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Original Article

Comparative evaluation of Direct Rapid Immuno-Histochemical Test (DRIT) with Direct Fluorescent-Antibody Test (DFAT) for laboratory diagnosis of animal Rabies in Ethiopia

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Summary
Direct rapid immuno-histochemical test (DRIT) is used as a gold standard method for rabies virus detection. The present study aimed to compare and evaluate DRIT with direct fluorescent antibody test (DFAT) to use equivalently as one of rabies diagnosing methods in areas where DFAT is not accessible. The method is based on the capture of rabies nucleoprotein (N) antigen in brain smears using a cocktail of biotinylated monoclonal antibodies specific for the N protein and color development by streptavidin peroxidase-amino ethyl carbazole and counterstaining with hematoxylin. The test was performed in parallel with the standard DFAT and mice inoculation test (MIT) using 100 brain specimens from various species of animals. The majority of them were dogs (n =88), followed by cats (n =8), cattle (n =3), and donkey (n =1), and also from those samples that were tested by DRIT and DFAT, we randomly selected and tested 12 brain samples by MIT. The results indicated that 63% of the tests were positive by DFAT and 64% were positive by DRIT. A slight difference was observed in such a way that one sample was negative by DFAT but positive by DRIT and MIT. Although, further laboratory and field examinations are essential, our findings were providing and remark the potential value of the DRIT for countries with limited diagnostic resources.
Keywords: DRIT, Rabies, MIT, DFAT, Surveillance

Introduction

Rabies is a lethal zoonotic disease with a worldwide distribution and is transmitted frequently by carnivores to humans and livestock. It is presented to cause a large number of deaths in animals and humans each year. Human rabies is present in 150 countries and territories and on all continents (Barecha et al., 2017). Of these countries, Ethiopia is one of the worst affected (Hampson et al., 2015). According to Tariku Jibat et al. (2018) research suggestions in most rabies endemic countries, reliable documents of occurrence data on rabies and rabies exposure are missing. However, estimates of burden have always been uncertain due to the absence of reliable data (Taylor and Nel, 2015). Official record usually underestimate the actual number of human rabies cases and hence the actual burden. For example, in sub-Sahara African countries such as Tanzania, the occurrence of human rabies predicted based on active surveillance information on bite occurrences was up to 100 times larger than the officially reported number of deaths. In Ethiopia, the national annual assesses from official

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records present 12 exposure cases per 100,000 population and 1.6 rabies deaths per 100,000 populations. However, the true numbers are expected to be higher as many cases are not reported (Beyene et al., 2018). Recently, rabies is a considerable disease that has been detected for many years in Ethiopia. The first rabies epidemic in Ethiopia reported in Addis Ababa in August 1903. It is believed that communication between the public and animal health sector were not enough to inexistent considering reporting and control of rabies cases (Pieracci et al., 2016). At present time, direct rapid immuno-histochemical test (DRIT) is mentioned as the gold-standard test by the World Organization for Animal Health (OIE) and World Health Organization (WHO)(Tekki et al., 2016) for rabies detection; however, the limitation of use of DRIT in developing countries is that the method is technically demanding and needs the use of a fluorescence microscope, which is expensive and difficult to maintain, and it also needs a specific rabies conjugate as well as technical expertise. Thus, there is a necessity for a rapid diagnostic test that has comparable sensitivity and specificity as fluorescent antibody (AB) test (FAT), which is economical and can be adapted to field as well as laboratory conditions in resource-constraint countries (Singh et al., 2017). The DRIT is an experimental assay, which is used in limited field trials in Africa, such as Nigeria and South Africa (Coetzer et al., 2014). It has been demonstrated to be as specific and sensitive as the gold standard direct fluorescent antibody test (DFAT). In Ethiopia, the surveillance system is very week due to lack of rabies laboratory and also the burden of rabies is under expected or under-reported. DFAT is currently available only in three regions of Ethiopia, because establishing DFAT in all regions requires high-level expertise, expensive laboratory equipment, reagents, and sophisticated laboratory setups. As in all other endemic countries, an establishment of a diagnostic laboratory set-up as the priority for the improvement of a national rabies control program is needed. Any technical advances that make diagnosis of rabies more rapid, accurate, and cost-effective, will consequently facilitate recruiting such programs in resource-limited countries. According to Middel et al. (2017), DRIT was presented to have a sensitivity and specificity of 100% and 98.2%, respectively. Moreover, positive and negative test agreement was reported to be 98.3% and 99.1%, respectively, with an overall test agreementof98.8%. The average cost to test a sample was $3.13 CAD for materials, and hands-on technical time to complete the test was estimated at 0.55 h. Also, DRIT procedure was proposed to be accurate, inexpensive, fast, easy to perform, and a great tool for monitoring the progression of rabies incursion. DRIT not only has a diagnostic efficacy equal to that of the gold standard DFAT, but is also faster, cheaper and easier to elucidate by an inexperienced reader. These findings highlight the versatility of the DRIT as a potential WHO and OIE accredited rabies diagnostic assay (DRIT SOP).The authors attempted to demonstrate which of the current laboratory techniques, being applied for rabies diagnosis worldwide, has unique advantages over the other by comparing the gold standard laboratory technique DFAT and DRIT so that the result of the study would serve to trigger government officials both in the ministry of health and agriculture to take further action in scaling up the suitable laboratory diagnostic techniques, provided the limited laboratory set up. For internal quality purpose, the researchers agreed to perform mice inoculation test (MIT) by selecting 12 randomly selected brain samples and inoculated into mice brain tissue. The rationale behind performing MIT was that there are indeterminate conditions, usually 1%, while performing both DRIT and DFAT. In situations where skills and facilities for other tests [*e.g.*, cell culture, polymerase chain reaction, (PCR)] are not available, MIT could be served as an optional confirmatory technique. In a country like Ethiopia, where rabies is highly prevalent, besides with a very limited access for laboratory diagnosis of rabies, the result of this research would have several implications like enhancing the rabies laboratory surveillance in Ethiopia and its

