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# Utilization of antibodies from experimental animals immunized with rabies vaccines for the development of in-house rabies ELISA kits: A preliminary study

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## Abstract

Rabies is a fatal zoonotic disease requiring prevention through early detection, such as via the Enzyme-Linked Immunosorbent Assay (ELISA). This study aimed to develop a homemade sandwich ELISA using antibodies against rabies vaccines. Local chicken anti-rabies antibodies served as the capture antibody, local rabbit anti-rabies as the secondary antibody, and goat anti-IgY conjugated with horseradish peroxidase as the detector. The assay was tested on commercial rabies vaccines—Defensor® 3, Nobivac, Rabisin, and a mixture of the three—as substitutes for natural rabies antigens. Results demonstrated that the assay could detect these vaccines with positive reactivity compared to a BSA negative control. Nobivac yielded the highest titer, followed by the vaccine mixture, Defensor, and Rabisin. Further validation is required to assess the assay's specificity and sensitivity, particularly in comparison to natural antigens and commercial ELISA kits.

**Keywords:** Rabies Vaccine; IgY Antibody; ELISA; *Rhabdoviridae*; *Lyssavirus*

## 1. Introduction

Rabies remains a major public health concern, posing a significant threat to both human and animal populations in over 150 countries and territories, particularly in Asia and Africa (WHO, 2024). It is a viral zoonotic disease classified as a neglected tropical disease (NTD), responsible for tens of thousands of human deaths each year—approximately 40% of which occur in children under the age of 15 (1,2). Despite the availability of effective vaccines and immunoglobulin therapies, access to these interventions is limited in many low-income regions. As of 2018, the estimated average cost of rabies post-exposure prophylaxis (PEP) was US$ 108, excluding indirect costs such as travel expenses and lost income an economic burden for individuals earning only US$ 1–2 per day (1).

Preventive measures have largely relied on the vaccination of animal reservoirs, particularly those carrying rabies virus from the *Rhabdoviridae* family and *Lyssavirus* genus (3). Ongoing research into rabies vaccine development continues to advance, with several vaccines already in use and recognized for their safety and efficacy in both humans and animals (4,5). In parallel, significant progress has been made in the field of rabies diagnostics. Surveillance and early detection are critical components of disease control strategies. Diagnostic approaches now range from immunoassay-based methods to advanced molecular techniques, including RT-PCR, qPCR, dRIT, dFAT, and LFA, all of which offer high sensitivity and specificity for rapid virus detection (6).

In our study, we focus on the development of an antibody-based diagnostic tool for rabies. We generated polyclonal antibodies using locally sourced chickens (7) and rabbits from Mataram, Lombok Island, in the West Nusa Tenggara Province of Indonesia. These antibodies were produced using commercially available rabies vaccines that are routinely administered to domestic animals. Here, we present our findings on the successful generation of these antibodies,

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evaluate their immunogenic properties, and assess their potential application in the development of a diagnostic kit for the detection of rabies antigens.

## 2. Materials and methods

### 2.1. Antibodies and Antigens

In this study, antibodies produced in-house were used, specifically purified antibodies from chicken serum that had been vaccinated three times with the commercial rabies vaccine Defensor®. Additionally, rabies antigen-capturing antibodies were used, which were homemade antibodies purified from rabbit serum that had been vaccinated with the rabies vaccine Nobivac®. The chickens and rabbits used in this study were sourced from local farms in Mataram, Lombok, West Nusa Tenggara, Indonesia.

All activities involving laboratory animals in this study were conducted in accordance with the guidelines set forth by the Animal Ethics Committee of the Faculty of Animal Science, University of Mataram, No. 362/UN18.F7/ETIK/2023.

Antibodies from chicken serum were purified using a combination of caprylic acid and ammonium sulfate salt (7). Meanwhile, antibodies from rabbit serum were isolated and purified using a combination of ammonium sulfate and protein an affinity column (8).

