues were smeared on the glass slide and allowed to dry before fixation in absolute acetone for at least 30 minutes at -20 °C. After fixation, the slides were incubated with FITC conjugate (FujireBio, USA) for 30 minutes at 35 °C. Then, the process continued by rinsing the slides two times with phosphate buffer saline for 2 minutes each and rinsing with distilled water one time for 2 minutes. The slides were then air-dried and mounted with fluorescein mounting media (Dako, USA). All slides were examined at 200X magnification under a fluorescent microscope (Nikon, Japan). Negative and positive control samples were run together with the test samples. The slide showing a specific fluorescent colour was confirmed as positive.

Histopathology

The selected brain parts were dissected and fixed in 10 % formalin for histology preparation. Following a standard processing procedure (Carleton *et al.,* 1980), the brain tissues were embedded in paraffin wax before sectioning of the brain tissues using microtome to obtain a thin film (3 - 4 μm) and finally stained with haematoxylin and eosin (H&E). Glass coverslip was put on the slide after mounting with DPX mounting medium. All slides were viewed under the light microscope Olympus BX41 (Olympus, Japan) at 4X to 40X magnification. The histopathological section was examined for Negri bodies, meningitis, meningeal infiltration, perivascular cuffing, parenchymatous infiltration, formation of encephalitic nodules, and ganglion infiltration with satellitosis and neurophagia.

Nested Polymerase Chain Reaction (Nested RT-PCR)

About 3 g of the right and left sides of the brain were homogenized with sterile sand and minimum essential media and then centrifuged at 3,000 rpm for 10 minutes. All homogenized samples were stored at -80 °C except for the current PCR work by adding 250 µl Trizol® to it for RNA extraction. Homogenized samples were then transported to the Virology Laboratory for extraction and amplification. Total RNA was extracted from the positive samples by TRIzol LS Reagent (Invitrogen, Brazil), following the manufacturer's instructions. The extracted samples then undergo nested RT-PCR amplification using redundant primer sets homologous to conserved regions of the *Lyssavirus* N gene (Ainani *et al*., 2014) which were able to detect other species of Lyssavirus. As previous reported by Smith *et al.* (1995) and De Mattos *et al.* (1999) reverse transcription was carried out M-MLV Reverse Transcriptase (Invitrogen, Brazil), dNTPs (10mM), DTT (0.1M), and RT buffer (5). 1 hour at 42 °C was spent incubating the mixture.

Table 1. Primer used in the study

	Primer Name	Sequence	Product Size (bp)	Working Stock (µM)	Reference
Primary RT-PCR	RVD007/F	ATG TAA CAC CYC TAC AAT G	606	20	Heaton *et al.* (1997) & AAHL Method QA/13-80-6 (2012)
	RVD008/R	CAA TTC GCA CAC ATT TTG TG			
	RVD009/R	CAG TTG GCA CAC ATC TTG TG			
	RVD010/R	CAG TTA GCG CAC ATC TTA TG			
Secondary PCR	RVD011/R	GTC ATC AAA GTG TGR TGC TC	498	20	
	RVD012/R	GTC ATC AAT GTG TGR TGT TC			
	RVD013/R	GTC ATT AGA GTA TGG TGT TC			
	RVD017/F	AGA TCA ATA TGA GTA YAA RTA YCC			

The following cycling parameters were used: a preliminary heating step lasting 5 minutes at 94°C, 35 cycles lasting 45 seconds each at 94°C, 55°C, and 72°C, and a final extension phase lasting 5 minutes at 72°C. Nested RT-PCR was carried out using GoTaq Green Master Mix (Promega) specific primer pairs RVD011, RVD012, RVD013 and RVD017 as described by Nadin-Davis (1998). After being stained with ethidium bromide and electrophoretically separated on 1.5% agarose gel, PCR results were examined. About 606bp and 498bp respectively fragment was then seen under an ultraviolet light source and captured on the camera. At least one more confirmatory test was run on negative samples.

Statistical Analysis of Sensitivity and Specificity

The number of samples with true-positive results as determined by the reference assay was denoted by the symbol TP, and the number of samples with false-negative results was denoted by the symbol FN. The formula used to assess the sensitivity was [TP/ (TP+FN)] X 100. The number of samples with true-negative findings and the number of samples with false-positive results

were used to calculate the specificity, which was calculated as [TN/ (TN+FP)] x 100 based on Yang *et al*. (2012).

