

Evaluation of Various Analytical Methods for Quantification of Rabies Antigen

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Abstract: *Background: Rabies is a zoonotic viral disease caused by rabies virus. Rabies virus has special inclination towards central nervous system (CNS) where it causes life threatening acute encephalitis. Perhaps being extremely fatal, rabies can be 100% prevented by early, effective and complete post exposure prophylaxis (PEP). PEP for rabies include immediate toileting of bite wound, administration of rabies immunoglobulins (severe exposures), and a full course of anti-rabies vaccination (ARV). As ensuring the absence of any variations in the primary nucleotide sequence of the viral backbone after successive propagation in human cell lines is crucial to ensure that the expression vector does not produce variants of the antigen during production of vaccine, the present study was conducted with an aim to evaluate various analytical methods for quantification of rabies antigen.*

Method: Various analytical methods the Western Blot technique, the Sodium Dodecyl Sulphate Agarose Gel Electrophoresis(SDS-PAGE), ELLA method, the Enzyme-linked immunosorbent assay (ELISA) and extinction coefficient were used for quantification of rabies antigen.

Results: When results of these tests were analyzed, it was observed that these analytical methods are easy, cost effective, accurate and most importantly rapid for quantification of rabies antigen.

Conclusion: On the basis of observations noted in this study, Western Blot technique, the Sodium Dodecyl Sulphate Agarose Gel Electrophoresis(SDS-PAGE), ELLA method, the Enzyme-linked immunosorbent assay (ELISA) and extinction coefficient can be reliably recommended as analytical methods for quantification of rabies antigen..

Keywords: Rabies, rabies antigen, quantification, immunological tests, ELISA, ELLA, SDS PAGE

I. INTRODUCTION

Zoonotic diseases are important among various public health concerns. As per definition zoonotic diseases or zoonoses, are infectious diseases that are transmitted naturally between humans and wild or domestic animals.¹ They are caused by a wide range of pathogens including bacteria, viruses, fungi and parasites. As per recent data, zoonotic diseases are responsible for nearly 2.4 billion cases and about 2.7 million human deaths per annum across the world.²

Among various zoonotic diseases, rabies is most dreadful with almost 99% mortality rate. Rabies is a zoonotic viral disease caused by rabies virus. This virus belongs to rhabdovirus family and has negative-stranded RNA genome.³ In man, rabies infection occurs through bite, saliva or scratches from infected animals.⁴ Although various animals like dog, bat, monkey, skunk, raccoon, or fox can transmit rabies, dog is the most important source for human rabies infection.⁵ Rabies virus has special inclination towards central nervous system (CNS) where it causes life threatening acute encephalitis. Incubation period of rabies varies from few days to several years depending on anatomical location and severity of wound, and viral load. Patients may present with symptoms like excitation, solicitude, anxiety, bewilderment, hallucination, and hydrophobia. ² Perhaps being extremely fatal, rabies can be 100% prevented by early, effective and complete post exposure prophylaxis (PEP).⁴ PEP for rabies include immediate toileting of bite wound, administration of rabies immunoglobulins (severe exposures), and a full course of anti-rabies vaccination (ARV).⁶

As compared to other parts of world, India has highest cases of rabies, primarily due to menace of stray dogs.⁷ As per the World Health Organisation (WHO), India accounts for approximately 36% of global rabies death. In India, as per

recent guidelines, the Thai Red Cross Updated Schedule for both intramuscular and intradermal vaccination is followed. This PEP schedule includes administration of a total of eight doses, intradermal (two sites per visit on days 0, 3, 7, 28) or five doses, intramuscularly 0, 3, 7, 14, and 28 days.^{7,8}

Confirming the absence of any variations in the primary nucleotide sequence of the viral backbone after successive propagation in human cell lines is crucial to ensure that the expression vector does not produce variants of the antigen during production of vaccine. The present study was conducted with an aim to evaluate various analytical methods for quantification of rabies antigen.

II. MATERIAL AND METHODS

In the current study various analytical methods for quantification of rabies antigen. These included the Western Blot technique, the Sodium Dodecyl Sulphate Agarose Gel Electrophoresis(SDS-PAGE), ELLA method, the Enzyme-linked immunosorbent assay (ELISA) and extinction coefficient.

Western Blot technique: This technique was performed by using Jess instrument quantification. This method is based on molecular size. The layout of the loading pattern is shown in figure1.

