Research Article

Evaluation of Direct Rapid Immunohistochemistry Test (DRIT) for Postmortem Diagnosis of Rabies

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Abstract

Direct fluorescent antibody test (DFAT) is considered as the gold standard for diagnosis of rabies in infected mammals as it has high sensitivity and specificity. However, high cost and technical demand limits its utilization, particularly in developing countries including India. Therefore, in this study we evaluated recently developed direct rapid immunohistochemistry test (DRIT) for diagnosis of rabies in comparison with the DFAT. A total of 109 brain samples received during the period of 6 years from different regions of India were tested following standard protocol. The results showed 100% correlation between the two tests.

Keywords: Rabies, Post-mortem diagnosis, Direct fluorescent antibody test, Direct rapid immunohistochemistry test

Introduction

Rabies is caused by single-stranded RNA virus (RABV) of the genus Lyssavirus of the family *Rhabdoviridae*. In India, it continues to be an important public health problem where an estimated 20,000 human deaths and 17.4 million animal bites are reported each year.[1] The primary vector of rabies in India is dog in over 95% of human cases but other animals like cats, monkeys, mongooses and wild animals also transmit the disease. Validated diagnostic tests that confirm the existence of rabies virus or a lyssavirus variant is the foundation of rabies control strategies in several countries.[2]

In past decades, wide range of methods have been developed for detection of rabies virus in clinical specimens.[3] Direct fluorescent antibody test (DFAT) is considered as the gold-standard test by the World Health Organization (WHO) and World Organization for Animal Health (OIE) for rabies diagnosis.[4] However, limitation of use of DFAT in developing countries is that the test is technically demanding and requires the use of a fluorescence microscope that is expensive and difficult to maintain.[5] Thus, there is a necessity for a rapid diagnostic test which has comparable sensitivity and specificity as DFAT, is economical, and can be adapted to field as well as laboratory conditions in resource-constraint countries.

At the Centers for Disease Control (CDC), USA, an easy and rapid method of rabies diagnosis has been developed to detect rabies virus antigen in fresh, fresh frozen, and glycerol-preserved brain tissue under the light microscope. The principle of immunohistochemistry has been used in which the rabies virus nucleoprotein (N) antigen within the brain smear is captured by a cocktail of biotinylated anti-N monoclonal antibody which is subsequently detected by color development.[2] This methodology is known as the direct rapid immunohistochemistry test (DRIT).

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The DRIT is an experimental method and in limited field trials done in Africa, China, Afghanistan, Iraq, South India, it has proved to be as specific and sensitive as the gold standard DFAT.[2,6,7] Therefore to further evaluate the utility of this test in diagnosing rabies in India, The Centers for Disease Control (CDC) USA, supplied the Rabies Diagnosis DRIT Kit and technical input to National Centre for Disease Control, Delhi, which is a WHO collaborating center for rabies epidemiology in India. Hence, this study was aimed to evaluate the Direct Rapid Immunohistochemistry Test (DRIT) in comparison with Direct Fluorescent Antibody Test (DFAT) for post mortem diagnosis of rabies.

Materials and Methods

Samples

As a WHO collaborating center for rabies epidemiology, National Centre for Disease Control, Delhi, provides laboratory services for postmortem diagnosis of rabies in mammals for Delhi and surrounding areas. The animal brain specimens, usually without preservative, received from various veterinary hospitals, government, non-governmental organizations and individual owners received from January 2011 to June 2016 constituted the material for the study, including 40 brain samples obtained from Central Research Institute, Kasauli, Himachal Pradesh, India.

A total of 109 brain samples from suspected cases of rabies from different mammals, viz., dog (55), spotted deer (36), cow (6), cat (5), buffalo (2), blue bull (1), monkey (1), sambar deer(1), squirrel (1) and human (1) were processed in the laboratory for detection of rabies by DFAT and DRIT as per the standard procedure. These specimens were received from 9 states and one Union Territory, viz., Delhi (64), Haryana (6), Utter Pradesh (2), Punjab (2), Chandigarh (2), Himachal Pradesh (10), Gujarat (2), Jammu & Kashmir (2), Manipur (18), and Sikkim (1). All brain samples were stored at −20°C until processed. The received brain specimens were found in different conditions and were differentiated as fresh (n=104) and partially decomposed (n=5) depending on the tissue condition.

