



Direct fluorescent antibody test for determination of $TCID_{50}$ potency for multiple batches of anti-rabies vaccine produced in Nigeria

AN Egbuji^{1*} & IS Tekki²

^{1.} Quality Control Division, National Veterinary Research Institute, Vom, Plateau State, Nigeria

^{2.} Rabies Diagnosis and Research, National Veterinary Research Institute, Vom, Plateau State, Nigeria

*Correspondence: Tel.: +2348035991674; E-mail: wisemenvet@yahoo.com

Copyright: © 2026

Egbuji & Tekki. This is an open-access article published under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Publication History:

Received: 06-08-2025

Revised: 08-01-2026

Accepted: 09-01-2026

Abstract

Strategic control of canine rabies amongst the domestic dog population in endemic regions requires the use of wholesome and potent rabies vaccine, together with strict adherence to a regimented vaccination programme, geared towards eradication. In this study, we applied the Direct Fluorescent Antibody Test (DFAT) to determine Tissue Culture Infectivity Dose₅₀ ($TCID_{50}$) potency values of five batches of dog anti-rabies vaccine produced at the National Veterinary Research Institute (NVRI), Vom, Nigeria. This was carried out after infecting suspension of Baby Hamster Kidney (BHK-21) cells at a concentration of 3.0×10^5 cells per mL in 96-well microtitre plates. Consequently, 100 μ L of serially diluted anti-rabies vaccine was dispensed in each well and incubated at 37°C in 5% carbon dioxide condition for three days. On the fourth day, the $TCID_{50}$ potency values of the vaccines were determined as $\log_{10} TCID_{50}$ 5.6; $\log_{10} TCID_{50}$ 3.5; $\log_{10} TCID_{50}$ 5.5; $\log_{10} TCID_{50}$ 4.2, and $\log_{10} TCID_{50}$ 5.5 per dose, respectively, against the standard reference titre value of $\log_{10} TCID_{50}$ 3.0 per dose. Our findings revealed that DFAT is reliable, rapid, and drastically reduces the time duration for $TCID_{50}$ potency value determination to 96 hours. Application of DFAT enables handling of multiple batches of anti-rabies vaccine concurrently and determining their $TCID_{50}$ per dose value simultaneously. To our knowledge, this is the first multiple assay of rabies vaccine batches in Nigeria using the DFAT. Furthermore, our study attests to the robust nature of the NVRI anti-rabies vaccine. The DFAT should be widely utilized for the rapid determination of anti-rabies vaccine $TCID_{50}$ potency value.

Keywords: Direct Fluorescent Antibody Test, Rabies Vaccine, Tissue Culture, $TCID_{50}$, Nigeria

Introduction

Rabies is a fatal zoonotic disease caused by rabies virus in the genus *Lyssavirus*, family *Rhabdoviridae*, and order *Mononegavirales*. The virus has a non-

segmented, negative-stranded RNA genome and is morphologically bullet-like in shape (Sarau, 1988; Quinn *et al.*, 2002; Acha & Szyfres, 2003; Pal, 2007).

The prototype genotype of Lyssaviruses is the classical rabies virus, genotype-1, which is found throughout the world (Sarau, 1988). Rabies virus transmission occurs via proximate contact of infected saliva through bites or scratches of infected animals (Fekadu *et al.*, 1982; Pal, 2005). Other transmission routes are known to exist; these include: contamination of mucous membranes (e.g., eyes, nose, mouth, etc.), aerosol transmission, corneal and organ transplants. However, transmission or infection through the latter routes is rarely documented (Fekadu *et al.*, 1983; Jackson & Wunner, 2007; Leung *et al.*, 2007). In rural Africa and Asia, where the majority of human cases occur, domestic dogs are the primary reservoir of the virus, and are responsible for up to 99% of rabies transmission to humans (WHO, 2013). It is on record that rabies is responsible for about 24,000 human deaths annually in Africa (WHO, 2001; Knobel *et al.*, 2005). In Nigeria, dog bite is of great public health concern because it is the most common mode of rabies virus transmission to humans and animals (Kolawole *et al.*, 2018). Although the true picture is difficult to determine because of high numbers of unreported cases, the prevalence rate of rabies in Nigeria is between 15% and 20% (Jahun *et al.*, 2012).

