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Assessment of Rapid Immunochromatographic Assay for detection of Rabies infection under field condition

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Abstract
Rapid and early diagnosis of rabies is of paramount importance in field conditions for control of the disease as well as for epidemiological surveillance of the prevalence of the disease. Techniques like Fluorescent Antibody Test and RT-PCR, although highly sensitive and specific, require laboratory facilities, trained laboratory personnel and are time consuming. Therefore there was an impending need to evaluate an essay for quick and accurate diagnosis of rabies under field conditions. The present study was carried out to study the efficacy of a commercially available diagnostic immunochromatographic kit for detection of rabies antigen from BioNote Inc. Seoul, Korea in comparison with conventional techniques viz. seller's stain and FAT as well as sensitive molecular techniques viz. RT-PCR. Also, the importance of rapid immunodiagnostic test was evaluated for rapid and accurate diagnosis of rabies under field conditions. A total of 25 brain samples of different species like cattle, buffalo, mongoose and sloth bear were collected from outbreaks and incidences of rabies in Gujarat State and examined in this study. Sensitivity of the kit was found to be 100% and specificity was also found to be 100%. Also, 100% agreement was observed between chromatographic assay, seller's stain, FAT and RT-PCR. The study implies that the immunochromatographic diagnostic test kit may be employed for diagnosis of rabies in field conditions.

Keywords: Rabies, Immunochromatographic assay, Seller's stain, FAT, RT-PCR

Introduction

Rabies (derived from Latin word *Rabere*, meaning 'Rage') is a viral disease caused by *Lyssavirus* rabies virus of the Rhabdoviridae family [1]. Although all species of mammals are susceptible to rabies virus infection, only a few species are important as reservoirs for the disease. Several distinct rabies virus variants have been identified in terrestrial mammals, including raccoons, skunks, foxes, and coyotes. In addition to these terrestrial reservoirs, several species of insectivorous bats are also reservoirs for rabies [2]. Rabies causes about 24,000 to 60,000 deaths worldwide per year [3, 4]. More than 95% of human deaths caused by rabies occur in Africa and Asia [5]. Rabies has been featured as one of the top ten diseases of livestock reported in India. An estimated 31,000 human deaths due to rabies occur annually in Asia [6] with the majority - approximately 20,000 - concentrated in India [7]. In Gujarat, during last five years, 28 outbreaks and incidences of rabies have been reported leading to death of 131 livestock population.

Although symptoms of rabies are quite characteristic, like changes in behaviour or difficulties in swallowing, the clinical examination alone cannot rule out rabies nor confirm the diagnosis. Therefore laboratory confirmation is must in case of rabies.

The diagnosis in animals depends upon proper collection and testing of appropriate specimens. Brain tissue is the preferred specimen for post-mortem diagnosis of rabies in livestock. Although Mouse inoculation test (MIT), is considered as the most effective tool for detection of the viability of the virus [8] with 97-98% sensitivity [9], is laborious, takes 2-3 weeks time and requires many experimental mice. Most of the laboratories follow standard seller's method- a preliminary diagnostic test, wherein about 75-80% cases shows Negri bodies [9-11]. World Health Organization (WHO) and World Animal Health Organization (OIE) have recommended FAT as the gold standard test for confirmatory rabies diagnosis. The direct fluorescent antibody test is a sensitive and specific procedure used in the routine diagnosis of

rabies. Despite high sensitivity of FAT in rabies diagnosis, FAT has many drawbacks, which limits its usefulness, including establishment of FAT laboratories requires expensive infrastructure, well-trained technician and getting fresh specimens to potentially distant laboratories in an adequate condition for testing is unfeasible [12, 13]. Recently, molecular techniques such as the reverse transcriptase polymerase chain reaction (RT-PCR) [14, 15] have been tried by various workers to improve the sensitivity and specificity of ante as well as post mortem diagnosis of rabies. Since, molecular approaches like RT-PCR also require sophisticated laboratory facilities and highly trained technical staff, thus, there is impending need of a commercially available diagnostic kit that could be employed for efficient diagnosis of rabies in field conditions. Thus, the present study was carried out to study the efficacy of a commercially available diagnostic immunochromatographic kit for detection of rabies antigen in comparison with conventional techniques viz. seller's stain and FAT as well as sensitive molecular techniques viz. RT-PCR. Also, the importance of rapid immunodiagnostic test was evaluated for rapid and accurate diagnosis of rabies under field conditions.