Table 1.Results of DFAT and DRIT on Brain Samples for Rabies Diagnosis

Brain Sample Source (Test Number)	DFAT+/ DRIT+	DFAT+/ DRIT−	DFAT−/ DRIT+	DFAT−/ DRIT-	Correlation (%)
Dog (n=55)	35	0	0	20	100
Spotted deer (n=36)	23	0	0	13	100
Cow (n=6)	4	0	0	2	100
Cat (n=5)	0	0	0	5	100
Buffalo (n=2)	2	0	0	0	100
Blue bull (n=1)	0	0	0	1	100
Sambar deer (n=1)	0	0	0	1	100
Monkey (n=1)	0	0	0	1	100
Squirrel (n=1)	0	0	0	1	100
Human (n=1)	0	0	0	1	100

(− Negative, + Positive, DFAT – Direct fluorescent antibody test, DRIT – Direct rapid immunohistochemistry test)

Table 2.Comparison of Results of DRIT and DFAT for Rabies Diagnosis

		DFAT		Total
		Positive	Negative	
DRIT	Positive	64	0	64
	Negative	0	45	45
	Total	64	45	109

Processing of Samples

DFAT, a gold standard test, was performed according to WHO guidelines.[8] Briefly, the impression smears were made on labeled glass slides from hippocampus and/or other areas, depending on availability of the material. The smears were dried in the air and fixed with chilled acetone for 4 hours. For internal quality control (IQC), positive and negative impression smears were made from rabid and normal mouse brains respectively. The fixed slides were stained with rabies conjugated anti-nucleocapsid (Bio-Rad, France) and incubated for half an hour at 37°C in a humid chamber. Finally, the slides were examined under UV microscope (Zeiss) using a 40X objective. Brain smears showing green fluorescent dotted particles as punctuate foci of varying sizes within or outside neurons were considered as positive.

The DRIT kit (Centers for Disease Control, USA) was used as per the instructions given in the manual for the same samples as used in the DFAT.[9] Briefly, the touch impression smears were air-dried at room temperature (RT) and then fixed in 10% buffered formalin for 10 minutes. Fixed slides were washed thoroughly with tween phosphate buffer saline (TPBS; PBS with 1% tween 80) then immersed in 3% hydrogen peroxide (H_2O_2) for 10 minutes. Excess H_2O_2 was removed by dip-rinsing in fresh TPBS.

The slides were then incubated with monoclonal antibody cocktail in humid chamber for 10 minutes at RT, washed by dip-rinse in TPBS and then incubated with streptavidin-peroxidase complex for 10 minutes in humid chamber at RT. This was followed by washing with TPBS. Slides were then incubated with fresh peroxidase substrate, aminoethylcarbizole (AEC) with the working dilution in a humidity chamber at RT for 10 minutes. After washing with distilled water, these slides were counterstained with hematoxylin for 2 minutes. The slides were examined under light microscope (Olympus) using a 20X objective to scan the field and a 40X objective for higher power inspection of red inclusion body against a light blue background .

Result Analysis

The results obtained from both DFAT and DRIT were entered in a Microsoft Excel 2010 spreadsheet. Sensitivity was calculated using the formula [True Positive (TP)/(True Positive (TP)+False Negative (FN))]×100, where TP was the number of specimens with true-positive results as based on the reference test and FN was the number of specimen with false-negative results. Specificity was defined as [True Negative (TN)/(True Negative (TN)+False Positive (FP))]×100, where TN was the number of specimen with true-negative results and FP was the number of specimen with false positive results. Confidence intervals for sensitivity and specificity were computed with the online MEDCALC® easy-to-use statistical software.

Results

Of the total of 109 brain samples tested, 64 (58.71%) were positive by DFAT and DRIT and 45 (41.28%) were negative by both the tests (Table 1 and 2). When we compared the performance of DRIT with the DFAT, the highest sensitivity (100%, 95% CI: 94.40–100) and specificity (100%, 95% CI: 92.13–100) values of DRIT were obtained.

One dog and four spotted deer brain samples that were partially decomposed were found to be weakly positive by DFAT, but were strongly positive by DRIT.

In our observation, interpretation of a smear stained by DRIT is easier than by DFAT. In case of fresh brains, the ease of interpretation was similar in both the tests, however, with brains preserved in 50% glycerol saline; interpretation was much easier when the smear were stained by DRIT technique.

