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Attempts to Manipulate Direct Fluorescent Antibody Test for Detection of Rabies Virus Antigen in Mouse Brain

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Abstract

Direct Fluorescent Antibody (DFA) Test for detection of rabies antigen was carried out with attempts in manipulating the standard protocols based on OIE. This study was aimed to achieve improvements of a more rapid detection by conducting modification that could help in reducing the time consume, and costs for obtaining results. The main purpose of this study is to modify the routine protocols for rapid viral detection of rabies virus in tissue. The manipulation that had been practiced was mainly on the fixation procedure with high grade cold acetone, temperature and time of incubation. The working dilution of the Fluorescein Isothiocyanate (FITC) was (10-1, 10-2, 10-3). Experimental trial on brain samples from 10 rats were obtained and spiked with highest concentration of rabies vaccine for the study. The staining intensity result was analyzed and calculated using T-test. The results indicate a variety of intensity for staining and grading of the fluorescent based of the manipulated protocol conducted.

Keywords: Direct Fluorescent Antibody Test, rabies, fluorescent staining grading

Introduction

Rabies is an acute viral inevitable zoonotic disease known worldwide that affects the central nervous system of animals and humans [1] It is known to be one of the longest known infectious diseases in human history since 2300 BC and in 1880s when Louis Pasteur identified the virus as the cause of the disease [2,3] The disease is caused by a neurotropic virus of the genus Lyssavirus of family Rhabdoviridae [4]. The virus is an enveloped single stranded negative sense RNA genome that encodes five genes; nucleoprotein (N), glycoprotein (G), matrix protein (M), phosphoprotein (P), and viral RNA polymerase (L). The most important proteins are the glycoprotein (G) and nucleoprotein (N) where by the glycoprotein is present in the projections of virions and stimulates the neutralizing antibodies however the nucleoproteins are group of specific antigens [5]. Negatively stained Rhabdovirus as seen through an electron microscope. Notice the bullet shape of the virus (A). See the "bee hive" like striations of the RNP (B). Notice the glycoprotein spikes in the outer member bilayer (C) Figure 1.

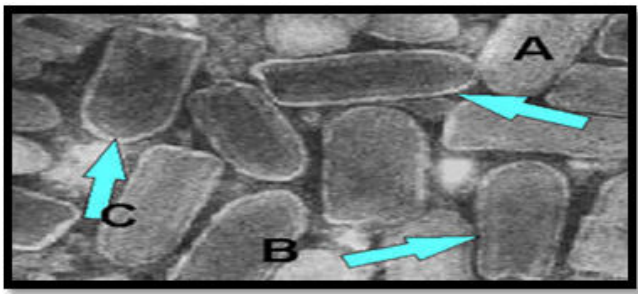

Figure 1: Rhabdovirus on electron microscope. (A) Shows bullet shaped virus, (B) bee hive like striations. (C) Glycoprotein spikes [5]

The classical rabies virus is Lyssavirus type 1 however there are another six rabies related viruses and others. Lagos bat virus (type 2), Makola virus (type 3), European Bat Lyssavirus 1 and 2 (type 5 and 6), Duvenhage virus (type 4), and Australian Bat Lyssavirus (type 7) [7].

Rabies is a public health concern in Malaysia due to the sporadic introduction of disease through infected dogs crossing over from Thailand along the Malaysian Thailand border [8,9]. Dogs appear to be the main reservoir of rabies in Malaysia and not much significant reservoir of rabies infection in wildlife [10]. Appropriate to its substantial importance of disease affliction and economic impact as to public health, Rabies disease is one of the many zoonotic diseases listed in the World Organization for Animal Health (OIE) Terrestrial Animal Health Code and any cases should be reported to OIE Terrestrial Animal Health Code in order to ensure public health safety [11] The epidemiology of rabies is complex and differs in each continent to country depending on the fauna or environmental factors and state of development. It is known that rabies most commonly occurs in the 3rd world countries in Asia and Africa due to the fact there is high population of stray dogs that serves as the virus reservoirs with more than 30000 of human cases each year [12].Thus, assumption has been made that more unreported case occurred in Asia and Africa [7]. Mode of transmission is commonly by the bite of infected animals as the virus is predominantly present in the saliva and the brain of infected animals most often in dogs [13].The incubation period for rabies contrasts, and considered to be weeks to six months however the infective period for dogs, cats is considered to start ten days before the onset of the primary clinical signs. Once symptoms are present, rabies are fatal in both animals and humans [14,15].

