SPECIAL ARTICLE

Modified Direct Fluorescent Antibody Assay: A safe and rapid approach

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Abstract

Rabies, present in many parts of the world is regarded as under reported in many regions, due in part, to a lack of surveillance and laboratory infrastructure, confounded by cultural or social taboos. Globally, the Direct Fluorescent Antibody assay (DFA) is the gold standard test for rabies diagnosis. However, routine DFA fixation at -20/-80 ˚C requires a deep freezer which may not be available in several laboratories. In addition, DFA involves chilled acetone as fixative which does not completely inactivate the rabies virus (RABV), thereby posing a possible biohazard to the laboratory personal.

In the present study, the DFA was modified to eliminate the biohazard involved in the test, which doesn't involve the tissue fixation at -20/-80 °C but involves the usage of 2.5% neutral buffered formalin for ten minutes and subsequent washes with Tween PBS and H2O2 which significantly reduces the time involved. Viability of virus in acetone and formalin fixed slides were checked by inoculating BHK-21 cell with scrapings from impressions. There was 100% inactivation of RABV in formalin fixed tissues. Fifty samples were subjected to both DFA and modified DFA, out of which 38 and 12 were found positive and negative by both the tests respectively. Further, grading was done. The results were compared and analyzed by Spearman's nonparametric correlation analysis. The "Correlation coefficient (Spearman r)" was found to be 0.98 ($P<0.001$) indicating very good positive correlation. In conclusion, the modified DFA was found safe for the laboratory personal and rapid than the conventional DFA.

Key words: DFA, Modified DFA, RABV, BHK-21 Cells

1. Introduction

Rabies is a fatal viral disease that affects all warm blooded vertebrates. Despite the availability of vaccines to prevent this disease, it is still a significant public and veterinary health problem in many countries particularly in Asia and Africa (Meslin et al., 1994; Cleaveland, 1998; WHO, 1999; Knobel et al., 2005).

Rabies virus belongs to the genus Lyssavirus of the family Rabdoviridae. The genus is composed of rabies virus (genotype 1) and rabies-related viruses, including Lagos bat (genotype 2), Mokola virus (genotype 3), Duvenhage virus (genotype 4), European bat lyssaviruses 1 and 2 (genotypes 5 and 6, respectively), and Australian bat lyssavirus (genotype 7). The virus has a negative-sense single-stranded RNA genome of approximately 12 kb containing coding information for nucleocapsid (N), phosphoprotein (P), matrixprotein, glycoprotein (G), and RNA polymerase (L).

Although it is difficult to distinguish field isolates of rabies virus serologically because of a single serotype, rabies isolates can be distinguished by genetic analysis

There are new developments in rabies research for enhancing the efforts towards the overall goal of rabies elimination. Laboratory confirmation of rabies in animals is important for suitable control measures. Different methods have been employed for the diagnosis of rabies which include Direct Fluorescent Antibody test (DFA), direct Rapid Immunohistochemistry (dRIT), Lateral Flow Assay (LFA), Polymerase Chain Reaction (PCR), Loop Mediated Isothermal Amplification Assay (LAMP) and Mouse Inoculation Test.

The DFA being the gold standard test for rabies diagnosis by the WHO (Dean et al., 1996; WHO, 2005) requires a -20˚C deep freezer for routine fixation of the slide which may not be available in several laboratories. In addition, DFA involves fixation of the brain impression using chilled acetone which does not completely inactivate the rabies virus thereby posing a possibility of biohazard and studies have demonstrated that intracerebral inoculation of acetone fixed tissues can cause disease in suckling mice (Umoh and Blenden, 1981). White and Chappell (1982) found that impression smears made out of brain samples suspected for rabies were fixed in acetone at -20 °C for 2, 4, 7, and 24 h and were further examined for virus viability. Tissues scraped from impression smears after acetone fixation were examined for virus viability in BHK-21/WI-2 cell cultures Scrapings from these smears contained infectious virus. The infectivity titers ranged from 1033/0.1 ml in suckling mice to 1051/0.1 ml in BHK-21/WI-2 cultures but acetone fixation at 50 °C for 30 min inactivated rabies virus without altering the outcome of DFA. An examination using the routine rabies direct fluorescent antibody test was performed on rabies or Eastern equine encephalitis positive mammalian brain tissue to assess inactivation of the virus. Neither the virus was inactivated with acetone fixation nor the routine test. Thus laboratory employees should treat all samples as rabies and when appropriate Eastern equine encephalitis positive throughout the whole procedure (Jarvis, 2016).

