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Screening of Rabies among Dogs in Kelantan Using Direct Fluorescence Antibody Test (DFAT)

Authors Names	ABSTRACT
<p><i>a-Imad Ibrahim Ali Al-Sultan</i> <i>b-Fawzia Jassim Shalsh</i> <i>c Abdul Rahman Bin Aziz</i> <i>d Salahudeen Muhamad</i></p> <p>Article History</p> <p>Received on: 4/6/2021 Revised on: 22/6/2021 Accepted on: 11/7/2021</p> <p>Keywords:</p> <p>Direct Fluorescent Antibody Test, rabies.</p> <p>DOI: https://doi.org/10.29350/jops.2021.26.4.1326</p>	<p>Inarguably, in several developing and in-transition countries, human are threatened by the occurrence of rabies in domestic dogs and it is posing a major concern. However, the infection of rabies in domestic livestock could definitely have economic consequences. This study aimed to determine the occurrence of rabies among dogs in Kelantan by the use of Direct Fluorescent Antibody Test (DFAT) to determine presence of rabies infection in dogs. The detected rabies cases were 12.5% (1 out of 8 samples). The finding might be inconclusive due to small sample size. It was generally difficult to obtain a satisfactory large number representing high sample size. The positive control used in this research work is made up of inactivated rabies vaccine (Rabisin®) whose antigenic determinant is the viral glycoprotein. It was sufficient to be used although the use of live rabies virus for inoculation in brain tissue smear would be a better option for positive control. The finding from the fluorescence microscopy examination reveals a good brilliant green fluorescence of high grade (+4) for the positive control. The results of the samples show all negative at conjugate dilution 1:100</p>

1. Introduction

Rabies is an infectious viral disease that is probably the oldest disease of zoonotic importance ever recorded in history. In more than 99% of human cases, the rabies virus is transmitted by domestic dogs. Domestic and wild animals are affected. Rabies may spread to human by getting bitten or scratched contaminated with infected saliva. It is caused by the virus genus *Lyssavirus* from the family *Rhabdoviridae*. It has a negative sense single-stranded RNA (ssRNA) with a nucleotide sequence of approximately 11-15 kb in molecular length (13). They are approximately 45–100 nm in diameter and 100–430 nm long, and consist of a helically coiled cylindrical nucleocapsid surrounded by an envelope with large (5–10 nm in length) glycoprotein spikes. The precise cylindrical form of the nucleocapsid forms the distinctive bullet or conical shape (7). Five structural proteins are encoded by the RNA: a nucleocapsid (N) protein, a phosphoprotein (P), a matrix (M) protein, a glycoprotein (G), and an RNA-dependent RNA polymerase (L) (8). All mammals are susceptible to rabies, but only a limited number of species also act as reservoir hosts. They include members of the families *Canidae* (dogs, jackals, coyotes, wolves, foxes and raccoon dogs), *Mustelidae* (e.g., skunks), *Viverridae* (e.g., mongooses), and *Procyonidae* (raccoons), and the order *Chiroptera* (bats) (16). Saliva of an infected animal is the primary mode of transmission caused by bite wounds that has rabies virus in its saliva (7). It may also occur when cut wound or mucous membrane comes into contact with infected saliva.