suitability regarding the time taken, technical ease, user acceptability, and the stability of the DRIT to perform the test makes the present study crucial.

Materials and methods

Addis Ababa, the capital city of Ethiopia, covers an area of 530 km^2 and is divided into 10 sub-cities. Ethiopian public health institute is located at Gulele sub-city. Currently, the institute is focusing on priority disease research and strengthening the national public health laboratory services in the country. It is also the technical hand of the Federal Ministry of Health. So, all dogs inflicting bite including laboratory diagnosis of rabies in and around Addis Ababa is managed through the national diagnostic center; which is Ethiopia Public Health Institute (EPHI) (Figure 1).

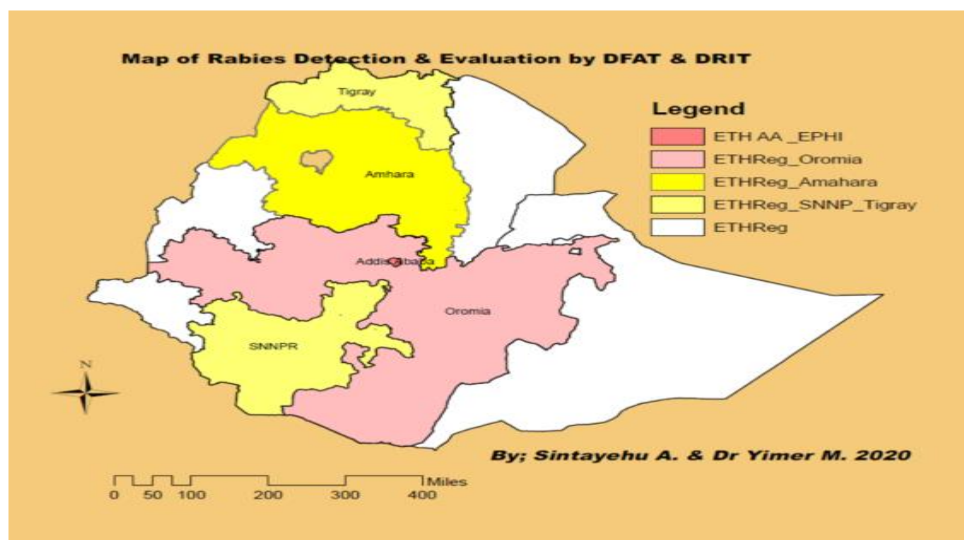

Fig.1. Map of the study area of rabies detection and evaluation by DFAT and DRIT

Sample Size

A total of 100 brain specimens were collected during the study period (December, 2020 – June, 2021) from various regions and different species of animals and brought to the Ethiopian public health institute rabies diagnostic laboratory.