It is recommended by the World Health Organization (WHO) that vaccination coverage of at least 70% amongst the domestic dog population should be implemented, and subsequent repeat of vaccinations and re-vaccinations over several annual campaigns, to achieve reasonable rabies control in the endemic regions (WHO, 2013). This level of control coverage is supported by established mathematical models of rabies dynamics (Coleman & Dye, 1996; Kitala *et al.*, 2002; Fitzpatrick *et al.*, 2012). However, a strict rabies vaccination control strategy and programme cannot be achieved without the use of safe, wholesome, efficacious, and potent vaccines that are readily available and rapidly deployed after duly determining the vaccine potency value post-production.

In a bid to control rabies, Nigeria has been producing live attenuated anti-rabies vaccine for vaccination of domestic dogs since 1956. Previously, mouse inoculation (MI) has been utilized in the determination of the TCID₅₀ value of each batch of anti-rabies vaccine produced, prior to passage for use. The MI, being *inv*Asive and slow procedure permits the assessment of only limited batches of the vaccine, its passage and final deployment to field for use. Thus, the rapid deployment of potent rabies vaccine through early and timely vaccine potency

assays prior to vaccine deployment for Veterinary field use, cannot be overemphasized.

Materials and Methods

Ethical clearance

This study focused on simultaneous determination of TCID₅₀ titre value for multiple batches of anti-rabies vaccine by the use of Direct Fluorescent Antibody Technique (DFAT) on BHK-21 cell monolayer. Consequently, ethical approval for use of laboratory animal, was not required.

Rabies vaccine source

Samples of five batches of rabies vaccine produced at National Veterinary Research Institute, Vom, Nigeria, were titrated to determine their TCID₅₀ potency values. The vaccine is lyophilized live attenuated, packaged in a single dose of 2.5ml per vial.

Culture media preparation

Complete cell culture media preparation was conducted according to the Eagle method (Eagle, 1955). Eagle's Minimum Essential Media (EMEM) with supplements were prepared and used for cultivation of BHK-21 cells and for the maintenance of the infected cells. The culture media was prepared by adding 10% heat-inactivated bovine fetal serum, 2% L-glutamine, 2% sodium bicarbonate and 1% antibiotic and antimycotic agent.

Incomplete media preparation and serial dilution of vaccine batches

The method of complete medium preparation as stated above was applied, except that in the incomplete media preparation, addition of 10% heat-inactivated bovine fetal serum was excluded. Consequently, 4.5mL of incomplete media was dispensed in bijou bottles marked 10⁻¹ to 10⁻⁶. The incomplete media was used for the lyophilized vaccine reconstitution and subsequent serial dilution sequel to BHK-21 cell infection in 96-well tissue culture microtitre plates.

Tissue culture infectivity dose 50 (TCID₅₀)

Preparation of the BHK-21 cell suspension consisting of 3.0 × 10⁵ cells per mL was made from confluent of BHK-21 cell monolayer. Confluent monolayer cell on 75 cm³ tissue culture flasks was washed thrice with pre-warmed phosphate buffered saline (PBS), pH 7.2. Afterwards, the washed cell monolayer was run over with 1.5ml of 0.5% trypsin-versine and incubated at 37°C for 5 minutes in a humidified incubator. After incubation, the detached cells were completely

dislodged into single cells by gently hitting the side of flask on the palm for about 10 seconds. The digestive effect of the trypsin-versine on the cell suspension was halted by the addition of 4mL of the complete medium. Cell count was carried out by adding 10 μ L of detached cells to 90 μ L of 0.2% trypan blue in a 1.5mL cryovial. Thereafter, 10 μ L of the 1:10 dilution of the cell and trypan blue were fixed in a Neubauer counting chamber, and viable cells (cells that did not retain the trypan blue stain) were counted using a tally counter. The cell concentration per mL was calculated and determined using the volumetric formula ($C_1V_1 = C_2V_2$) (Rooijackers *et al.*, 1996a). The required cell volumes for the vaccine titration were also determined to be 3.0×10^5 cells per mL. Consequently, 100 μ L of the 3.0×10^5 cells per mL suspension was dispensed into 96-well tissue culture microtitre plates. Cells were dispensed in wells A to F and from wells 1 to 10 in each row. Well 11 was left empty, while wells 12 and H were filled with diluent and uninfected cells as controls, respectively. Prior to titration, the lyophilized vaccines were reconstituted with 1mL of the incomplete medium and vortexed for complete rehydration. Consequently, 0.5 mL of the reconstituted vaccine was dispensed into the bijou bottle marked 10^{-1} , containing 4.5 mL of incomplete medium. Serial dilution of the reconstituted vaccine after rehydration was carried out from dilution 10^{-1} to 10^{-6} (columns A to F). This procedure was repeated for the other four vaccine batches.