	1	2	3	4	5	6	7	8	9	10	
A	Biot...	Rabies_Std					S1	S2	S3	S4	
		0.5 IU	0.25 IU	0.125 IU	0.25 IU	0.125 IU	1:50	1:50	1:50	1:50	
B	Antibody Diluent										
C	Bloc...	Anti Rabies									
		1:1000	1:1000	1:1000	1:1000	1:1000	1:1000	1:1000	1:1000	1:1000	
D	Stre...	Anti Mouse									
E	Luminol / Peroxide										

Figure 1. Layout of the loading pattern.

The SDS-PAGE: In brief, the glass plates were placed in the gel holder and checked for leakage by the adding water for injection in between the plates. The level of water was observed. Once it was confirmed that the water for injection was not leaking, it was removed from in between the plates with the help of tissue. The resolving gel solution (10%) was thoroughly vortexed and then added in between the two gel plates up to a certain level. Followed by addition of butanol (for making gel preparation even) and allowed to set for 30 min. The butanol was then removed after setting of gel and stacking gel was prepared. Further, dilution of samples was done. After setting of stacking gel, the various dilutions of samples were loaded and then the gel ran at 100 V- 150V.

*ELLA:*ELLA is a combination of performance and automated ELISA workflow. For ELLA 48-Digoxigenin Cartridge was used. Manufacturer instructions for use were adhered to through the test.

Enzyme-linked Immunosorbent Assay (ELISA): Sandwich ELISA was performed for quantification of rabies antigen. It was used to quantitatively assess rabies antigen potency during production of polymeric particles for the single injection (SI) vaccine technology. To explain in brief, the rabies viral vaccine ELISA is a double-sandwich ELISA technique in which the viral glycoprotein in the sample is immobilized between an antibody (mAb, clone 1112-1) adsorbed onto a 96-well plate and a biotinylated mAb (clone D1-25). The immobilized glycoprotein was detected by a 2-step system: streptavidin, coupled to peroxidase ('conjugate'), binds to the biotin (conjugated to the detection mAb D1) and a peroxidase substrate (OPD) was then added to form a colored product in the presence of peroxide. After stopping the reaction with sulfuric acid, the intensity of the colored product (which is proportional to the quantity of RABV GP in

the sample) was measured by spectrophotometry. The manufacturer’s instructions for use were adhered to throughout the entire procedure.

Extinction coefficient: Solo Variable Path length Extension (VPE) was used for Extinction Coefficient establishment study. It is an innovative instrument for UV-Vis-NIR spectroscopy. It provides analysts easy access to another dimension of measurement using the Beer-Lambert Law, specifically fine path length control. The SoloVPE Analysis tool enables the user to perform data regression on the wavelength cross section plot. The linear regression analysis provides three key parameters: the slope and y-intercept of the best fit equation and the coefficient of determination.

VPE software was used to determine a set standard Extinction coefficient by using various protein samples with known protein concentration. This Established Extinction Coefficient was further used to determine unknown protein concentration. Calculations were based on the expected sample concentration and used for determining the level of dilution. If the expected concentration of the sample were unknown, then several scouting runs and experimental dilutions were performed to determine the approximate level of protein present.

III. RESULTS AND DISCUSSION

When 4 different batch samples (S1, S2, S3, and S4) were analysed by Western Blot technique along with a standard, R^2 for standard curve was found to be 0.997. The results of Western Blot technique are shown in figure 2 (complete lane), figure 3 (graphical view of molecular weight marker profile) and figure 4 (sample results).