RESULTS AND DISCUSSION

Many diagnostic assays are available for identifying rabies in the field (Veasna *et al*., 2016). In this study, FAT, histopathology, and nested RT-PCR were selected as they play an important role in the detection of the RABV (Table 1). The study is focused on the sensitivity and specificity of the tests. A total of 51 brain samples were tested positive by FAT. The positive FAT samples were then further tested using histopathology and nested RT-PCR. Overall, 17.65 % FAT-positive samples (9 out of 51) were detected positive in histopathology and 23.53 % FAT-positive samples (12 out of 51) were detected positive in the nested RT-PCR test.

Table 2. Comparison results of FAT, histopathology, and nested RT-PCR

		Histopathology			**Nested Polymerase Chain Reaction (nested RT-PCR)**		
		P	N	T	P	N	T
Fluorescent Antibody Test (FAT)	P	9	42	51	12	39	51
	N	0	556	556	0	556	556
	T	9	598	607	12	595	607
Sensitivity (%)			17.65			23.53	
Specificity (%)			100			100	

P = positive, N = negative, T = total

A comparison on the sensitivity and specificity of FAT and histopathology indicated that histopathology showed 17.65 % sensitivity and 100 % specificity. Comparison between histopathology and FAT showed less sensitive results as FAT is generally very subjective. Advanced training should be conducted to familiarize the examiner with changes in the brain region related to rabies infection, such as multifocal and mild polioenchephalo-myelitis, craniospinal ganglionitis, mononuclear perivascular infiltrates, diffuse glial proliferation, and regressive changes in neuronal cells and glial nodules as well as the presence of Negri bodies (Appolinario *et al*., 2015). Even though Negri bodies are known as pathognomonic lesions of rabies, not in all cases can they be seen. According to Shankar (2009), histological technique is no longer recommended as a detection method of rabies as it is very subjective. Interpretation by the examiners might vary depending on their competency.

The nested RT-PCR result indicates 23.53 % sensitivity and 100 % specificity. It is less sensitive possibly due to the nested RT-PCR primers being designed for specific rabies virus genotype 1 and rabies virus genotype 2-6 (Lagos bat virus, Mokola virus, Duvenhage virus, and European bat virus 1 and 2, Australian bat *lyssavirus*) based on the *Lyssavirus* N gene (Heaton *et al*., 1997). Perhaps, the virus present or circulating in Malaysia possessed a different N gene sequence than the one targeted by the primer for nested RT-PCR as referred to the WOAH list, about 12 species of virus under the genus Lyssavirus had been distinguished currently (WOAH, 2022).

Specific primer for rabies plays a crucial role which was designed to amplify rabies nucleocapsid (N) sequence in successful nested RT-PCR for future rabies detection. Furthermore, real-time PCR may be a very useful diagnostic tool in the future due to its fast detection and not requiring the presence of live viruses.

Besides FAT, histopathology, and nested RT-PCR, a few other methods of detection of rabies antigen are available such as real-time polymerase chain reaction (qPCR) (Nadin-Davis *et al.,* 2009). Besides that, cell culture tests using neuroblastoma cells as well as BHK cells have also been established (WOAH, 2022). Mice inoculation tests could be conducted accompanied by animal care and use committee approval (Sarah *et al., 2008)*. Another method for the detection of rabies antigen known as rabies immunoperoxidase antigen detection (RIAD) can also be applied especially in laboratories without a fluorescent microscope facility (Ibnu *et al.*, 2017).

Besides early and accurate diagnostic testing, rabies control programs play a major role in controlling the spreading of the disease (Baker, 2015). In Malaysia, immune belt areas referring to a 10 km radius bordering Thailand were set up to control the spreading of rabies from the neighbouring country that is already endemic to rabies (Loke *et al*., 1998). All dogs within these immune belt areas must be vaccinated against rabies (Navanithakumar *et al.*, 2019). Countries like the Philippines, Namibia, Haiti, and Tunisia have demonstrated that vaccination could reduce rabies infection in dogs (WOAH, 2022). A dog population control program needs to be established to prevent further infection. As such, a proactive public awareness campaign for dog-biting cases is needed to obtain fast and accurate treatment. These are to ensure the infected saliva does not transmit the virus through peripheral nerves ascending to the brain and eventually start the transmission of the Lyssavirus.

CONCLUSION

In conclusion, neither histopathology nor nested RT-PCR could produce good sensitivity results. Meanwhile, both histopathology and nested RT-PCR produce high specificity compared to FAT. Other alternative tests in conjunction with both of these rabies diagnostic tests should be established for future rabies investigation.

CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare related to this article.

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