Infection of cell in tissue culture plates

The cells in the wells were infected in suspension with the serial dilutions of the vaccine, beginning from dilution 10^{-6} to 10^{-1} i.e., columns F to A of tissue culture microtitre plates, while columns H and all wells in row 12 were used as controls. The plates containing the infected cells were incubated at 37°C in a humidified incubator with 5% CO₂ conditions. This procedure was repeated for the other four vaccine batches. The infected and control wells were viewed daily using a Carl Zeiss inverted microscope to observe for any sign of cell contamination.

Staining of infected BHK-21 Cells

The media covering the cell monolayers was discarded after 72 hours of incubation, and the monolayer was rinsed with 80% acetone. To each infected and control wells 200 μ L of 80% acetone was added using a multi-channel micropipette. These were incubated at -20°C for 30 minutes to fix the infected monolayer in the wells of the plate. After fixation, acetone was discarded, and the plates were

air-dried at room temperature. Subsequently, 50 μ L of working dilution of rabies fluorescein isothiocyanate (FITC) conjugate (Anti-rabies monoclonal globulin; Fujirebio AB, Goteborg, Sweden) was added to each infected well and control well for staining. Thereafter, the plates were incubated in a humidified incubator at 37°C for 45 minutes. Afterwards, the plates were washed thrice with phosphate-buffered saline (PBS) of pH 8.5 according to the manufacturer's instructions. The plates were blotted on a clean absorbent paper towel and air-dried. Subsequently, the infected and control wells were examined under an inverted fluorescent microscope for the presence of brightly coloured, apple-green or greenish-yellow appearance of virus particles against a dark background. Wells that showed fluorescence were considered positive, while wells that did not were considered negative. The amount of observed fluorescence per row for each infected well was determined, using the methods of Spearman-Kärber (Karber, 1931). This was subsequently applied to determine the TCID₅₀ titre for each vaccine batch per dose.

Results

Plates I and II depict the microscopic fields of uninfected and infected BHK-21 cell monolayers, respectively, before staining. Plates III and IV depict images of uninfected and infected BHK-21 cell line monolayers after staining with rabies FITC conjugate 72 hours post-infection and incubation. While there was no presence of characteristic apple-green immunofluorescence in the uninfected control wells, the infected cell monolayer showed an abundance of immunofluorescence, indicating the presence of rabies virus antigen. The TCID₅₀ value of each of the vaccine batches tested (samples A to E) is presented in Tables 1, 2, 3, 4, and 5, respectively, and summarized in Table 6:

Discussion

Canine rabies is endemic in Nigeria, with a reported prevalence rate of about 15% to 20% (Jahun *et al.*, 2012). In an effort to control the rabies scourge in Nigeria, the NVRI, Vom in the last sixty-seven years, has been producing high-quality and potent anti-rabies vaccine for vaccination of domestic dogs. The strategic vaccination control approach or option will be unachievable without a steady release of field-fit, safe, wholesome and potent anti-rabies vaccine for veterinary use post production and quality assessment. Thus, the use of MI, considering its long duration for anti-rabies vaccine titre value

determination, will hinder the steady release of anti-rabies vaccine batches.