In this study, gold standard DFA was modified where different concentrations of Neutral Buffered Formalin (NBF) were used as fixatives and compared. Suspected brain samples of animals collected from different parts of India were subjected to gold standard DFA and Modified DFA. Fifty preserved and freshly collected post-mortem brain samples collected from different Rabies suspected animals were subjected to both the tests. We observed 100 per cent inactivation of virus using 2.5 percent NBF which takes only 10 minutes instead of 30 minutes. There was 100% correlation between DFA and Modified DFA. This study enhances the use of Modified DFA as a simple test that can be adopted for diagnosis for Rabies.

2. Materials and Methods

2.1. Samples

Preserved and freshly collected post-mortem brain samples (n=200) from rabies suspected animals from different species viz., Canine-44, Bovine-02, Bubaline-2, Equine-01, Bat-1 from different geographical locations of India like Karnataka (n=42), Manipur (n=04), Rajasthan (n=01), Jammu & Kashmir (n=1), Gujarat (n=1) and Tamil Nadu (n=01) were used in the study (Table 4.1).

2.2. DFA Protocol

All the 50 samples were subjected to DFA and was done essentially following the method as described by the CDC, Atlanta.

2.3. The Modified DFA

Initially tissue impressions were fixed at 10 per cent Neutral Buffered Formalin (NBF). To remove formalin effect and to unmask the RABV antigen, Tris EDTA was used by immersing slide and heating in microwave over at 30°C for 30 seconds. Later tried with hydrogen peroxide and TPBS washes. Tissue impressions were fixed at different

concentrations of 5%, 2.5% and 1%. Neutral Buffered Formalin. Then impressions were stained with Rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) and examined under a fluorescent microscope at 200X and 400X magnification.

2.3.1. Protocol

1. Touch impression were prepared from brain tissue samples on microscope slides.
2. For each of the test samples, three impressions were made one each for the anti rabies nucleoprotein IgG- FITC conjugate (Millipore-Light Diagnostics, Rabies DFA III, Cat # 5600), negative control FITC conjugate (Millipore-Light Diagnostics, Cat # 5102) and normal goat serum FITC conjugate (Millipore-Light Diagnostics, Cat # 5202).
3. A known healthy brain sample (as negative) was also included in the test as an internal control.
4. The smears were blotted onto paper towels to remove excess of moisture, tissue remains and the blood stains.
5. The smears were initially air dried for 5 min.
6. Impressions were then fixed by immersing in varied concentrations of buffered formalin at room temperature for 10 min.
7. The slides were removed and dip-rinsed twice in wash buffer TPBS (PBS with 1 % Tween 80).
8. Further, the slides were immersed in 3 per cent hydrogen peroxide (H2O2) for 10 min. to neutralize the endogenous peroxidases.
9. Later, the slides were removed and dip-rinsed twice in TPBS.
10. The slides were washed twice in 1X PBS.
11. The fixed smears were briefly air dried to ensure that the 2.5% NBF traces on it evaporated and were stained using 1:20 dilution of the above said FITC conjugate by incubating in a humid chamber at 37 °C for 45 min.
12. The smears were washed with 1x PBS for 5 min.
13. The stained smears were observed under a fluorescent microscope.
14. Presence or absence of typical granular intra-cytoplasmic apple green fluorescence of aggregated nucleocapsids was used as a criterion in declaring positive and negative samples respectively.