As for the pathogenesis firstly the organ of affinity of the virus is the brain. From the point of entry which is from the bite wounds, the virus will start to replicate at site of infection and binds to Nicotinic Acetylcholine (NAC) receptors at peripheral nerve terminals to enter and spread [7,2]. There is no evidence of transmission or distribution through hematogenous route. It will then reach into the spinal cords and lastly to the brain. The virus within the CNS will travel again but this time through anterograde and retrograde that will alter the mood of the infected animals seeing them being furious stage or dumb/ paralytic stage [16]. There are many methods in diagnosing rabies such as the use of central nervous system tissues which is detected using fluorescent antibody test (FAT) known to be the gold standard test most frequently used and approved by both WHO and OIE

[17,19]. Others include Mouse Inoculation Test (MIT), samples like CSF, saliva are inoculated into the mouse under specific conditions and observed approximately 30 days and confirmed by extracting the brains [17]. Also, Direct Rapid Immunohistochemical Test (DRIT), immunochromatography, RT-PCR protocols, Rapid Latex Agglutination Test, ELISA have been used to aid in detection of rabies throughout the years (20,5). With the most frequently and most reliable method of Direct Fluorescent Antibody Test (DFAT) the results of the test using immunofluorescence technique on fresh brain smears showed evidence of the rabies virus Nucleoprotein Antigen (N). However, the diagnostic test conduct takes quiet sometime to obtain the result therefore modification on some aspect of managing the test need to be improved to obtain a more rapid result of the infected animal or person for treatment and prevention control measures to be carried out. The DFAT is known to be sensitive, specific and cheap though, the sensitivity of the DFAT depends on the specimen's degree of autolysis and how meticulously the brain is sampled (21). The methods sensitivity and specificity also depend on the type of Lyssavirus and the competency of the diagnostic operation conducted [17,22]. Glycerol-preserved can be applied to the DFAT specimens after the washing step. Specimens preserved in formalin solution may only use the FAT after the specimen has been treated with a proteolytic enzyme [23]. However, the DFAT fixed with formalin and digested samples are always less consistent and more cumbersome than when performed on fresh tissue [24]. In circumstances of unsound outcomes from DFAT, it is recommended to conduct additional tests on the same sample or repeat FAT. It is important in order to determine the confirmatory or suspected autolysis of the sample that could disrupt the results or test conducted.

Aimed of this study was to achieve set of objectives that were to determine an attempt for improvements and modification of the Fluorescent Antibody Test (FAT) procedures to ensure a more reliable detection and to manipulate or modify the factors used in the standard test for better outcome and rapid results of detection.

Materials and Methods

Sampling and data collection

Approximately 10 rat brain samples were obtained and collected accordingly as the brain sampling collection was done by opening the skull of the rat and gentle removal of the brain. The location of the study was conducted in the Faculty of Veterinary Medicine - University Malaysia Kelantan that had been conducted from March up till May 2016

Identification of rabies virus antigen

Direct Fluorescent antibody Test (DFAT)

Antisera and conjugates

The Anti-rabies fluorescent conjugates are available commercially either polyclonal or Monoclonal Antibodies (MABS) which is specific to the whole virus or to the rabies nucleocapsid protein, conjugated to a fluorophore such as fluorescein isothiocyanate (FITC) [24]. The conjugate of 25□g was diluted to 500 □L as a working stock and diluted again to obtain working dilution. (10^{-1},10^{-2},10^{-3}) was the working dilution for the conjugate by using 60 □l of stock added with 540 □l PBS and diluted accordingly to the concentration markers. Each working dilution was then placed in -4 °C refrigerator and used within a week. Fresh dilution was made each week until samples were stained. Working stock was kept in -20 °C.

Microscopic equipment

Examination of the FAT slides require specific usage of fluorescent microscope with appropriate filter in order to obtain sufficient wavelength of the fluorescent conjugate to be used. The wavelength is excited at 490nm and re-emits at 510 nm. The nucleocapsid protein aggregates was examined and identified by the specific fluorescence of bound conjugates [24].