Materials and Methods
Collection of samples
A total of 26 brain samples of species like cattle, buffalo and sloth bear were received in the Office of the Deputy Director of A.H., F.M.D Typing Scheme, Ahmedabad for diagnosis of rabies over a period of five years (2012-2017). Table 1 shows the outbreaks and incidences of rabies in Gujarat state along with the number of animals attacked and died, during the year 2012-2017.

In all the above mentioned outbreaks and incidences, the clinical symptoms were quite characteristic of rabies, as observed by the field veterinarians. In most of the cases a history of dog bite was there, but in few cases there wasn't any history of dog bite but the clinical symptoms were characteristic of rabies. Symptoms usually observed in case of rabies in cattle include- abnormal movement of posterior extremity, foamy white froth, wandering, salivation, decrease yield of milk in milking animal, frequent micturition and abnormal bellowing.

Part of samples were processed at Office of the Deputy Director of A.H., F.M.D Typing Scheme, Ahmedabad for Seller's stain and immunochromatograhic assay and remaining samples were submitted to different laboratories including- 1) Department of Microbiobiology, Sheth Vadilal Sarabhai General Hospital, Ahmedabad, 2) Department of Veterinary Microbiology, Veterinary College, Anand, 3) National Institute of Veterinary Epidemiology and Disease Informatics, Bengaluru and 4) R & D Department, National Dairy Development Board, Hyderabad.

The samples were submitted to these laboratories in two parts- one on ice and another one on 50% glycerol saline, in wide mouth leak proof sterile containers. While submitting brain samples, care was taken to include part of hippocampus and cerebrum in each sampling. Samples were transported in three layered packing and maintaining cold chain.

Seller's stain
Fresh (not fixed) whole brain samples were submitted for confirmation of rabies in all the above mentioned cases. Fresh smears were prepared from a full cross-section of the mid-cerebellum and brain stem. Wet smear was stained with Seller's stain (methylene blue 2 parts and basic fuschin 1 part).

Analysis
Positive smears revealed presence of negri bodies as magenta to bright red bodies with well-defined dark blue to black inner granules. All parts of the nerve cell stained blue and the interstitial tissue stained pink. Erythocytes stained copper red and could be easily differentiated from magenta tinged red negri bodies.

Immunochromatographic diagnostic test
Test was obtained using commercially available immunochromatographic diagnostic test kit. The immunochromatographic assay employed direct sandwich method wherein 10% homogenate of each brain sample was formed in phosphate buffered saline (PBS). Brain homogenate was collected using a swab and mixed in the diluent tube provided in the test kit. Purple colored band moved across the result window in the center of the test device indicating proper loading of the samples. Results were interpreted within 5-10 min.

Analysis
Test kit revealed positive control band in the left section of the result window and test band in the right section of the window. In positive cases, both positive control color band as well as test sample band appeared, whereas, in negative cases, the test band failed to appear. Absence of positive control band rendered the test invalid.

Flurescent Antibody Technique & RT-PCR
The samples were submitted to Rabies Unit, V.S Hospital, Ahmedabad; Department of Veterinary Microbiology, Veterinary College, Bengaluru; NDDB, Hyderabad; and National Institute of Communicable Disease, New Delhi for further confirmation of rabies. These institutes performed other more sensitive techniques like RT-PCR and Fluorescent Antibody Technique (FAT) for detection of rabies virus.

Table 1: Reported outbreaks and incidences of rabies in Gujarat state along with the number of livestock attacked and died, during the year 2012 to 2017 and confirmation by laboratory test

Sr. No.	Village	Taluka	District	Species of Animal from which Brian sample collected	No. of livestock attacked & died	Detection by			
						Seller's stain	Chromatograhic assay	FAT	RT-PCR
1	Fatehpura	Vijapur	Mehsana	Cattle	7	Yes (A)	Yes (A)	Yes	Yes (E)
2	Kubadharol	Vadali	Sabarkantha	Cattle	29	Yes (A)	Yes (A)		
3	Foolchhatrapura	Kathlal	Kheda	Buffalo	15	Yes (A)	Yes (A)	Yes	Yes
4	Shivrajpur	Jasdan	Rajkot	Buffalo	2	Yes	Yes	Yes	Yes