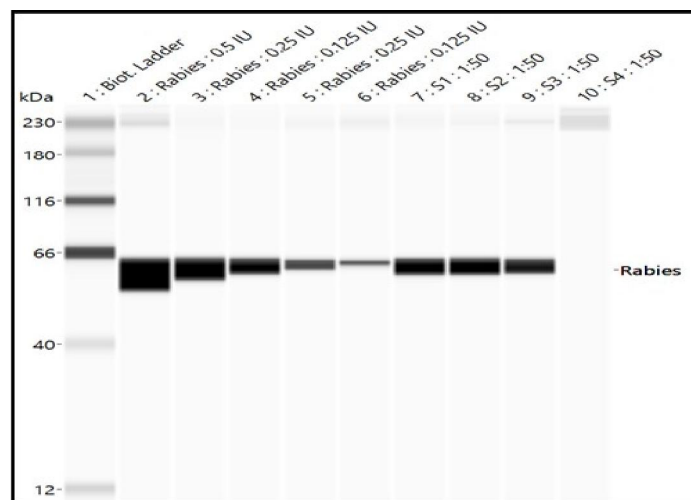


Figure 2: Simple Western: Complete Lane view of results

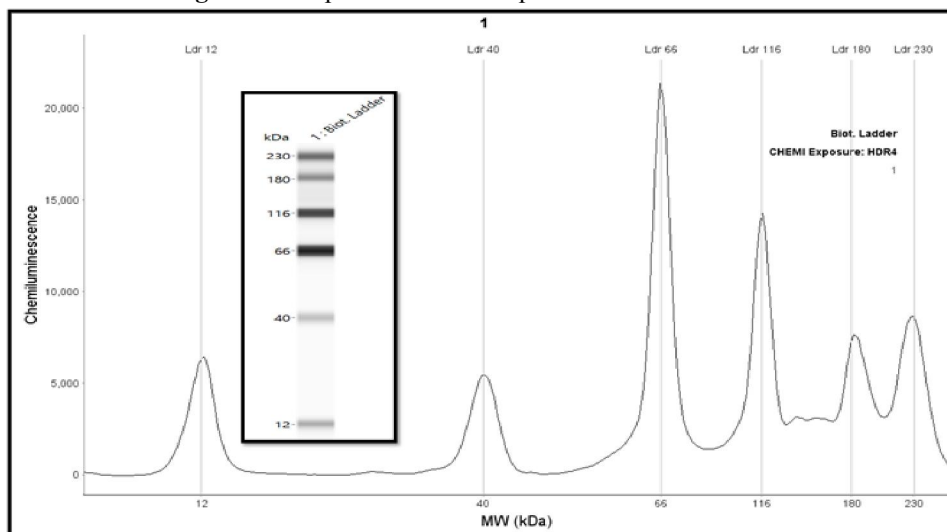


Figure 3: Graphical view of Molecular weight Marker profile.

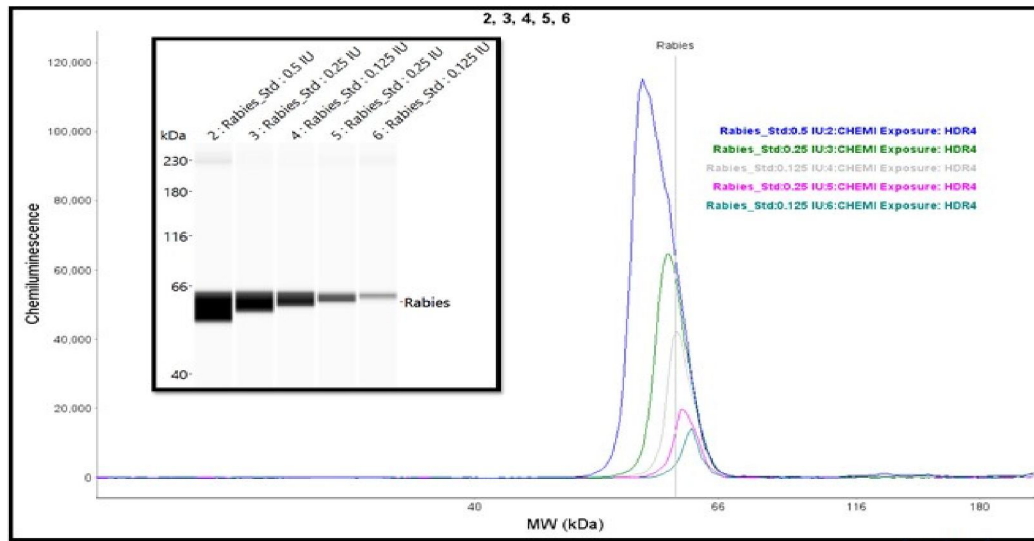


Figure 4: Graphical & lane view: Overlay of ALL 4 Samples results (S1, S2,S3,S4 with 1:50 fold dilution). The bands seen in SDS PAGE are shown in figure 5. All of the sample proteins were mainly in the size range of 75 kDa to 63 kDa.