Direct Fluorescent antibody test methodology

Slide preparation

The glass slides were cleaned and degrease with two circles of approximately 1 cm in diameter was drawn on the labeled slide for smear area.

Specimen preparation

The brain material tissue collected and added killed rabies vaccine to enable presence of antigen in the samples. One vial of the rabies vaccine contains /1ml virus and the preparation of brain sample were done by mixing the brain tissue samples with vaccine. One hundred microliters of vaccine were spiked into 1000mg of brain sample which made the concentration of virus 104/1 mg brain sample. The samples were then lightly pressed on the slide using wooden tongue depressor and dried with absorbent papers. Positive and negative controls were also prepared and included in each staining session. On days the specimen is unable to be examined, it is stored in freezer (-20 °C) and unstained.

Acetone fixation

After smearing the samples on the slides, the slides were air-dried thoroughly and a 100% high grade cold acetone was used. The smears were placed in containers containing cold acetone fully submerge the slides in the container and placed in -20 °C for 2, 5 and 10 minutes respectively.

Conjugate FITC staining

Slides are removed from acetone and air-dried to reach room temperature. Working dilution of the conjugate prepared were added and layered onto each area of the smears and placed in the incubator at 39 °C for 10 and 15 minutes. PBS of pH 7.2 to 7.4 is used to rinse the slides and thorough draining was done to ensure excess stains washed off. A drop of mounting medium 50% glycerol. Prepared was placed onto the smears and slides were arranged for reading.

Reading

Both control and specimen slides were observed using fluorescent filter microscope within 2 hours to obtained accurate results. Findings of staining intensity and amount of antigen present were conducted by grading from +4 to +1 Table 1.

Staining intensity	Description
4	High glare, apple green brilliance, abundant antigen in every field and not possible to count amount per field.
3	Staining intensity slightly diminished, less glare, abundant antigen with

	one or more particles per field and able to count
2	Dull stain with scanty antigen with one or more particles in few than 100% field but more in 50% field
1	Noticeably dull stain, antigen very scanty, one or more particles in fewer than 50% of microscope fields
Negative	No presence of antigen throughout whole smear/field

Table 1: Direct Fluorescent Antibody Test (DFA) staining intensity grading (25).

Statistical Analysis

The results analyzed by using Linear Model General by SAS ready program to study the effect of factors according to complete random design (CRD) also Duncan test was done to defining the significant differences between means of effected factors on parameters at levels $P<0.05$.

Results

The impression (smears) slides examined under fluorescence technique were evaluated on the basis of fluorescence intensity of rabies virus. The results revealed a positive FAT with evidence of foci areas on smears indicating presence of viral antigen-antibody binding as fluorescence apple green Figure 2.

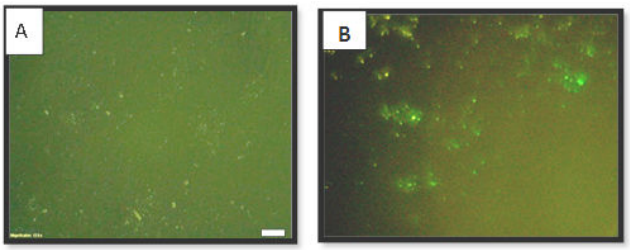

Figure 2: Impression smear of brain tissue showing positive result for FAT.

A-positive result, B-positive result

The effect of fixation and incubation time modification showed positive outcome (Figures 3 and 4) of detecting virus antigen with complete correlation with the standard DFA protocol as in OIE observation showed large oval to small dust like particles within the samples smear areas. Through the rapid modification method, the intensity of the stain and amount of antigen detected were lower than the standard method (Figures 3 and 4).

Different groups of dilution factor with time settings were used and calculation revealed with the highest dilution factor of 10-1, acetone fixation of 10 minutes and time set for 15 minutes incubation had highest intensity of positivity comparatively (Figure 5). The fluorescence stain varied in size and intensity which ranged from +1 being the lowest to +4 as the highest grade.