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Transmission via inhalation has been reported from bats (4). The virus will either replicates in non-nervous tissue or directly enters peripheral nerves (11). According to Greene et al. (8) at this stage the incubation period varies reportedly ranging from 3 to 24 weeks (average 3 to 8 weeks) several days to months. The viral load in the inoculum as well as the proximity of entry site from the brain also influence the disease progress (11). Once the virus enters the peripheral nerves, it then moves via retrograde axoplasmic flow up to the CNS at the rate of 10-400mm per day into the brain (8). The virus multiplies vigorously inside the brain tissue which in turn causes the primary rabid clinical. Then, it will transmit along the nerve tissue down into the salivary gland where it will continue its transmission via salivary shedding to the next host. Terminal stage of the disease is death within a few days of clinical signs onset. Furious form usually occurs within 1-7 days. Dogs will show signs of aggressive, excitable, restless and anxious behaviour. They become hyperaesthetic with vicious respond towards auditory and visual stimuli. Muscle incoordination, disorientation, and generalised grand mal seizure soon develop and the dog would eventually die (9). Diagnosis rabies is often suspected because of neurological symptoms in an affected animal. However, because of the recognised atypical nature of the clinical signs, rabies should be in the suspected list in any animal that suddenly develops unusual behavioral changes or features of LMN paralysis, or both. Demonstration of lyssavirus antigen by the direct fluorescent antibody test (DFAT) test in suitable brain tissue of dead animal is conformatory diagnostic test (8). It is rapid and reliable diagnostic test. According to (2) the sensitivity and specificity of FAT are 98.26% and 97.29% respectively. FAT is based on the concept that the virus is in the neurone and in particular the brain unlike many other virus tests. The brain stem, cerebellum, cerebrum, hippocampus, medulla, thalamus and are the paramount areas of the brain for examination (3). Rabies suspected brain tissue is added with fluorescently-labelled anti-rabies antibody. The antibody theoretically binds to antigenic site of the rabies virus (nucleoprotein or glycoprotein). Unattached antibodies will be rinsed away (2). The antigen-antibody bound areas will be seen as a brilliantt fluorescent apple green color when observed with a special fluorescence microscope. No stain is indication of negative result (15). (Fig. 1)

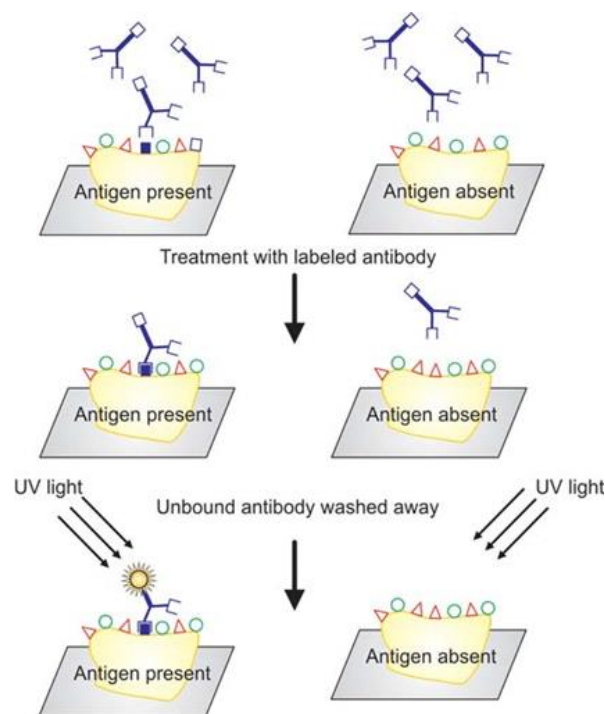


Fig. 1 Direct fluorescent antibody test (DFAT) (15).

Other rabies virus antigen detection include immunochemistry, rapid immunodiagnostic test (RIDT), and enzyme-linked immunosorbent assay (ELISA). Apart from that, cell culture and mouse inoculation

can be done to identify the replication of live rabies virus. Molecular identification and histological identification of characteristic cell lesions may also be elected (1).

Rabies ranks 12th on the WHO list of major killer diseases (13). As an OIE list B disease, rabies remains an ongoing threat to human populations and animals. Rabies is estimated to cause 35,000–60,000 human deaths worldwide each year, and an estimated 10 million people had post-exposure treatments (7). Rabies is present on all continents with the exception of Antarctica, but more than 95% of human deaths occur in Asia and Africa. Malaysia, located in Southeast Asia is surrounded by countries endemic with rabies such as Thailand, Indonesia, and Philippines. Malaysia was previously declared free from rabies in 2013. However, an outbreak of rabies infection had occurred among dogs on July 2015 (5). The little epidemiological data on the occurrence of rabies outbreak in Kelantan prompted the need for similar studies to be carried out. This study aimed to determine the occurrence of rabies among dogs in Kelantan by carry out Direct Fluorescent Antibody Test (DFAT) to determine presence of rabies infection in dogs.