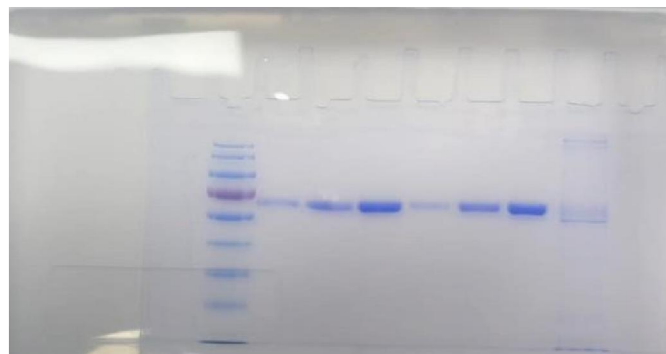


Figure 5: SDS PAGE.

In case of Rabies antigen quantification by ELLA method, the Intra assay precision for Standard between triplicate glass nonreactors (GNR) of sample was less than 5%. The Inter assay precision between 2 different standard curve was less than 10% (table 1). The inter assay precision for all 11 samples analyzed 4 times was less than 10% (The samples were with independent dilution and at different days) (table 2).

Table 1. Inter Assay Precision between 2 Standard Curve of different preparation

Sample Name	GNRs	Mean RFU	RFU %CV
5 IU	3	206.02	2.22
2.5 IU	3	161.6	4.14
1.25 IU	3	123.18	2.26
0.63 IU	3	93.15	2.55
0.31 IU	3	62.75	3.48
0.16 IU	3	53.06	2.53
0.08 IU	3	45.52	2.13

RFU: Relative Fluorescent Unit

Table 2. Standard Curve Sample Concentration (11 Samples- 4 analysis).

Sample ID	Sample Name	1st Replicate	2nd Replicate	3rd Replicate	4th Replicate	Average	SD	%CV
Conc. (IU/ml)	Conc. (IU/ml)	Conc. (IU/ml)	Conc. (IU/ml)					
S1	M067	NA	5.26	5.34	5.01	5.20	0.17	3.31
S2	M068	NA	4.53	4.7	4.68	4.64	0.09	2.00
S3	M069	NA	4.94	5.00	5.36	5.10	0.23	4.45
S4	V1707	NA	3.96	4.29	4.00	4.08	0.18	4.41
S5	V156	NA	9.19	10.1	10.7	10.00	0.76	7.61
S6	V15601	NA	12.6	12.9	12.8	12.77	0.15	1.20
S7	V15001	NA	13.2	12.6	11.1	12.30	1.08	8.79
S1-1	M060	5.39	5.68	5.77	5.16	5.50	0.28	5.07
S2-1	M061	4.35	4.38	4.98	4.38	4.52	0.31	6.75

Figure 6, 7, 8 and 9 shows results of ELISA. The absorbance of the sample and standard was in the range of 0.05-2.00 and the blank's absorbance was less than 0.3. The percentage CV of four of the standards was less than 20%, therefore there were less inconsistencies between duplicates. The regression value of 5 parameter curve was more than 0.98 (r value =1).