Table 1. Details of samples collected

	Karnataka	Manipur	Rajasthan	Jammu & Kashmir	Gujarat	Tamil Nadu	Total
Canine	38	04	01	-	-	01	44
Bovine	02	-	-	-	-	-	02
Bubaline	-	-	-	01	01	-	02
Equine	01	-	-	-	-	-	01
Bat	01	-	-	-	-	-	01
Total	42	04	01	01	01	01	50

2.3.2 Retrieval of rabies virus antigen in formalin fixed brain impressions from BHK-21 cells

2.3.2.1. Protocol

- Routine touch impressions of primarily tested positive and negative brain tissue were made and fixed in varied concentrations NBF for 10 minutes at room temperature.
- Slides were allowed to air dry.
- Scrapings were taken from impressions made on slides and added to 400µl of 10% GM (Tissue suspension)

- 200 µl of tissue suspension was added to Lab-tek slide chambers
- 100 µl of BHK-21 cell suspension was added to Lab-tek slide chambers containing 200 µl of tissue suspension
- Co-cultivation of virus with cells was carried out in 5% CO2 incubator for 45 hours.
- After 45 hours of incubation, contents were discarded.
- 100 µl of 70% chilled acetone was added and kept for incubation at -20 °C for one hr.
- Contents were discarded and air dried for 5 min.
- Lab-tek slide chambers were stained using 1:20 dilution of the aforementioned said FITC conjugate by incubating in a humid chamber at 37 °C for one hour.
- Contents were discarded and washed with 1X PBS twice.
- The stained slide chambers were observed under a fluorescent microscope.

3. Results

All the 50 samples were subjected to modified DFA protocol as described in the Section 2.3.1.1. A good fluorescence was seen at 2.5% NBF as compared to the other concentrations of formalin (Plate 4 to 7). There was 100 percent inactivation of the virus when inoculated to BHK-21 cells. The stained slides were examined under a fluorescent microscope at 200X and 400X magnification. It was compared with the slides prepared from same impression, processed by gold standard DFA. Samples were declared positive when bright apple green fluorescent particles of varying size were noticed either scattered or within the neurons, whereas, in the negative samples no such fluorescence was observed.

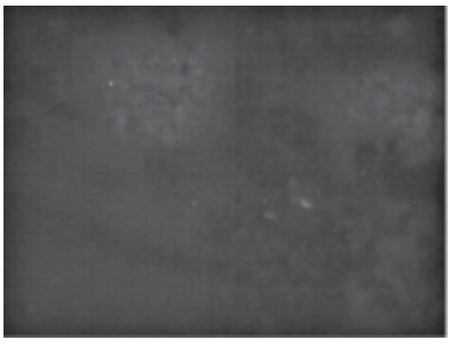

Plate 1: Rabid animal brain impression showing no fluorescence after fixing the slide in Tris EDTA.

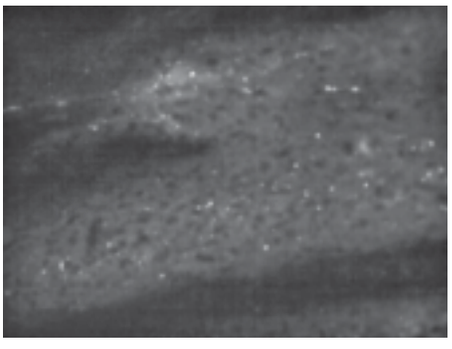

Plate 2: Rabid brain impression showing less fluorescence after fixing the slide in 10% Neutral Buffered Formalin.