The commercial antibody used was Goat anti-Chicken IgY (H+L) Secondary Antibody, HRP (Invitrogen, USA). For immunogenicity testing, three commercially available rabies vaccines were used: Defensor® 3 (Zoetis), Nobivac® Rabies, and Rabisin.

### 2.2. ELISA-based Testing Process

The testing process for antibody immunoreactivity against rabies antigen was performed using a homemade indirect sandwich ELISA modified from (9–11) using a 96-well high-binding ELISA microplate with 8-well strips and flat-bottom wells. Each well of the plate was coated with 10 µg/ml (100 µl per well) of antibodies isolated and purified from rabbit serum vaccinated with Nobivac® vaccine in carbonate buffer (pH 9.5), as the rabies antigen capture antibody. After incubation overnight at 4°C, each well was washed three times using PBS containing 0.05% Tween 20 (PBST), then the wells were blocked using PBST containing 2% skimmed milk. After blocking for 60 minutes at 37°C, each well was washed again three times using PBST. Next, the vaccines Defensor® 3, Nobivac® Rabies, Rabisin, and a mixture of the three were diluted using phosphate-buffered saline (PBS) to final dilutions of 50 and 500 times each, and then each of diluted antigen was added 50 µl per well, and incubated for 60 minutes at 37°C. After three washes, each well was filled with purified Chicken anti-vaccine Defensor® 3, 50 µl per well, and incubated again at 37°C for 60 minutes. After three washes, each well was filled with Goat anti-Chicken IgY (H+L) secondary antibody-HRP, 50 µl per well, and incubated again at 37°C for 60 minutes. Finally, after three washes, each well was filled with 50 µl/well of TMB substrate and incubated at room temperature in the dark for 10–20 minutes, then the reaction was stopped using 50 µl of $H_2SO_4$ per well, followed by reading at 450 nm.

### 2.3. Data Analysis

Quantitative data were analyzed using simple statistical analysis, with the help of Microsoft Excel 365. Meanwhile, information in the form of images was analyzed descriptively. To determine the positive or negative value of the ELISA results, it was done based on the cut off value, with the formula of the mean value of the negative control plus three times the standard deviation value of the negative control, as suggested by Lunn et al. (12) and Lardeux et al. (13)

## 3. Results and discussion

This study is essentially a continuation of our previous research, which showed that chickens vaccinated with commercial rabies vaccines can produce antibodies against rabies vaccines (7). Based on this information, we intend to explore the possibility of creating a homemade ELISA to detect rabies antigens. In this series, we utilized local rabbits to produce antibodies against rabies, which we designed as antibodies to capture rabies antigen in an ELISA format. Since we already had antibodies against rabies from the vaccination of chickens using the Defensor vaccine, we used the Nobivac vaccine to expand the range of responses that could be produced by different species (14). Our preliminary study results indicate that local rabbits have the potential to produce antibodies against Nobivac. After the booster, rabbit serum was harvested, and antibodies against Nobivac were isolated and purified from the serum using a

combination of ammonium sulfate salt and Protein A affinity columns. The purification results, as shown in Figure 1, demonstrate adequate purity levels (Lane 2) relative to unpurified antibody (Lane 1)

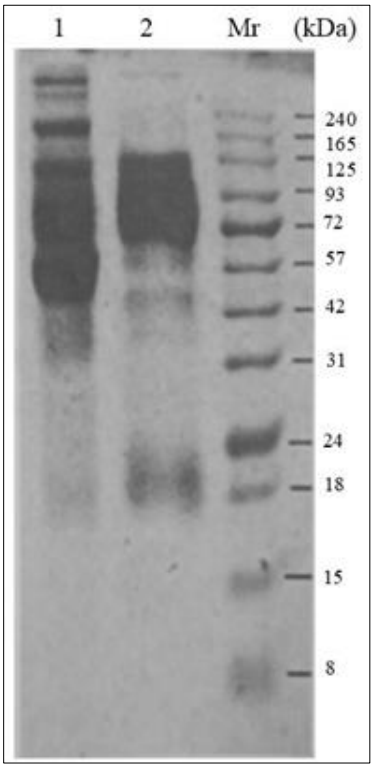


**Figure 1** Illustration of the purity level of rabies antibodies isolated from the serum of rabbits vaccinated with Nobivac on 10% SDSPAGE