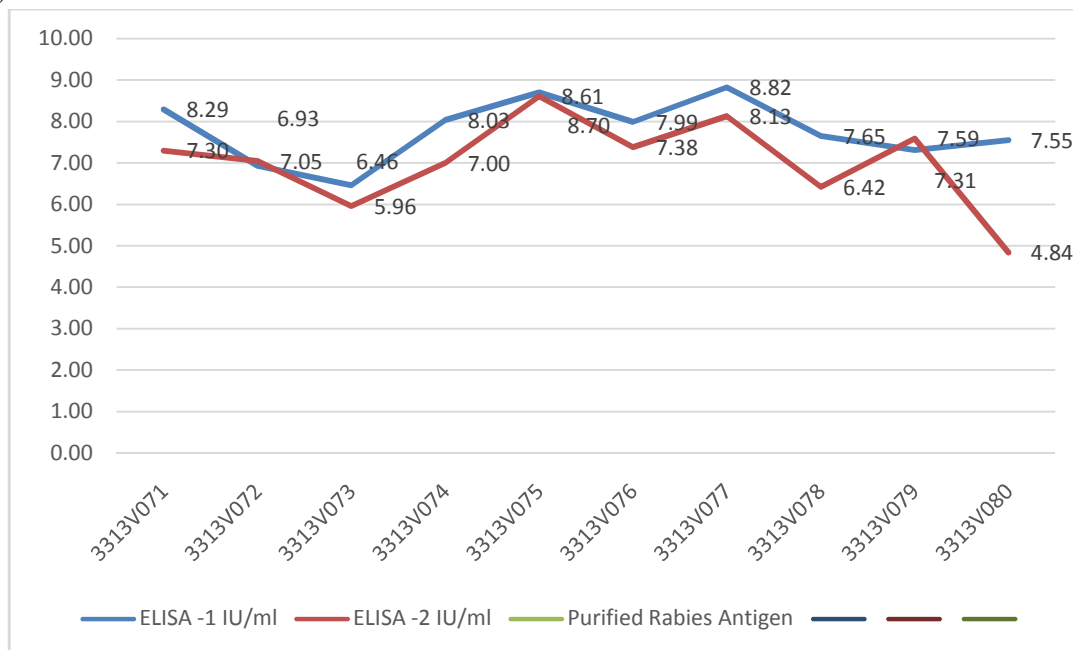


Figure 6: Results of ELISA for lot no 3313V071 to 3313V080.

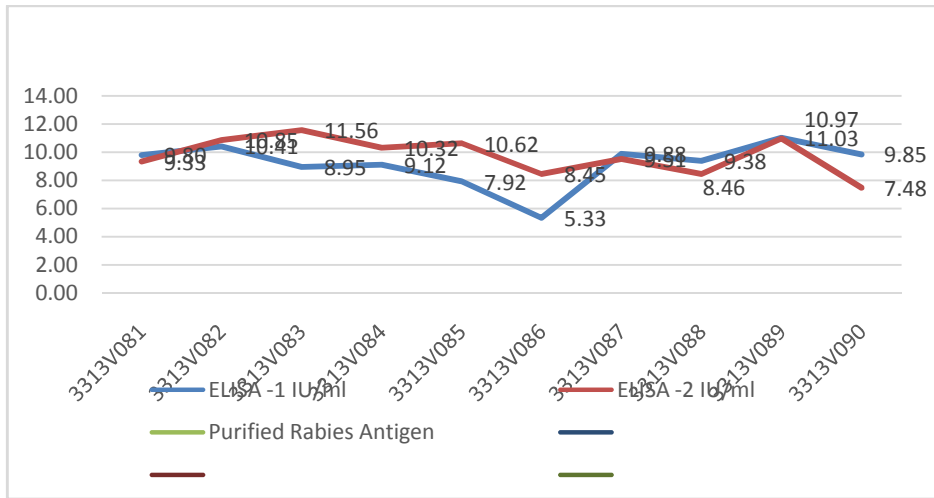


Figure 7: Results of ELISA for lot no 3313V081 to 3313V090.

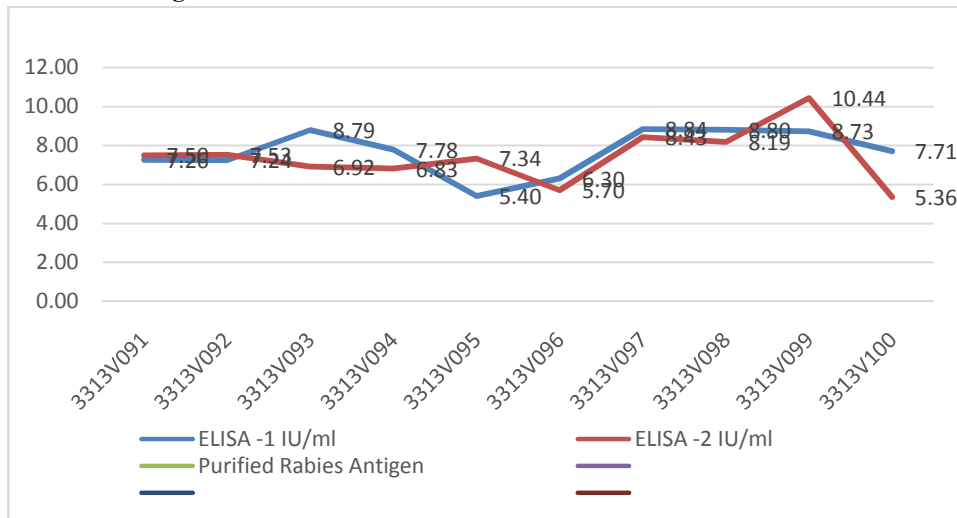


Figure 8: Results of ELISA for lot no 3313V091 to 3313V100.