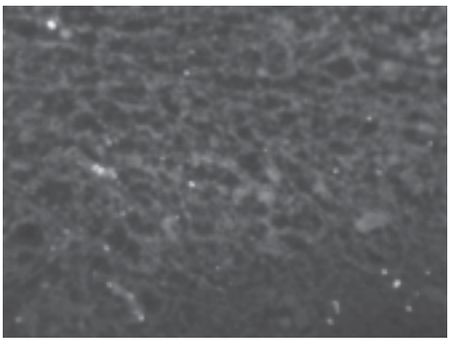

Plate 3: Rabid animal brain impression showing less fluorescence after fixing the slide in 5% Neutral Buffered Formalin.

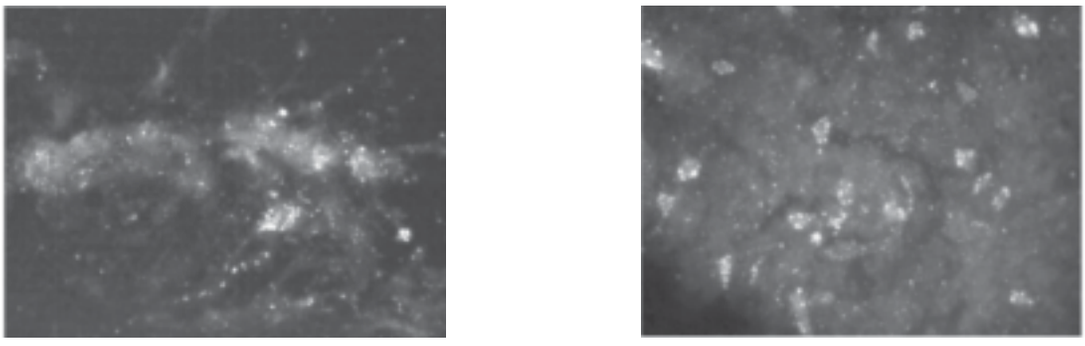

Plate 4: Rabid brain impression showing fluorescence (++++) after fixing the slide in 2.5% Neutral Buffered Formalin (Left) and chilled acetone (Right).

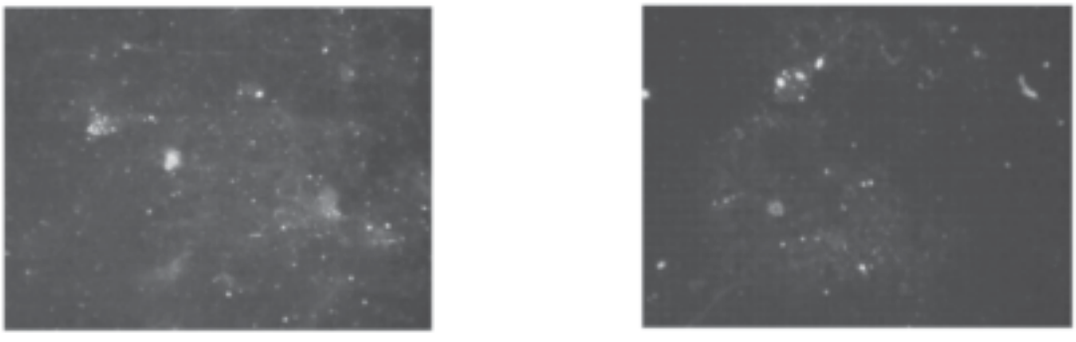

Plate 5: Rabid animal brain impression showing fluorescence (+++) after fixing the slide in 2.5% Neutral Buffered Formalin (Left) and chilled acetone (Right).