Discussion

The true beginning of rabies diagnosis was the description of inclusion bodies in the cytoplasm of nerve cells by Adelchi Negri in 1903. The use of Sellers stain for detection of intracytoplasmic inclusion bodies, i.e., Negri bodies, has sensitivity ranging from 53 to 75%.[10] This remained the mainstay of diagnosis for more than half a century and its gradual replacement by the DFAT after Gold Wasser and Kissling in 1958 described immune-fluorescent antibody technique, which was modified by Dena and Ableseth in 1973 and subsequently by Kissling in 1975.[11] This is the most widely used test procedure for diagnosis of rabies. Webster and Dawson developed Mouse Inoculation Test (MIT) for isolation of the virus,[12,13] which is considered to be an ideal test but yields delayed results. Other tests like Rabies Tissue Culture Infection Test (RTCIT),[14] Rapid Rabies Enzyme Immuno Diagnosis (RREID), Rapid Immunochromatographic diagnostic test and Reverse-transcriptase Polymerase Chain Reaction (RT-PCR)[15-17] have also been developed and standardized for the diagnosis. However, there is no single test which fulfills ideal test requirement.

In this study, a total of 109 brain samples from nine states of India were tested and we observed that DRIT had 100% sensitivity and specificity as compared to DFAT. Our results were comparable with other authors, who reported sensitivity and specificity of DRIT to be 100%.[7,18-23] DFAT is the “Gold Standard” for rabies diagnosis all over the world. It is the WHO and OIE recommended test for the diagnosis of rabies in fresh or frozen brain samples. However, in tropical countries preserving fresh samples at 4°C is often a challenge.[12,17] FAT is based on attaching fluorescein isothiocyanate (FITC) to polyclonal antibodies targeting the RABV ribonucleocapsid, or monoclonal antibodies targeting the RABV nucleoprotein (N), which is observed under high-priced fluorescence microscope.[4,7,13,24] The monoclonal antibodies used in DRIT are non-specific and recognize all variants of the rabies virus.[25] The sensitivity and specificity of DFAT nears 99% in an experienced laboratory, but is observer-dependent especially in weak positive and preserved and decomposed samples.[14,26] When compared with DFAT, the estimated sensitivity and specificity of DRIT approach 100%.[24,27] This correlation of the detection capacity of monoclonal antibodies in both tests might be the possible explanation for the high sensitivity and specificity of the DRIT.[28] As DFAT is best performed on fresh brain samples; the reliability of this assay to diagnose rabies in decomposed animal brain samples is low.[13] In such cases, the DRIT would be a superior test that is less sensitive to microscope issues. The sensitivity and specificity of the DRIT has been shown to be comparable with that of the DFAT, yet an overarching benefit of the DRIT is the ease of differentiation between a positive and a negative result by trained personnel.[29] This is because of the reduction in visible interfering background staining and the reduced reliance upon stringently calibrated equipment. Furthermore, archival samples are subject to degradation, resulting in a larger proportion of background fluorescence when using the DFAT.[16,30,31]

On the contrary, the DRIT does not rely on fluorescence and thus largely eliminates any potential misinterpretations

of results because of the limited interfering background staining caused by degraded or archival samples. In our study, one dog and four spotted deer brain samples, which were partially degraded, were weakly positive by DFAT, and strongly positive by DRIT.

DRIT is simple to perform though the numbers of steps are more than DFAT. In the DRIT, air-dried smear requires fixation for 10 min in formalin which inactivates the virus without affecting the antigenicity but in DFAT, air-dried smear requires at least two hours fixation in cold acetone.[32]

The earlier reports and our study demonstrate that the operational characteristics, including the time taken to perform the test, technical simplicity, user acceptability and the stability of the DRIT under laboratory condition were found to be better than that of DFAT.[7,18,19,24]

Furthermore, testing procedure of DRIT has distinct advantage over the DFAT, that it can be performed by laboratories other than the referral laboratories as a very adequate surveillance and screening tool in the field. Studies conducted in Chad, [27] Tanzania,[19] Iraq and Afghanistan[18] demonstrated the applicability of DRIT under field conditions, even at ambient temperature. Although further laboratory and field evaluations are required, our results highlight the potential value of the DRIT to improve laboratory-based surveillance for countries with limited diagnostic resources.[2,22] DRIT appears to be a promising, economical and rapid diagnostic test which gives comparable results with DFAT.

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Conflict of Interest: None

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