This study, which was based on simultaneous multiple screening of rabies vaccine batches using DFAT, revealed that multiple batches of rabies vaccine can be screened concurrently within a span of 96 hours, compared to the MI, which requires a minimum of three weeks of post-infection observation. There was

clear and abundant evidence of rabies virus antigen presence in the infected BHK-21 cells (Plate I) and complete absence of rabies antigen in the control wells Plate II), as obtained after microscopy. The application of the 'Spearman Karber' (Karber, 1931) method (Table 6) showed that all the screened vaccine batches were well within the acceptable $TCID_{50}$ titre value range for rabies vaccine intended



Plate I: Uninfected BHK-cells monolayer before staining with FITC Conjugate



Plate II: BHK- cells monolayer infected with vaccine virus prior to staining with FITC Conjugate



Plate III: Uninfected BHK-21 cell monolayer showing no immunofluorescence appearance after staining with FITC conjugate

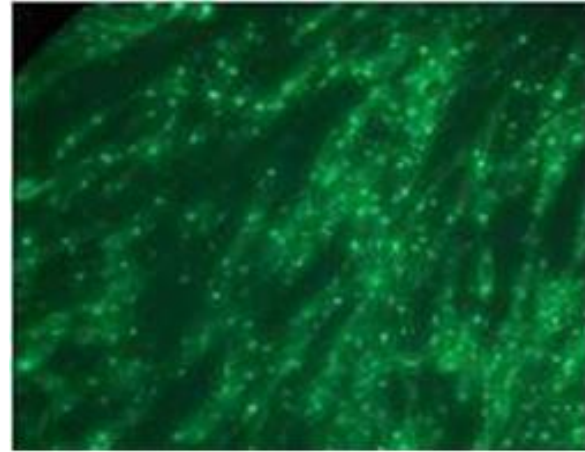


Plate IV: Infected BHK-21 cell monolayer showing apple green Immunofluorescence appearance of rabies virus antigen after staining with rabies FITC Conjugate

Table 1: Determination of $TCID_{50}$ value per dose of vaccine sample A in BHK-21 cell Monolayer using DFAT

Column No. in 96-well tissue culture plate	Log_{10} titre of virus dilution	Observed fluorescence per dilution (%) per well per column	Incubation Duration per dilution in Hours
A	-1	100	72 hours
B	-2	100	72 hours
C	-3	90	72 hours
D	-4	70	72 hours
E	-5	0	72 hours
F	-6	0	72 hours
G	Empty	Empty	Empty
H	Control	0	72 hours

Vaccine Sample A = $TCID_{50}$ 5.6 per dose value

Table 2: Determination of *TCID₅₀* value per dose of vaccine sample B in BHK-21cell Monolayer using DFAT

Column No. in 96-well tissue culture plate	<i>Log₁₀</i> titre of virus dilution	Observed fluorescence per dilution (%) per well per column	Incubation Duration per dilution in Hours
A	-1	100%	72 hours
B	-2	80%	72 hours
C	-3	20%	72 hours
D	-4	0%	72 hours
E	-5	0%	72 hours
F	-6	0%	72 hours
G	Empty	Empty	Empty
H	Control	0%	72 hours

Vaccine Sample B = *TCID₅₀* 3.5 per dose value

Table 3: Determination of *TCID₅₀* value per dose of vaccine sample C in BHK cell Monolayer using DFAT

Column No. in 96-well tissue culture plate	<i>Log₁₀</i> titre of virus dilution	Observed fluorescence per dilution (%) per well per column	Incubation Duration per dilution in Hours
A	-1	100	72 hours
B	-2	100	72 hours
C	-3	100	72 hours
D	-4	90	72 hours
E	-5	10	72 hours
F	-6	0	72 hours
G	Empty	Empty	Empty
H	Control	0	72 hours

Vaccine Sample C = *TCID₅₀* 5.5 per dose dose

Table 4: Determination of *TCID₅₀* value per dose of vaccine sample D in BHK cell Monolayer using DFAT

Column No. in 96-well tissue culture plate	<i>Log₁₀</i> titre of virus dilution	Observed fluorescence per dilution (%) per well per column	Incubation Duration per dilution in Hours
A	-1	100%	72 hours
B	-2	70%	72 hours
C	-3	60%	72 hours
D	-4	40%	72 hours
E	-5	0%	72 hours
F	-6	0%	72 hours
G	Empty	Empty	Empty
H	Control	0%	72 hours

Vaccine Sample D = *TCID₅₀* 4.2 per dose value

Table 5: Determination of *TCID₅₀* value per dose of vaccine sample E in BHK cell Monolayer using DFAT