Protocol for DFAT

In the present study, DFAT, monoclonal antibody-conjugate, were applied. The working (reagent) dilution after titration was prepared at 1:40 in accordance with the manufacturer's recommendations (Standard Operating Procedure for DRIT, center for disease control (CDC); Reagent for the study was manufactured and supplied by CDC, Atlanta). A small piece of the brain tissue specimens was smeared using a wire loop on one part of a slide, which was subsequently air-dried and fixed in cold acetone at -20°C for one hour. Then, the slides were air-dried and the rabies conjugate was used at 1:40 and incubated at 37°C for 30 minutes in a humid chamber after which excess conjugate was removed from the slides by rinsing it with 7.4 pH PBS solution about 3-5 minutes and was conducted to air-dry. The coverslips were mounted with buffered glycerol mounting medium and the slides were evaluated by a fluorescence microscope within two hours after staining. When brilliant apple-green fluorescence color or greenish-yellow objects a represented against a black background, the test slide is positive. If no specific apple green fluorescence was presented, the test slide is negative (Figure 3B).

Protocol for DRIT

1. Routine touch impressions of suspect CNS tissues were made on labeled glass microscope slides (Standard

positive and negative controls were included).

2. Slides were air-dried for 5 minutes at room temperature.
3. Slides were immersed in 10% buffered formalin at room temperature for 10 minutes (Dish I).
4. Slides were removed and dip-rinsed several times to wash off any excess fixative in wash buffer TPBS (PBS with 1% tween 80; Dish II).
5. Slides were immersed in 3% hydrogen peroxide for 10 minutes (Dish III).
6. Excess hydrogen peroxide was removed by dip-rinsing slides in TPBS (Dish IV). Then slides were transferred to the next rinse, Dish V (after dipping, excess buffer was shaken off, and blotted from the slide edges surrounding the impression). One slide at a time was done leaving the remaining slides immersed within TPBS rinse.
7. Slides were incubated in a humidity chamber (Plastic top to a 96-well plate was used), at room temperature with primary antibody - biotinylated anti-rabies mAb for 10 minutes (Enough of this primary antibody were added by dropping to cover the impression).
8. After incubation, excess conjugate was shaken off. Slides were Dip-rinsed with TPBS, Dish V (The same wash buffer was used through step 10. Slides were incubated with streptavidin-peroxidase complex (enough of this complex was added to the slide by dropping to cover the impression) in a humidity chamber at room temperature for 10 minutes. After incubation, excess of the complex was shaken off.
9. Slides were dip-rinsed with TPBS, Dish V (excess buffer was shaken off and also blotted from slide edges surrounding the impression).
10. Slides were incubated with peroxidase substrate, amino-ethyl carbizole (AEC) – The working dilution was prepared just prior to use. Enough of this substrate was added to the slide by dropping to cover the impression in a humidity chamber at room temperature for 10 minutes. Excess substrate was shaken off after incubation.
11. Slides were dip-rinsed in deionized/distilled water (Dish VI).
12. Slides were counterstained with Gills Hematoxylin (diluted 1:2 with deionized/distilled water) for 2 minutes (Dish VII).
13. Immediately, the stain was dip-rinsed with deionized/distilled water (Dish VIII). A second dip-rinsing was made of slides with fresh deionized/distilled water (Dish IX) to ensure removal of excess stain.
14. Slides were transferred to fresh distilled water (Dish X). Slides were mounted with water-soluble mounting medium and cover-slipped. (One slide at a time was made; Excess deionized/distilled water was shaken off and blotted from slide edges surrounding the impression). Slides were allowed to air-dry prior to cover slipping.
15. Slides were viewed by light microscopy, using a 20x objective to scan the field, and a 40x objective for higher power inspection (rabies virus antigen appeared as red inclusions against the blue neuronal background).
16. Finally, results were recorded.