|									
						(A)	(A)	(B, D)	(E, D)
5	Rati Devadi	Wankaner	Rajkot	Buffalo	3	Yes (A)	Yes (A)	Yes (B, D)	Yes (E, D)
6	Ishanpur	Mangrol	Surat	Cattle	8	Yes (A)	Yes (A)	Yes (B)	
7	Lavet	Mangrol	Surat		13				
8	Badpar	Rajkot	Rajkot		5				
9	Dhoria	Kadi	Mehsana	Buffalo	12	Yes (A)	Yes (A)	Yes (B, C)	Yes (C)
10	Nadasa	Mehsana	Mehsana	Buffalo	2	Yes (A)	Yes (A)	Yes (B, C)	Yes (C)
11	Gokalpura	Mehsana	Mehsana	Buffalo	2	Yes (A)	Yes (A)	Yes (B, C)	Yes (C)
12	Tundav	Unjha	Mehsana	Buffalo	1	Yes (A)	Yes (A)	Yes (C)	Yes (C)
13	Kadrana	Olpad	Surat	Buffalo	9	Yes (A)	Yes (A)	Yes (C)	Yes (C)
14	Dashela	Gandhinagar	Gandhinagar	Buffalo	3	Yes (A)	Yes (A)	Yes (E)	Yes (E)
15	Janjmer	Dhoraji	Rajkot	Buffalo	1	Yes (A)	Yes (A)	Yes (D,)	Yes (C,D)
16	Bilodara	Mansa	Gandhinagar	Buffalo	1		Yes (A)		
17	Kamalpur	Chanasma	Patan	Cattle	1		Yes (A)	Yes (B)	Yes (D)
18	Navabhatvas	Satlasan	Mehsana	Cattle	1		Yes (A)		
19	Pratapgadh	Unjha	Mehsana	Buffalo	1		Yes (A)		
20	Machhava	Kheralu	Mehsana	Buffalo	1		Yes (A)		
21	Samoja	Kheralu	Mehsana	Cattle	1		Yes (A)	Yes (D)	
22	Kuda	Kheralu	Mehsana	Cattle	1		Yes (A)		
23	Badad	Kheralu	Mehsana	Cattle	1		Yes (A)	Yes (D)	
24	Nortol	Kheralu	Mehsana	Cattle	1		Yes (A)		
25	Shahpur	Vadnagar	Mehsana	Buffalo	1		Yes (A)	Yes (C)	Yes (C)
26	Forest Department			Sloth Bear	1		Yes (A)		Yes
27	Kutch		Kutch	Cattle & Buffalo	3		Yes (A)		
28	Tankara	Tankara	Morbi		5		Yes (A)		
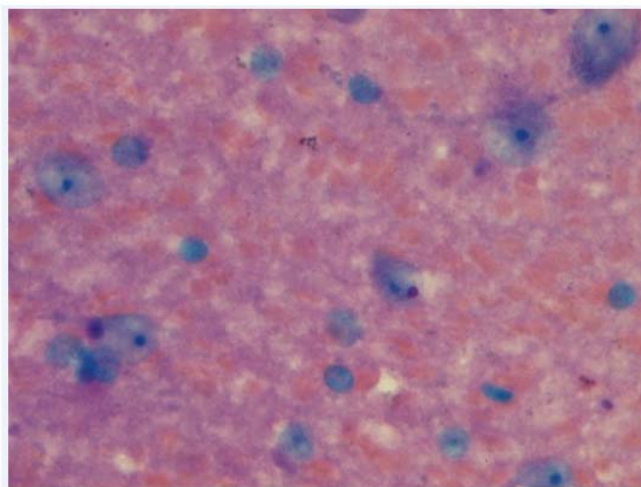
Image 1: Negri bodies in the demonstrated in the cytoplasm of neuron (1000x) by Seller's Stain
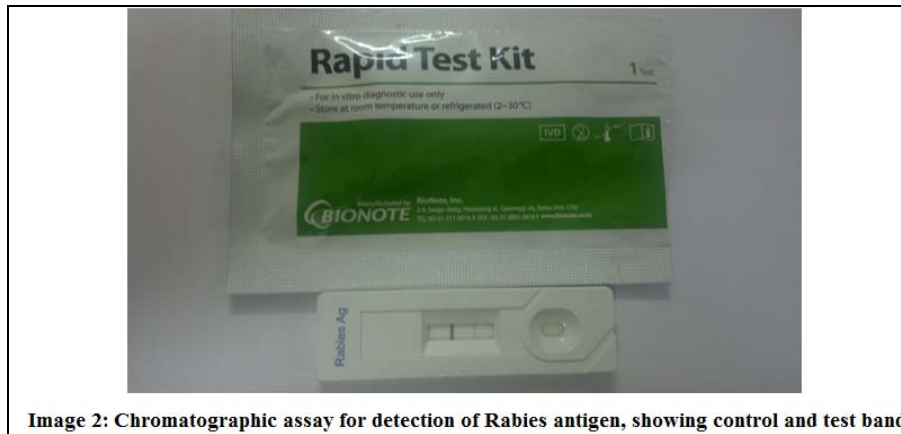
Image 2: Chromatographic assay for detection of Rabies antigen, showing control and test band