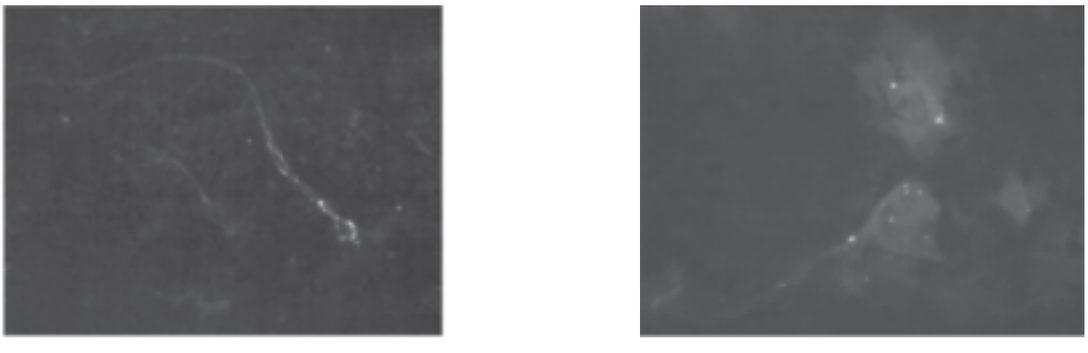

Plate 6: Rabid brain impression showing fluorescence (++) after fixing the slide in 2.5% Neutral Buffered Formalin (Left) and chilled acetone (Right).

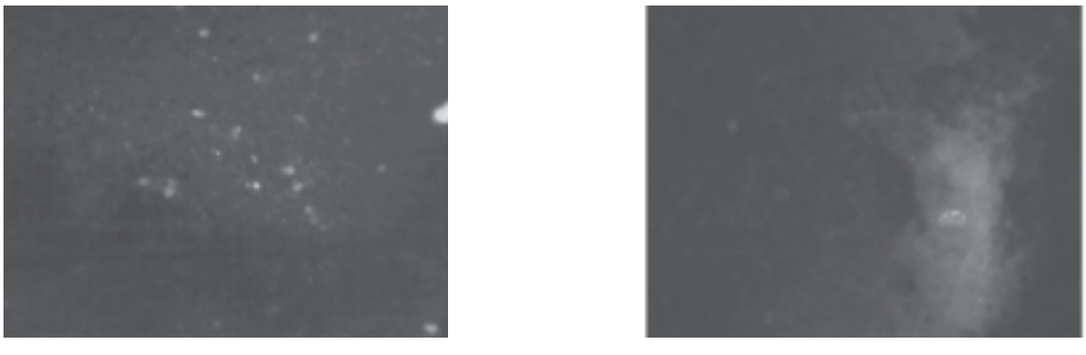

Plate 7: Rabid animal brain impression showing fluorescence (+) after fixing the slide in 2.5% Neutral Buffered Formalin.

The results were compared and analysed by Spearman's nonparametric correlation analysis. The "Correlation coefficient (Spearman r)" was found to be 0.98 (P<0.001) indicating very good positive correlation. Scoring of Modified DFA provided in **Fig. 1**