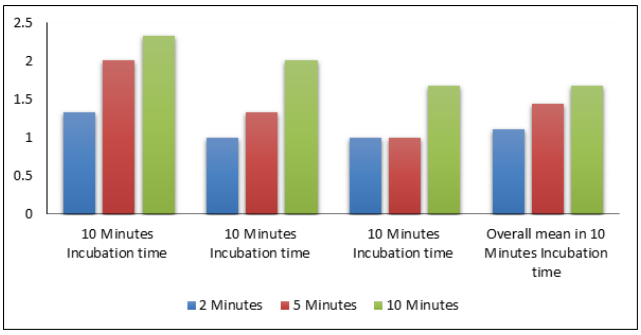

Figure 3: Result of staining grading intensity within Group 1 (incubation at 39°C for 10 min)

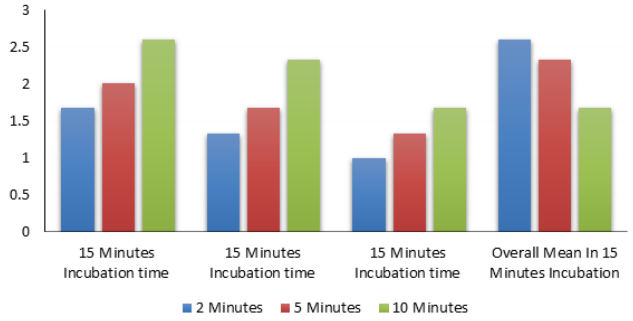

Figure 4: Result of staining grading intensity within Group 2 (incubation at 39 °C for 15 min)

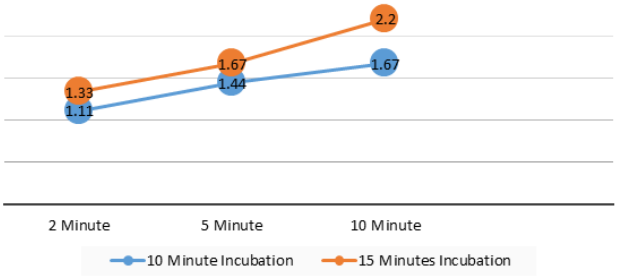

Figure 5: Comparison of staining intensity with different time intervals for FA test

The mean incubation for 10 minutes with different set fixation time is summarized in (Table 2). Fixation time of 2 minute showed the least intensity with 1.11 + 0.110 followed by 5 minutes with 1.44 + 0.110 and 10 minutes with 1.67 + 0.1905. All groups revealed significant difference of P-value 0.0011, 0.0085, and 0.0141 respectively.

Group 1	Mean ± SE
2 Minutes acetone fixation	1.11 ± 0.110
5 Minutes acetone fixation	1.44 ± 0.2942
10 Minutes acetone fixation	1.67 ± 0.1905

Table 2: Smear intensity grading in 10 minutes incubation time

The mean incubation for 15 minutes with different set fixation time is summarized in (Table 3). Fixation time of 2 minute showed the least intensity with 11.33 + 0.1934 followed by 5 minutes with 1.67 +

0.1934 and 10 minutes with 2.20 + 0.2762. All group revealed significant difference between each group with the p-value 0.0031, 0.0062, and 0.045 respectively.

Group 2	Mean ± SE
2 Minutes acetone fixation	1.33 ± 0.1934
5 Minutes acetone fixation	1.67 ± 0.1934
10 Minutes acetone fixation	2.20 ± 0.2762

Table 3: Smear intensity grading in 15 minutes incubation time

Based on the result revealed in this study and data analysis, it is shown that the results fluctuate with the use of various conjugate dilutions along with different fixation time intervals (2, 5 and 10 minutes). Increase in time of fixation, incubation and high concentration of diluent factor will result in increased staining intensity.

Discussion

In this study, we have a successful trial and evidence that positive rabies antigen can be detected using direct FA test first. The second attempt was in manipulating the standard protocol for FA test which was conducted and lead to positive presence of various fluorescent staining intensity according to the procedure of manipulation done with various working dilution of FITC [22].

The manipulation conducted from the standard protocol, showed positive intensity of fluorescent staining from the sample tested in all working dilution with different fixation and incubation periods duration time settings (Fig. 5 and 6). This is due to the fact that each sample in this study was impregnated with vaccine in order to obtain virus antigen within the brain tissues for detection using FA test as no positive case sample were obtained during the study. The grade of intensity is not consistent, somewhat varies and lower from the standard protocol depending on the modification of fixation time, conjugate dilution and incubation time similarly reported by [26] in previous attempts of modification.