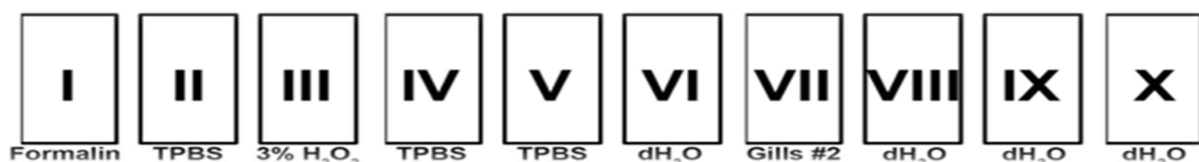

Fig. 2. Staining dish set - up for DRIT

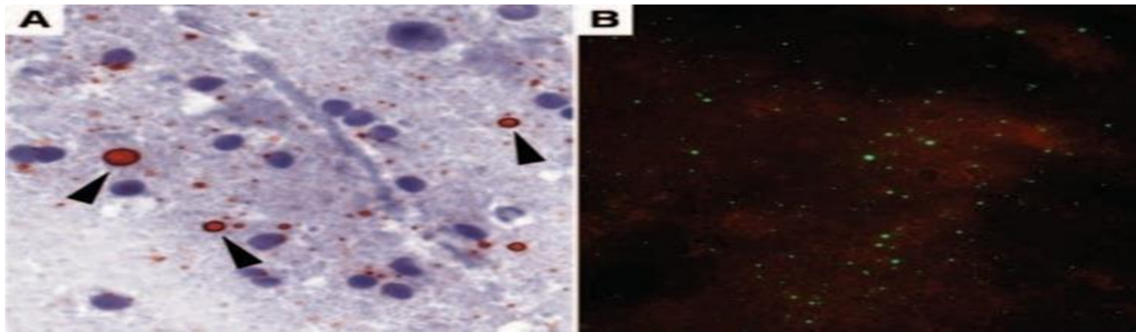

Fig. 3. (A): Brain stained by DRIT: rabies virus antigen appears as magenta inclusions (arrow heads) against the blue neuronal hematoxylin counterstain, Magnification 630. Figure 2Immunofluorescent apple-green viral inclusions in the same brain processed by DFAT, Magnification200 (Lembo et al., 2006).

Mice Inoculation Test (MIT)

In the present study, five-to-ten mice, 3-4 weeks old (12-14 g), or a litter of 2-day-old newborn mice, were inoculated intracerebrally. It is suggested, though not strictly required, to apply specific pathogen-free (SPF) mice. The young adult mice were monitored daily for 28 days, and every dead mouse was evaluated for rabies by the DFAT. Another advantage of this low-tech test is that it can be easily and practicably be used in situations where skills and facilities for other tests (*e.g*., cell culture) are not available (Trimarchi et al., 2007).

Data analysis

The results obtained from both DFAT and DRIT were analyzed by SPSS version 25. Sensitivity was calculated by the formula (Miodrag et al., 2014) [True Positive (TP)/(True Positive (TP)+False Negative (FN))]×100. Indeed, TP is the number of samples with true-positive results as based on the reference test and FN is the number of a sample with false-negative results. Specificity was defined as [True Negative (TN)/ (True Negative (TN) +False Positive (FP))] ×100, where TN was the number of a sample with true-negative results and FP was the number of a specimen with false-positive results. Confidence intervals for sensitivity and specificity were computed with SPSS statistical software.

Results

A total of 100 samples were collected and tested by the two techniques from different species of animal and from different regions of the country. The majority of the samples were from dogs (n = 88), followed by cats (n = 8), cattle (n = 3) and donkey (n = 1). Additionally, we randomly selected and tested 12 brain samples by MIT (Table 2) technique from those samples that were tested by DRIT and DFAT for internal quality control of the tests. Among the total samples tested, we found similar results, i.e., 63% were positive by DFAT and 64% were positive by DRIT. A slight difference was observed in a way that one sample was negative by DFAT but positive by DRIT and MIT (Table 1, Figure 4).

Table 1. Results of rabies virus diagnosis by DFAT and DRIT

Sample scores(n)	DFAT test result		DRIT test results		Correlation
	Positive	Negative	Positive	Negative	
Dog (88)	56	32	57	31	99%
Cat (8)	5	3	5	3	100%
Cattle (3)	1	2	1	2	100%
Donkey (1)	1	0	1	0	100%
Total(100)	63	37	64	36	100%

Table 2. Diagnosis result of MIT for rabies virus detection

Sample scores and number	MIT test		Total
	Positive	Negative	
Dog (9)	4	5	9
Cat (2)	1	1	2
Cattle (1)	1	0	1
Total (12)	6	6	12