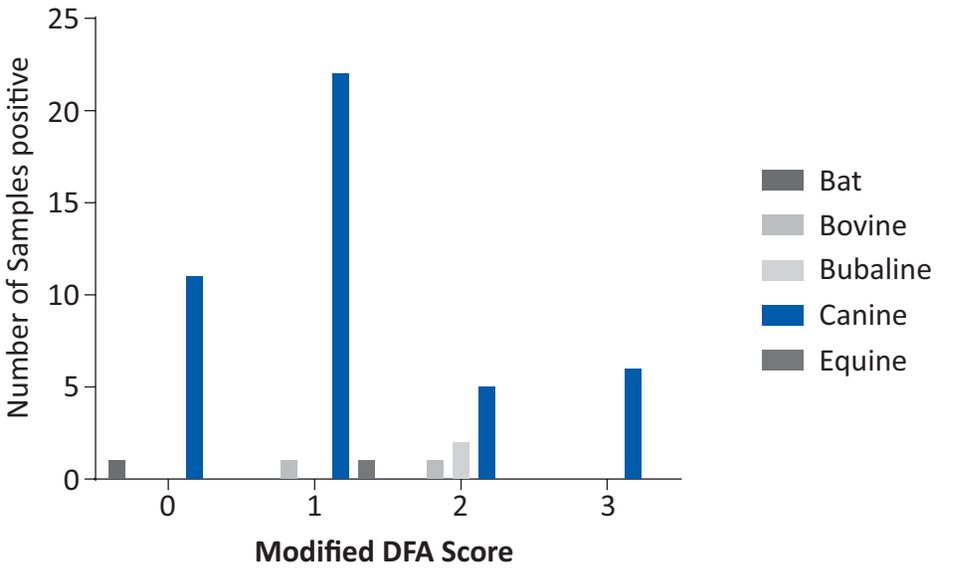

Fig. 1: Details showing Modified DFA score

3.1. Retrieval of rabies virus antigen in formalin fixed brain impressions from BHK-21 cells

The presence or absence of virus in different fixatives (Tris EDTA, 10%, 5%, 2.5%, 1% NBF) were confirmed by infecting BHK-21 cells. (Plate 15-22). The tissue impressions which were fixed in 2.5% NBF showed 100 % inactivation of virus in BHK-21 cells as indicator system (Plate 22). It reduces potential biohazard to laboratory personnel involved in handling the infected brain sample.

Plate 8: Cell control

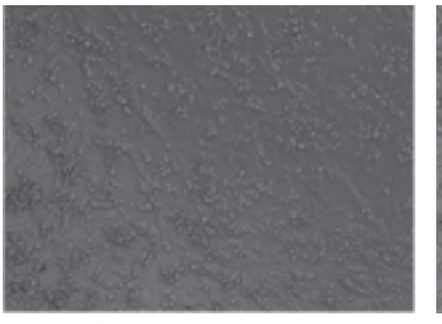

Plate 9: Positive control

Plate 10: Negative control

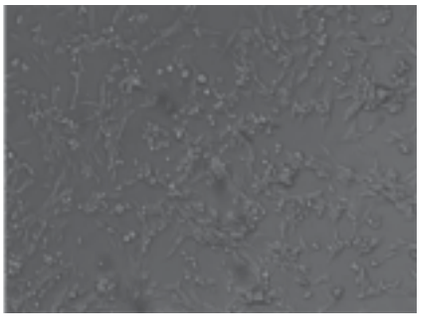

Plate 11: 2.5% NBF

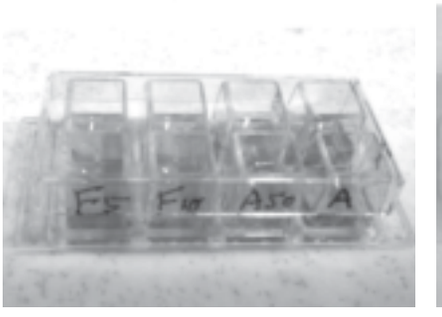

Plate 12: Lab-tek slide chambers; Day 1

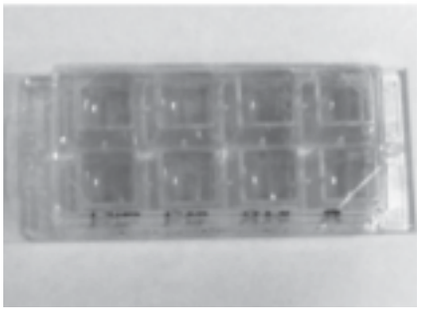

Plate 13: Lab-tek slide chambers; Day 1

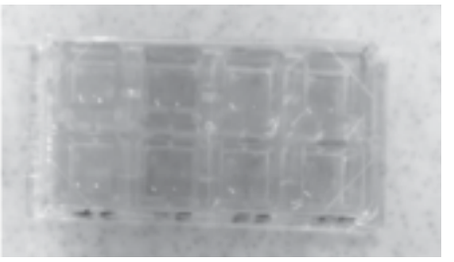

Plate 14: Lab-tek slide chambers; Day 2

Plate 15: Cell control stained in Lab-Tek slides with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue).