Column No. in 96-well tissue culture plate	<i>Log₁₀</i> titre of virus dilution	Observed fluorescence per dilution (%) per well per column	Incubation Duration per dilution in Hours
A	-1	100%	72 hours
B	-2	100%	72 hours
C	-3	40%	72 hours
D	-4	40%	72 hours
E	-5	30%	72 hours
F	-6	0%	72 hours
G	Empty	Empty	Empty
H	Control	0%	72 hours

Vaccine Sample E = *TCID₅₀* 5.6 per dose value

Table 6: Rabies Vaccine Batches $TCID_{50}$ per Dose Values Summary

S/N	$TCID_{50}$ per dose value	Remark
1	Vaccine Sample A = $TCID_{50}$ 5.6 per dose value	Pass/Fit
2	Vaccine Sample C = $TCID_{50}$ 5.5 per dose value	Pass/Fit
3	Vaccine Sample C = $TCID_{50}$ 5.5 per dose value	Pass/Fit
4	Vaccine Sample D = $TCID_{50}$ 4.2 per dose value	Pass/Fit
5	Vaccine Sample E = $TCID_{50}$ 5.6 per dose value	Pass/Fit

Note: Rabies vaccine reference $TCID_{50}$ per dose values = $\log_{10} TCID_{50}$ 3.0 per

for field veterinary use. The obtained $TCID_{50}$ titre values were either within or well above the standard recommended $TCID_{50}$ value of 3.0 per dose as required.

In a similar study on six batches of commercial rabies vaccine in the Netherlands, virus glycoprotein (GP) and nucleoprotein (NP) contents were determined in an antigen competitive ELISA (Rooijackers *et al.*, 1996b). In our study, however, we only assayed for NP and recommend that further study be carried out on the GP content of the vaccine. In the study in the Netherlands, it was reported that two of the vaccine types screened did not meet the minimum requirement for potency as expected, whereas in our study, all the vaccine batches assayed met the recommended potency standard requirement. This attests that the anti-rabies vaccine produced in NVRI in Vom, Nigeria, is efficacious and of high quality.

Our findings further revealed that DFAT can be applied simultaneously on batches of rabies vaccine intended for field veterinary use and that the expected vaccine $TCID_{50}$ results can be obtained within 96 hours. The strength of the study is that the potency titre values of multiple batches of anti-rabies vaccine can be determined simultaneously within 96 hours. This favours steady releases of potent anti-rabies vaccines for field veterinary use. Thus, the use of DFAT greatly minimizes time expectancy for $TCID_{50}$ value determination for the anti-rabies vaccine, unlike the conventional MI method. The use of DFAT equally limits possible bio-risks and hazards associated with handling of infected laboratory animals; however, the limitation of the study is that the DFAT kit is quite expensive and requires a steady electric power supply for cold-chain storage and maintenance.

Our study demonstrates the robustness of the potency of the NVRI, dog anti-rabies vaccine. In comparison to MI, the DFAT is faster, and $TCID_{50}$ values for multiple batches of rabies vaccine are accurately determined within the shortest possible time. We therefore suggest that DFAT be employed

widely in the assessment of $TCID_{50}$ values of live attenuated rabies vaccines wherever they are produced and used. Therefore, we recommend that DFAT be used widely for rabies vaccine potency value determination, considering its short turnaround time and safety, especially for live attenuated rabies vaccines. The authors also wish to recommend that governments at all levels should ensure strict adherence to routine vaccination, licensing of dogs by owners and severe penalties for non-compliance. Considering the robust nature of the NVRI anti-rabies vaccine, we further recommend its deployment towards regimented rabies control and eradication programmes in Nigeria and beyond.

Acknowledgement

We appreciate the contribution of Mr. Sati Lokason and Mrs. Fatima Mukaila who assisted with the vaccine sterility check. Miss. Helen Gyan is appreciated for carrying out the vaccine molecular identity assay. Mr. Haruna Rabo, Mr. Ponfa Zhakom Ndem, Mr. Stephen Habila Luka and Mr. Frances Maisaje are also greatly appreciated for diligently reading and recording the fluorescent results for each vaccine batch that were tested.

Funding

No funding was received.

Conflict of Interest

The authors declare no conflict of interest.