This study revealed that with modification of 2 minutes acetone fixation and 10 minutes incubation, there is evidence of low staining intensity with +1 in all dilutions indicating stain is dull with scanty amount of antigen and within 50% microscope field less particles are observed and interpreted as in (Figure 5). Besides that, in 5 and 10minutes acetone fixation showed slight increase in stain intensity with some trials within average of +2 and few +3 and with indication of slightly diminished glaring of stain when abundant antigen per field was able to be counted.

In comparison with the standard protocol of 30 minutes acetone fixation with 30 minutes incubation at 37°C, our study was able to obtain positive outcomes from the modification protocol despite the inconsistent stain intensity due to the presence of rabies virus antigen in the tissue with increase temperature for incubation that generally increases the maximum level of reaction of antigen antibody binding time and multiplication process [6]. In addition to that, increase in temperature could result in more rapid reaction of agents that could shorten the time of reaction in normal protocol however the temperature should not exceed 40°C as thermal deactivation of organism can occur leading to inaccurate result [27]. As for incubation time setting for 15 minutes, evidence of increase staining intensity depending on dilution factors used were observed with high amount +2 and +3 respectively (Figure 6). In comparison with the normal standard OIE protocol of FA test, most staining observed in positive cases are within +4 intensity indicating true and high positive value of the antigen present [28].

Based on the results,there is variation in findings that show high concentration of working conjugate and increase in fixation and incubation time that resulted increase in staining intensity thus the grade reaches +2 to +3 with the criteria of dull stain with scanty antigen of one or more particles in few than 100% held but more in 50% field (+2), slightly diminished stain with less glare, abundant antigen with more particles per hield able to count (+3) compared to OIE gold standard protocol for rabies virus detection obtaining +4 grade. In accordance to the above statement, the technique used in this study is vaccine impregnated tissue resulting in a not genuine or classical case of rabies that might be one of the reasons of unable to obtain high number of +4 staining grade. It is also mainly due to insufficient or no thorough specific working dilution protocol to be used in the DFAT for rabies virus whereby it is usually through try, error and determined by the end user of the experiment conductor (29). Grading of the staining intensity is highly subjective therefore required more than 2 personnel to independently evaluate the slides but the grading was only done by 1 person in this study.

Technical skills are to be highly emphasized in the success rate as it depends largely on how procedures are being carried out with the appropriate preparation especially fluorescent conjugates (FITC) working dilution factors and conduction of the FA test itself [30]. Reagents, microscopic examination using fluorescent microscope is commonly advisable to be done with experience personnel in attempts for the test [31].

Although FAT is widely known to be sensitive and specific for detection of rabies, recently new test has been used in several diagnostic laboratories using direct Rapid Immunohistochemical Test dRIT for detection of rabies with similar sensitivity and specificity as per FA test Accordingly, dRIT has been studied to be useful for rapid detection of rabies virus under various circumstances and usage of each diagnostic tool depends on preferences of the personnel and rapidity of the test with accurate result [32,35]. It is highly important to ensure the efficacy of each test is well determined and tested on field to be evaluated of its potential.

Presence of positive outcome from the modification of fixation and incubation settings suggests that the DFA test can be modified into a more rapid detecting kit for rabies virus antigen based on the study theory with the significant of the p-value of each group study and further studies can enable the usage and calculate sensitivity and specificity of this modified test and other test such as dRIT.

Conclusion

In conclusion, the alterations, modification attempts in the standard protocol for FA test in diagnosing rabies antigen resulted in obtaining potential positive results with inconstant and slightly lower grading from the standard OIE protocol. These circumstances highly due to the inadequate time of thorough conduction and professional personnel looking into the study. There is no thorough or specific working dilution of the protocol as it is mainly determined by the end user. Grading system used was also subjective and questionable at times therefore further intensive study can be done in future for a more conclusive test.

Recommendation

A more intensive study and wider application of method suggested obtaining more specific and accurate result for a more rapid test to be used in future. Determination of whether the standard protocol for diagnosis of rabies used currently is able to be replaced with a more sensitive and rapid detection test.

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Conflict of interests

The authors declare no conflict of interest.

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