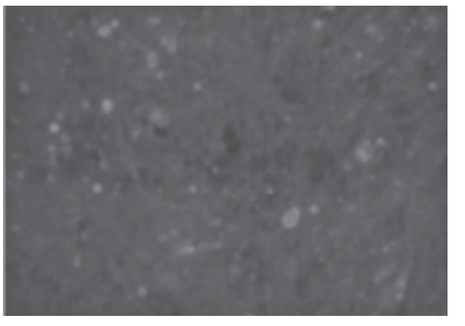

Plate 16: Negative control stained in Lab Tek slides with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue).

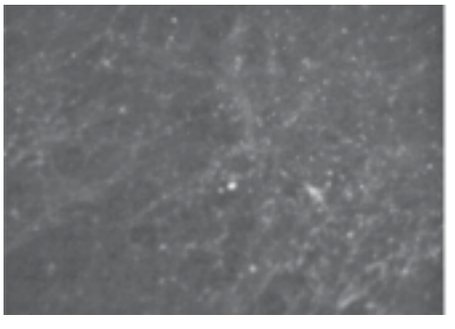

Plate 17: Positive control stained in Lab Tek slides with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue).

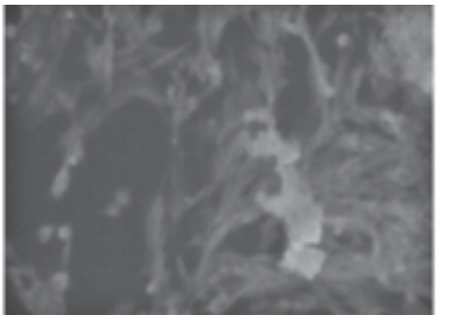

Plate 18: No fluorescence at 10% NBF fixation in Lab Tek slides with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue).

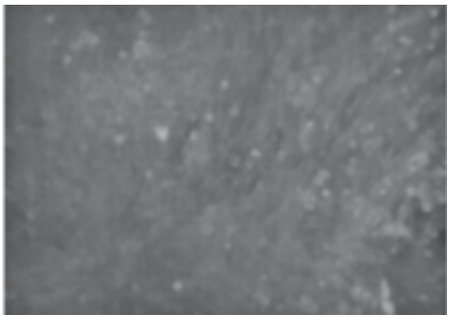

Plate 19: No fluorescence at 5% NBF fixation in Lab Tek slides with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue).

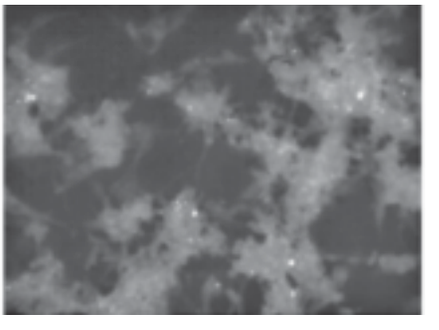

Plate 20: Presence of fluorescence at 1% NBF fixation in Lab Tek slides with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue).

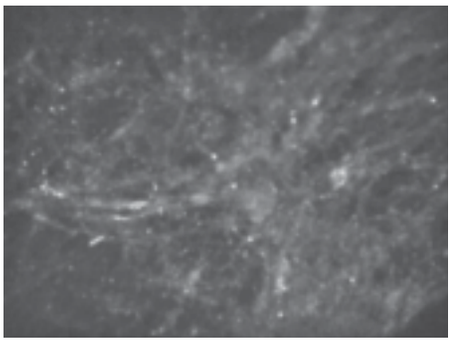

Plate 21: Presence of fluorescence at Acetone at -20 °C in Lab Tek slides with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue).

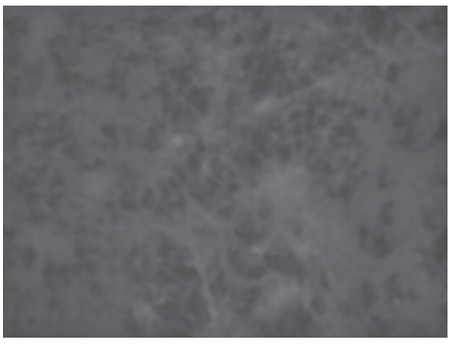

Plate 22: No fluorescence at 2.5% NBF fixation in Lab Tek slides with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue).