2. Material and method

2.1 Sample collection

Sampling was done in Kota Bharu, Kelantan. Collected from six dead stray dogs necropsied at the Faculty of Veterinary Medicine University Malaysia Kelantan send by the Department of Veterinary Services Kelantan. Dogs were captured and euthanized in two separate operations were conducted in Industrial Area and downtown. Another 2 samples were retrieved from necropsy case from Pathology Department, Faculty of Veterinary Medicine UMK. All samples are kept fresh in -18 °C freezer prior to use. Study was conducted in February 2016 until May 2016

2.2 Fluorescent antibody test

In this study, the FAT protocol by (10) was employed. Firstly, the slides are prepared by making two circles demarcation of approximately 1 cm in diameter with a pencil on a labelled slide. Next, specimen preparation is done by impression of a fresh section of hippocampus and medulla oblongata or any other available brain material was made by lightly pressing the slide on the brain pieces. It is then air dried. Positive and negative controls are included in every staining session; they are prepared and fixed regularly. Next, the slides are then fixed in 100% pure cold acetone at -20°C for 30min. After that, the slides are removed from the acetone, air dried and allowed to reach room temperature. Next is staining with antibody conjugate with its working dilution onto the smears within each of the circles. The slides are placed in a humid chamber and incubated at 37°C for 30min. After that, they are rinsed in 1X PBS for 5 minute and repeated once. After draining the slides, they are cover with mounting medium 50% glycerol. Controls and specimen slides are examined using x20 lens with a total magnification of 200X. Specific apple green fluorescence of bound conjugate identifies positive aggregates of glycoprotein of the rabies virus. Staining intensity is graded from +4 to +1. Positive control slides in all tests should always contain staining of +4 intensity (a glaring, apple green brilliance). For each area of the brain examined, staining is graded by the amount of antigen present as follows:

+4, a massive infiltration of big and small inclusions of varying shape in almost every field of view of the impression.

+3, inclusions of varying size and shape are found in almost every field of view, the number of inclusions per field varies, but inclusions are mostly numerous.

+2, inclusions of varying size and shape are present in 10% to 50% of the field of view and most fields contain only a few inclusions.

+1, inclusions of varying size and shape are present in <10% of the field of view and only a few inclusions are found per field (usually only one or two inclusions per field). Determination of positive fluorescence result is only at grade +3 and +4.

3. Results

In this study, one out of 8 brain samples show positive for rabies at both 1:10 conjugate dilutions (Table 1). All sample shows negative result when stained with conjugate at dilution of 1:100. A result of negative is considered for slides which fluoresce at grade +2, +1, and fluoresce other than brilliant apple green e.g., yellow. In the positive control slide (Fig. 2) under the 400X magnification fluorescent microscope, at several microscopic field, there are presence of multiple dust-like particles of $< 1 \mu\text{m}$ in diameter, and there are also large, round to oval masses (red arrows) and strings 2 to $10 \mu\text{m}$ in diameter. This determine that, when specifically stained with an FITC-labelled antibody, these inclusions which is the vaccines antigens used in the positive control appear smooth, with very bright margins, and a somewhat less intensely stained central area. Observations made for each test slide are recorded as staining intensity and antigen distribution. Meanwhile, in the negative control slide (Fig.3), the bright green fluorescence is absence, which mean the conjugate was not picked up or washed away due to absence of vaccine antigen for the FITC to spike on.

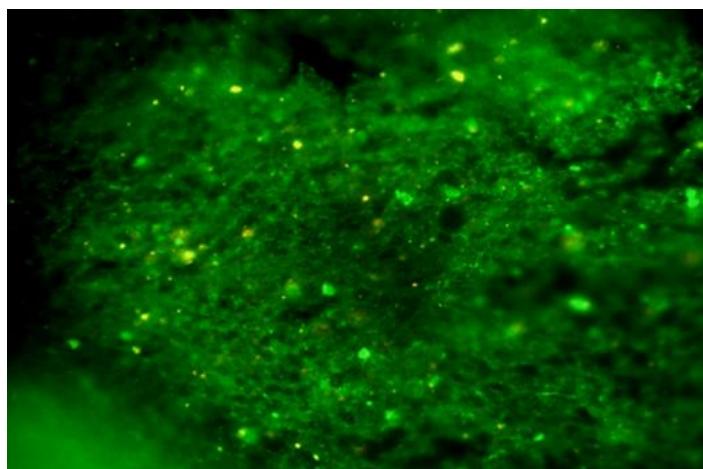


Fig. 2 Positive Control 1:10 (400X)

Table 1: Result of DFAT on brain dog samples as graded by staining with different dilutions of conjugate.