References

- Acha PN & Szyfres B (2003). *Zoonoses and Communicable Diseases Communicable to Man and Animals*. Pan American Health Organization, Washington D.C. USA, No.580. Pp 1-2.
- Coleman PG & Dye C (1996). Immunization coverage required to prevent outbreaks of dog rabies. *Vaccine*, doi.10.1016/0264-410x(95)00197-9.

- Eagle H (1955). Minimum essential medium for animal cell culture. *Science*, doi.10.1126/science.122.3168501.
- Fekadu M, Shaddock JH & Baer GM (1982). Excretion of rabies virus in the saliva of dogs. *Journal of Infectious Diseases*, **145**(5): 715–719.
- Fekadu M, Shaddock JH, Chandler FW & Baer GM (1983). Rabies virus in the tonsils of a carrier Dog. *Achieves of Virology*, **78**(1-2): 37–47.
- Fitzpatrick MC, Hampson K, Cleaveland S, Meyers LA, Townsend JP & Galvani AP (2012). Potential for rabies control through dog vaccination in wildlife-abundant communities of Tanzania. *PLoS Neglected Tropical Diseases*, **6**: e1796. doi.10.1371/journal.pntd.0001796.
- Jackson AC & Wunner WH (2007). *Rabies: Scientific Basis of the Disease and Its Management*. Academic Press by Elsevier: London, UK. Pp 341-381.
- Jahun BM, Ehimiyein AM, Audu SW, Orasetin AT & Adawa DAY (2012). Rabies in Nigeria: A new paradigm shift? *Revista de Educação Continuada em Medicina Veterinária e Zootecnia do CRMV-SP*, **10**(2/3): 74.
- Karber G (1931). 50% endpoint calculation. *Archive für Experimentelle Pathologie Pharmakologie*, **162**: 480-483.
- Kitala PM, McDermott JJ, Coleman PG & Dye C (2002). Comparison of vaccination strategies for the control of dog rabies in Machakos District, Kenya. *Epidemiology and Infection*, **129**(01), 215–222.
- Knobel DL, Cleaveland S, Coleman PG, Fèvre EM, Meltzer MI, Miranda ME, Shaw A, Zinsstag J & Meslin FX (2005). Re-evaluating the burden of rabies in Africa and Asia. *Bulletin of the World Health Organization*, **83**(5): 360–368.
- Kolawole GO, Umoh JU, Kia SN & Dzikwi A (2018). Demographic factors influencing the rabies antibody prevalence of dogs in Federal Capital Territory, Nigeria. *Folia Veterinaria*, **62**(3): 9–23.
- Leung AK C, Davies HD & Hon K LE (2007). Rabies, epidemiology, pathogenesis, and prophylaxis. *Advances in Therapy*, doi.10.1007/BF02877781.
- Pal M (2005). Importance of zoonoses in public health. *Indian Journal of Animal Sciences*, **75**(5): 558-591.
- Pal M (2007). *Zoonoses*. Second edition. Satyam Publishers, Jaipur, India. Pp Pp 234-235.
- Quinn PJ, Markey BK, Carter ME, Donnelly WJC & Leonard FC (2002). *Veterinary Microbiology and Microbial Disease*. Blackwell Science, Oxford, UK. Pp 668-675.
- Rooijackers EJM, Nieuwenhuija JHM, Vermeulen AA & va Steanis G, Osterhaus ADME (1996a). Potency of veterinary rabies vaccines in the Netherlands: A case for continued vigilance. *Veterinary Quarterly*, doi.10.1080/01652176.1996.9694637.
- Rooijackers EJM, Uttenbogaard JP, Groen J & Osterhaus ADME (1996b). Rabies vaccine potency control: comparison of ELISA system for antigenicity testing. *Journal of Virological Method*, **58**(1-2): 111- 119.
- Sarau P (1988). History of rabies: Advances in research towards rabies prevention during the last 30 years. *Reviews of Infectious Diseases*, **10**(Supplement_4), S581-S584.
- WHO (World Health Organization) (2001). Rabies Fact Sheet No.99 Geneva WHO pp 4.
- WHO (World Health Organization) (2013). *World Health Organization Expert Consultation on Rabies*. World Health Organization Technical Report Series **931**: 1–88.