(Lanes: 1 unpurified-, 2 purified antibodies; Mr: molecular weight marker, GangNam-STAIN™)

As per the initial design, antibodies isolated from rabbit serum were used as rabies antigen capture in a sandwich ELISA format, while antibodies isolated from chickens were used as secondary antibodies. In this study, we did not label the secondary antibody but instead utilized a commercial product, namely antibodies against Chicken IgY (H-L) labeled with horseradish peroxidase (HRP) as the detector. Due to the limitations of facilities for testing natural rabies samples from victims, during the development of the ELISA format, rabies antigen from a commercial vaccine was used, with results as presented in Table 1.

**Table 1** Results of testing three commercial rabies vaccines as "replacements" for natural rabies antigens, using the ELISA developed in this study

| **Rabies vaccine (as a "replacement" antigen)** | **OD450 value (n=3)** | | **Cut off value (CoV)*** | **Decision** |
|---|---|---|---|---|
| | Mean | SE | | |
| Defensor®3 (D) | 0.675 | 0.017 | 0.607 | (+) |
| Nobivac® (N) | 2.132 | 0.027 | 0.607 | (+++) |
| Rabisin (R) | 0.648 | 0.054 | 0.607 | (+) |
| Mix of D, N and R | 1.206 | 0.044 | 0.607 | (++) |
| Negative control (BSA) | 0.519 | 0.029 | 0.607 | (-) |

*) CoV: Calculated based on the mean value of the negative control plus three times of the standard deviation of the negative control (12,13). BSA: bovine serum albumin; (+): positive results, and (-) negative results.

From table 1, it can be seen that in general the ELISA format developed in this study worked adequately as reflected by the significant difference between positive samples and negative controls. The highest positive value was produced

when Nobivac was used as a substitute for the natural rabies antigen, while the other two vaccines (Defensor® and Rabisin) showed low positive values. The low value for the Rabisin vaccine can be understood because the ELISA format developed does not involve antibodies from Rabisin directly, but rather antibodies to Nobivac as a catcher and Defensor® as a secondary antibody. Interestingly, the value produced by Defensor is similar to Rabisin, this result needs to be studied further. The mixture of the three vaccines showed predictable results, which were between the values of Nobivac and the other two vaccines. When examined from the absorbance value of OD 450, it is generally similar to the OD450 value obtained by Yuliani et al. (15) who analyzed natural rabies antigen from saliva and brain using monoclonal antibody protein G rabies by indirect double antibody sandwich ELISA technique. What needs attention is that Yuliani and her colleagues (15) analyzed the natural antigen and conducted an analysis of the antigen series dilution. This analysis needs to be done in the future because it has not been done in this study.

## 4. Conclusion

This preliminary study developed a homemade sandwich ELISA using local chicken and rabbit anti-rabies antibodies to detect rabies antigens. Commercial vaccines—Defensor® 3, Nobivac, Rabisin, and their mixture—were used as antigen substitutes tested using the developed homemade ELISA. The assay successfully detected all vaccines, with Nobivac yielding the highest titer, followed by the mixture, Defensor, and Rabisin. Further validation is required to evaluate specificity and sensitivity, particularly against natural rabies antigens and in comparison to commercial ELISA kits.

### *Disclaimer*

This study did not aim to evaluate commercial vaccines; their use was solely due to limited access to facilities equipped to handle natural rabies antigens.

## Compliance with ethical standards

### *Disclosure of conflict of interest*

No conflict of interest to be disclosed.

### *Statement of ethical approval*

All activities involving laboratory animals in this study were conducted in accordance with the guidelines set forth by the Animal Ethics Committee of the Faculty of Animal Science, University of Mataram, No. 362/UN18.F7/ETIK/2023.

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