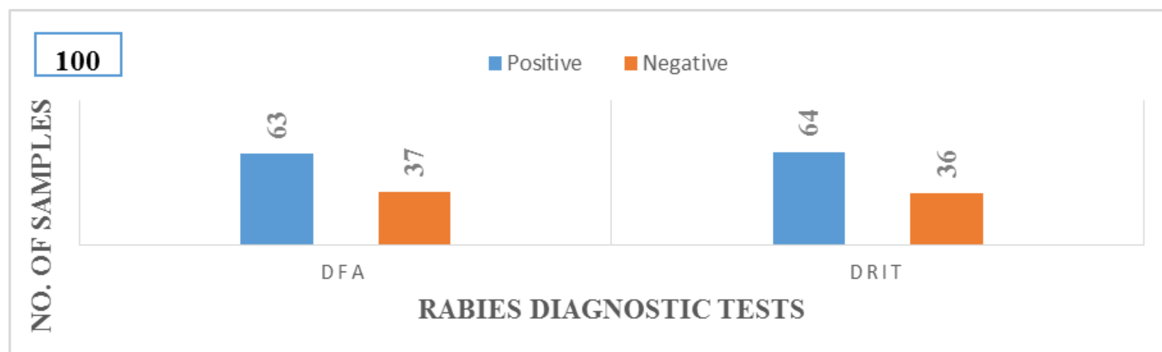

Fig.4. Comparison results of rabies diagnostic tests DFAT and DRIT

Discussion

The rabies virus can be diagnosed by using laboratory techniques such as DFAT, DRIT, PCR, MIT, and others. From those tests, DFAT is used as a gold standard method; however, DFAT is currently available only in three regions of Ethiopia, because establishing DFAT in all regions requires high-level expertise, expensive laboratory equipment, reagents, and sophisticated laboratory setups. So that, to strengthen the surveillance system in our country; we believed that it is important to assess, to evaluate, and to apply other less costly, accessible, and easier laboratory tests that can equivalently be sensitive and specific with DFAT. Thus, this study aimed to compare and to evaluate DRIT with DFAT in order to be equivalently used as one of rabies diagnosing methods in areas where DFAT is not accessible. Since, DFAT is a very expensive type of method that requires highly trained expertise, sophisticated laboratory setup, and costly reagents it becomes challenging to be accessible to all regions of Ethiopia. As WHO emphasis that rabies prevention and control requires an integrated and functional surveillance system that is supported by easily applicable and extensively accessible laboratory tests. Thus, our study aimed to compare and evaluate a simple and easy alternative laboratory test other than DFAT; such test can be used for the diagnosis of rabies and will help to strengthen the rabies surveillance system in animals. Laboratory testing is desirable; however, it is extremely limited in are source-poor country and rural areas of endemic. Animal surveillance requires in the disease endemic setting with high caseloads may therefore be satisfied by resorting to syndrome case definitions and history of a dog bite. The findings of our study strongly prove that DRIT should potentially be used as an alternative diagnostic test for rabies in areas where DFAT laboratory setups are not available. The study results were compared and evaluated with the gold standard test and out of the total 100 animal samples tested using DRIT and DFAT, 64% and 63% tested positive, respectively.

In the current study, we found that DFAT had 100% sensitivity and specificity as compared to DRIT. Our finding was comparable and in line with the study conducted by G. Singh et al. (2017) who have demonstrated that the sensitivity and specificity of DRIT were 100%.. The positivity among submitted samples tested using DFAT stood at 63%. In comparison, a slight variation in positivity (64%) was seen in samples tested using DRIT, showing a significant agreement between DFA and DRIT results. This is in line with previous reports elsewhere (Dürr et al., 2008). There were 1.56% false-negative results showed by DFAT as compared to DRIT, with some variation in specificity; however, this is happened due to sample decomposition. Thus, DFAT is best done on fresh brain specimens than DRIT; the reliability of this method to diagnose rabies in decomposed animal brain samples is low (Mani and Madhusudana, 2013).In such cases, the DRIT would be a superior test that is less sensitive to microscope issues and provides accurate results. DRIT is simple to perform, though the numbers of steps are more than DFAT. DFAT requires air-dried smear at least two hours for fixation in cold acetone (Dürr et al., 2008),whereas DRIT requires only 10 minutes for fixation by using formalin. This also has another advantage as formalin inactivates the virus without impacting the antigenicity, whereas DFAT use acetone as a fixative, which does not completely inactivate the virus as presented by the infectivity of acetone-fixed tissue for neuroblastoma cells; if available, would be a potential biohazard to laboratory personnel (Prabhu et al., 2018). DRIT requires only light microscope, which isabout10 times cheaper than the fluorescent microscope that is used in DFAT. Also, DFAT requires a refrigerator, due to storage condition of the kit, various chemical reagents, as well as for the acetone fixation, which is a critical point for the field use. Another concern about DFAT is the need for incubator (Madhusudana et al., 2012).

Conclusion

The DRIT showed sensitivity and specificity equivalent to those of the DFAT.DRIT is simple to perform, and does not require expensive equipment and sophisticated laboratory setup or infrastructure. The earlier reports and the present study proved that the time taken to perform the test, technical ease, user acceptability, and the stability of the DRIT laboratory condition makes DRIT as a very suitable laboratory test to strengthen the rabies surveillance in Ethiopia. To conclude, DRIT should be an alternative approach for rabies diagnostic testing, which can be done to decentralize rabies laboratory in the field or in those areas that have no access to DFAT.

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Conflicts of Interest Statement

The authors declare that they have no conflicts of interest.

Ethical approval

The study was approved by the zoonoses research case team of the institute. All clinical and laboratory investigations were conducted according to the principles expressed in the declaration of Helsinki 1975, as revised in 1983.

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