Slide	Staining Intensity Grade		Interpretation
	1:10	1:100	
Positive control	+4	+4	Positive
Negative control	-	-	Negative
jpv 1	+1	+1	Negative
jpv 2	+3	+1	Positive
jpv 3	+2	+1	Negative
kb 1	+2	+1	Negative
kb 2	+1	+1	Negative
kb 3	+2	+1	Negative
p28	+1	+1	Negative

p33	+2	+2	Negative
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Table 2. Detection of Rabies Using DFAT with conjugate dilution 1:10

SampleNo.	Positive (%)	Negative (%)
8	1(12.5%)	8(87.5%)

Table 3. Detection of Rabies Using DFAT with conjugate dilution 1:100

SampleNo.	Positive (%)	Negative (%)
8	0 (0%)	8(100%)

4. Discussion

The occurrence of rabies in this study is at 12.5% (1 out of 8 samples). The overall finding might not be conclusive due to several factors. The first aspect of discussion is the small sample size of 8. It was generally difficult to obtain a satisfying sample size because the scarcity of stray dogs in Kota Bharu relative to regions in the west coast Malaysia. The planned sample size was 30 dog brains. Furthermore, Department of Veterinary Services Kelantan did not do regular culling program for controlling of stray dogs in Kelantan hence no consistent large number of dog sample was readily available. Next Issue to discuss is the technical possible errors during the application of DFAT procedure. According to (16) the DFAT requires the use of FITC which binds to the nucleocapsid antigen of the rabies virus, in this study FITC used is a polyclonal antibody that binds to viral glycoprotein. This attempt may yield different result as oppose to FITC that binds to nucleocapsid but the possible significance difference is unknown. It is also recommended to use minimum two types of polyclonal FITC antibody conjugate for better comparison justification.

Next, the positive control used for this study is made up of inactivated rabies vaccine (Rabisin®) whose antigenic determinant is the viral glycoprotein. This is sufficient to be used although the use of live rabies virus for inoculation in brain tissue smear would be a better option for positive control. According to CDC (2015) during the fixation process of brain smear with 100% cold acetone, positive control slides are recommended to be process in a different container with the sample slides because false-positive result may be contributed by the cross transfer of tissue between slides in a common container. During the study a same container was used for fixation of slides. A counter stain is recommended but optional. It is used by adding it to the working solution of the conjugate to provide contrast and lowering background intensity. It also serves as a marker for accidental omission of diagnostic reagent (17,1). During the interpretation of the slides, a minimum of two readers is advised to avoid individual biased. The finding from the florescence microscopy examination reveals a good brilliant green fluoresce of grade +4 for the positive control (Table 1). However, the negative control slide also fluoresces but the color is more of yellowish in nature. It was interesting to note that a direct examination of empty clean slide also revealed some degree of yellowish fluoresce of dirt and debris(14). Hence the presence of fluoresce other than the characteristic brilliant apple green color is justified as negative finding in this study. The results of the samples show all negative at conjugate dilution 1:100. (Table 3). The outcome of the result may be also influence by the fact that different dogs may naturally incubate different amount of viral load. However, according to (8) the incubation period of rabies in dogs may range from 3 weeks up to 24 weeks (average 3-8 weeks). Hence, the viral progression might not reach the brain completely.

5. Conclusion

In conclusion, the current study shows that the occurrence of rabies among dogs in Kelantan is existed at 12.5%. The Direct Fluorescent Antibody Test (DFAT) can be used to determine presence of rabies infection in dogs.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript

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