Results and Discussion

All the 26 specimens, received for the rabies diagnosis were found positive after subjecting to various diagnostic tests. Smears showing round, oval, discrete sharply demarcated magenta coloured Negri bodies in Seller's Stain were suggestive of rabies infection. Out of 26 specimens received, 13 were subjected to seller's staining as they were collected from recently dead animals. Negri bodies were demonstrated in all the 13 (100%) brain samples (Figure 1). All the 26 samples (100%) revealed positive results when subjected to chromatographic assay suggesting that rabies rapid test kit is good tool for field diagnosis of the rabies giving quick and accurate results (Figure 2). Results of RT-PCR and FAT were also found positive in all the cases thus confirming these outbreaks

The present study evaluated the efficacy of immunochromatographic kit to be used for rapid and accurate diagnosis of rabies under field conditions and found 100% sensitivity and specificity. In the present study, 100% agreement was observed between chromatographic assay, seller's stain, FAT and RT-PCR.

Sharma *et al* [16] evaluated the efficacy of immunochromatographic kit to be used under field condition for rabies diagnosis and obtained 91.7% sensitivity, which is comparatively lesser than the findings of the present study. The study of Sharma *et al* is in complete agreement with the earlier findings [17], when rapid immunodiagnostic test was applied on 54 brain samples. In this study, the performance of the rapid immunodiagnostic test was compared to fluorescent antibody test, and reported 91.7% sensitivity and 100% specificity of immunodiagnostic test [17].

In another study, indirect fluorescent antibody test, virus isolation, reverse transcriptase polymerase chain reaction (RT-PCR), and rapid immunodiagnostic assay were compared to detect rabies and observed the sensitivities of VI, RT-PCR, and RIDA to be 100, 100, and 95%, respectively, and specificities of VI, RT-PCR and RIDA to be 100, 100, and 98.9%, respectively, compared to FAT [18].

Servat *et al* [19] studied the performance of immunochromatographic test as compared to the conventional gold standard method (FAT) and demonstrated an overall specificity of 100% and a sensitivity of more than 88%. A total agreement between the Rapid Immunochromatographic Diagnostic Test and conventional technique results has been obtained.

In another study, Reta *et al* evaluated the sensitivity and specificity of a commercially available Rapid Immunodiagnostic Test kit using 115 brain samples collected from rabies suspected dogs, cats and cattle compared to FAT [20]. Compared to the Fluorescent Antibody Test the sensitivity and specificity of the Rapid Immunodiagnostic Test was found to be 96.5% and 100%, respectively.

Ahmed K. *et al* compared the efficacy of rapid immunochromatographic test with that of FAT [21]. The sensitivity (0.74–0.95), specificity (0.98–1.0), positive predictive value (0.98–1.0), negative predictive value (0.75–0.97), accuracy (0.91–0.98), and kappa measure of agreement (0.79–0.93) were all found satisfactory for animal samples.

Kasempimolporn S. *et al* evaluated an immunochromatographic test strip for *Rabies virus* with dog saliva samples and found 94.4% specificity and 93.0% sensitivity compared to the gold standard fluorescent antibody test [22]. The sensitivity and specificity of a nested polymerase chain reaction (nPCR) assay using saliva were 100% compared to the FAT results. The performance of strip test with field saliva samples from street dogs had a specificity of 98.7% in comparison to nPCR as the reference method.

Panda *et al* examined a total of ten rabies suspected cattle brain sample from different parts of West Bengal, India, through Rapid Immunochromatographic diagnostic test and found one sample to be positive [23]. Their observations stated that RIDT can be employed as a reliable and quick approach for diagnosis and control of rabies under field condition.

Sophisticated techniques for detection of rabies virus require highly equipped laboratories and well-trained personnel. Since rabies is a disease of grave concern, in order to achieve effective control on the disease, rapid and reliable diagnosis is desirable. Immunochromatographic kit can be used for rabies surveillance study and for diagnosis in field condition. As immunochromatographic kit is less time consuming, less hazardous and requires no special equipment and trained personnel to provide results.

Conclusion

For effective diagnosis of rabies under field condition, immunochromatographic kit used in the present study may be used to serve as a tool for rapid diagnosis of rabies, and is also conveniently feasible in the field conditions. However, the samples found negative by the kit may be investigated further by FAT or other molecular approaches for authenticating the diagnosis of rabies.

Author's Contributions

VC, PS, DK and AK designed and performed the experiment, sample collection was performed by DK and SD. FAT was performed under the supervision of PS and BB. RT-PCR was performed under the supervision of SR, GM and BB. Manuscript preparation was supervised reviewed and edited

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