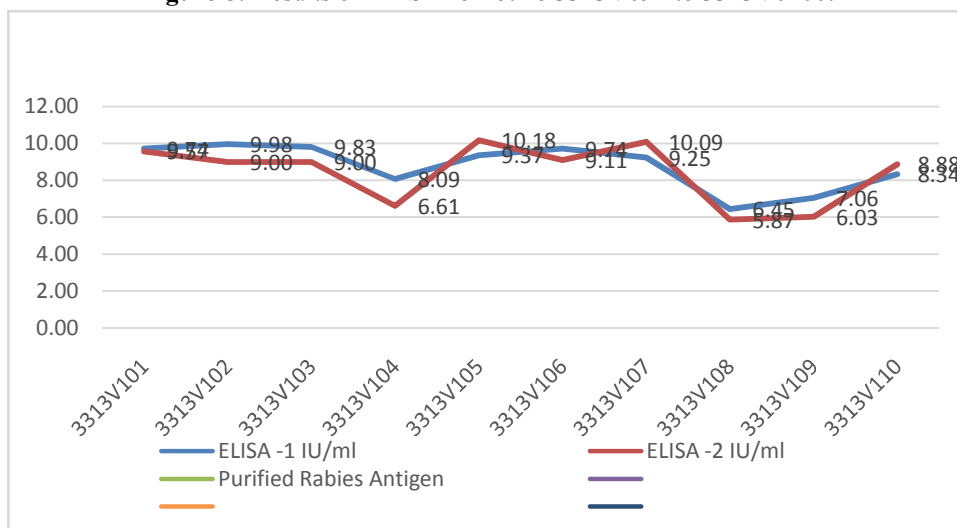


Figure 9: Results of ELISA for lot no 3313V0101 to 3313V0110.

The results of Solo VPE system is shown in table 3. Measurement accuracy is represented through data points taken at multiple path lengths and the R2 value of the regression-line slope, which is typically at least 0.999.

Table 3: Results of Solo VPE system

Samples	Known conc.	EC	Unknown conc.	% RSD	R sq.
1	4.9	1.15	8.2589	0.2367	0.9999
2	5.06	1.15	8.0498	0.4554	0.9999
3	5.01	1.15	8.4532	0.6091	0.999
4	4.98	1.15	7.9665	0.6019	0.9998
5	8.12	1.15	8.0614	0.8306	0.9992
6	7.46	1.15	8.2463	0.0863	0.9999

Rabies disease in human beings can be prevented by using pre and post exposure prophylactic vaccines. In the process of vaccine production, potency testing is an important step that ensures the capacity of vaccines to induce a protective immunity in vaccinated individuals.⁹ Similarly it should also be ensured the absence of any variations in the primary nucleotide sequence of the viral backbone after successive propagation in human cell lines. Historically, potency testing of inactivated rabies vaccines is done by intra-cerebral inoculation in mice. This method was first introduced by the National Institute of Health (NIH) and is still a mandate for release of vaccine.¹⁰ However this method recommended by NIH is cumbersome and time consuming. In addition to other limitations associated with use of mice and virulent strain of rabies virus.¹¹ On the other hand, various immunological, non -animal based techniques are gaining importance in recent years. Experts in the field of agreed upon utilization of immunological methodologies like ELISA, Single Radial Immunodiffusion assay and other tests that are based on antibody binding both for assessing the potency of vaccine and content of antigen in place of NIH method.⁹ In the present study, various analytical methods for quantification of rabies antigen were evaluated. These included Western Blot technique, the Sodium Dodecyl Sulphate Agarose Gel Electrophoresis(SDS-PAGE), ELLA method, the Enzyme-linked immunosorbent assay (ELISA) and extinction coefficient. When results of these tests were analyzed, it was observed that these analytical methods are easy, cost effective, accurate and most importantly rapid for quantification of rabies antigen. Similar observation was noted in the study of Chabaud-Riou *et al* (2017), where ELISA based on was found be capable of distinguishing between potent and various types of sub-potent rabies vaccine lots.⁹ Lin et al (2010) underscored the importance of Western Blot technique in quantification of rabies antigen for vaccine preparation.¹² In the study of Katayama et al SDS PAGE was recommended as an appreciable method for quantification of rabies antigen.

IV. CONCLUSION

On the basis of observations noted in this study, Western Blot technique, the Sodium Dodecyl Sulphate Agarose Gel Electrophoresis(SDS-PAGE), ELLA method, the Enzyme-linked immunosorbent assay (ELISA) and extinction coefficient can be reliably recommended as analytical methods for quantification of rabies antigen.

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