4. Discussion

The DFA has few limitations. The major drawback is that the acetone as fixative does not completely inactivate the infective virions, posing potential hazard to laboratory personnel (Velasco-Villa et al., 2005, OIE, 2011 and Jarvis, 2016). Studies have demonstrated that intracerebral inoculation of acetone fixed tissues can cause disease in suckling mice (Umoh and Blenden, 1981 and Velasco-Villa et al., 2005). But formalin inactivates the RABV (Velasco-Villa et al., 2005, OIE, 2011) within 10 min and this property is explored in case of direct rapid immunohistochemistry (dRIT). Furthermore, chilled acetone fixation of tissue is time consuming and is dependent on availability of deep freezer since the brain impressions have to be fixed for at least one hour in -20oC deep freezer. In view of these advantages of formaldehyde based fixation of brain impressions DFA was modified.

In this study, viability of virus in acetone and formalin fixed slides were checked by inoculating BHK-21 cell with scrapings from the impressions. There was 100 per cent inactivation of virus in formalin fixed slides (10%, 5% and 2.5%), whereas 80 per cent fluorescence was found in acetone fixed slides when compared with positive control. A similar study was performed in impressions made from RABV fixed in acetone at -20 °C for 2, 4, 7, and 24 h and examined for virus viability. Tissues scraped from impression smears after acetone fixation were examined for virus viability in BHK-21/ Winster Institute-2 (WI-2) cell cultures. The infectivity titers ranged from 103.3/0.1 ml in suckling mice to 105.1/0.1 ml in BHK-21/ (WI-2) cultures. Scrapings from these smears all contained infectious virus, thus it was concluded that acetone fixation at -20 °C does not inactivate rabies virus. (White and Chappell, 1982). Further, tissues scraped from acetone fixed rabies or Eastern equine encephalitis positive mammalian brain tissue impression were inoculated to mouse neuroblastoma C-1300 cells and incubated for 72 hours in a 34 °C humidified incubator with 5% CO2. It was found that the virus was not inactivated with acetone fixation, thus it was suggested that laboratory employees should treat all samples as rabies and Eastern equine encephalitis positive throughout the whole procedure (Jarvis, 2016).

In the present study, initially several impressions of a DFA confirmed brain sample were fixed with varying concentrations of NBF from 10%, 5%, 2.5% and 1 % for 10 minutes (Section 3.3.3.6). The criteria considered to select the particular concentration of Formaldehyde for fixing the brain impressions were complete inactivation of the virus after fixing the impression without compromising with the fluorescence due to viral inclusions. It was observed that at 2.5% NBF there was complete inactivation of the virus and appreciable fluorescence of the viral inclusions based on three repetitions. In all, 50 brain samples which were found to be positive by DFA were subjected to modified DFA. This approach is completely biohazard free to the laboratory personnel and significantly minimized the fixation time from minimum 1hr to just 10 min. This is an important finding in view

of the safe handling of brain impressions from rabid animals and rapid testing without compromising with the Sensitivity and specificity of modified DFA.

5. Conclusion

In conclusion, the laboratory based confirmatory diagnosis of rabies in animals serves as the basis for the downstream post exposure prophylactic measures. However, one of the limitations of the currently employed gold standard DFA is that there is a biosafety concern with respect to handling of brain impression slides and the longer duration of fixing the slides in Chilled acetone. In this context, the modified DFA is user-friendly in view of its bio safety and rapidity. The outcome of the present study reveals the statistically significant correlation between the DFA ad modified DFA, LFA and LAMP. Hence, this tests could be employed for diagnosis of rabies in animals laboratory or field level.

6. References

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