

## Original Papers

# Evaluation of Rapid Neutralizing Antibody Detection Test against Rabies Virus in Human Sera

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**Abstract:** Rapid and easy determination of protective neutralization antibody (NAb) against rabies in the field is very important for an early and effective response to rabies in both animal and human health sectors. The rapid neutralizing antibody detection test (RAPINA), first developed in 2009 and then improved in 2012, is a quick test allowing detection of 0.5 IU/ml antibodies in human and animal sera or plasma. This study aimed to assess the RAPINA test by comparison with rapid focus fluorescence inhibition test (RFFIT), using 214 sera of vaccinated and unvaccinated professional dog butchers, laboratory workers and rabies patients in Vietnam. The sensitivity, specificity, false negative rate, false positive rate and concordance of the RAPINA test as compared to RFFIT were 100%, 98.34%, 0%, 1.66% and 98.6%, respectively. The positive predictive value and negative predictive value were 91.7% and 100%, respectively when RAPINA test was used. With its remarkable sensitivity, specificity and easy implementation, RAPINA test can be used for rapid determination of NAb in the field.

**Key words:** rapid test, immunochromatography, rabies, neutralization antibody

## INTRODUCTION

Rabies is a zoonotic disease mainly transmitted to humans through direct contact with an infected animal [1]. The rabies virus presents in a diverse range of animal reservoirs and transmitters including dogs, cats, bats and wild carnivores [1, 2]. The virus has been detected in more than 150 countries, territories, and all continents except a few countries and Antarctica [1]. In humans, the virus infects the peripheral nerves and spreads to the central nervous system resulting in encephalomyelitis and hydrophobia which are the most classical clinical signs of rabies. Once clinical signs appear, fatality is almost 100% [2].

In Vietnam, rabies remains a serious problem with approximately 100 human deaths and 400,000 people receiving rabies post-exposure prophylaxis annually. Human

rabies has been mainly seen in 30/63 provinces/cities throughout the country, particularly poor rural and mountainous areas [3, 4]. The main transmitters are dogs (95–97%) followed by cats [3, 4]. From 2008 to 2013, a total of 497 human rabies deaths were reported in Vietnam. Of the total rabies deaths, 22 victims (4.4%) were atypically exposed to rabies virus via the butchering and processing of dog meat [3, 4]. Several measures are available, including the mass vaccination of dogs which offers a safe and effective means to control rabies [1]. However, only about two million dogs are vaccinated nationally in annual dog vaccination campaigns, equivalent to less than 40% of the estimated dog population [3, 5]. As a result, pre-exposure prophylaxis (PreP) for people at high risk of rabies and post-exposure prophylaxis (PEP) for people bitten by rabies-suspected animals are the most common interven-

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**Abbreviations:** FAVN, fluorescent antibody virus neutralization; IU/ml, international unit per milliliter; NAb, neutralization antibody; OIE, World Organization for Animal Health; PreP, pre exposure prophylaxis; PEP, post exposure prophylaxis; RAPINA, rapid neutralizing antibody; RFFIT, rapid fluorescent focus inhibition test; RIG, rabies immunoglobulin; WHO, World Health Organization.

tion methods currently implemented in Vietnam. Annually, millions of anti-rabies vaccine doses and rabies immunoglobulin (RIG) are consumed. However, anti-rabies vaccine and RIG are limited in availability and supply. Therefore, rapid screening of neutralization antibodies (NAb) in animals and people with occupational exposure such as laboratory workers, clinicians, veterinarians and professional dog butchers facilitates as to whether to give booster vaccinations or a full course of PEP. This decision can help to eliminate the unnecessary use of RIG and vaccine. Given this scenario, an easily performed and rapid technique that does not require expensive equipment is needed.

The rapid neutralizing antibody detection test (RAPINA) was first developed and evaluated by Shiota et al. in 2009. The sensitivity and specificity of the first version was 88.7% and 91.9%, respectively in comparison with RFFIT [6]. The RAPINA test was further improved in 2012 by Nishizono et al., and the second version was evaluated using dog and human sera collected in Japan, Sri Lanka and Thailand [7]. The sensitivity, specificity and accuracy of the second RAPINA test compared with RFFIT was found to be 99.5%, 98.6% and 98% respectively, much higher than those of the first version [6, 7]. However, in order to apply the RAPINA test widely for NAb detection, it is important to evaluate the test in different target populations, geographical regions and laboratories. Our purpose is to evaluate the second version of RAPINA test using sera of vaccinated and unvaccinated professional dog butchers, laboratory workers and rabies confirmed patients in Vietnam.

## METHODS

### Sample collection

This study was approved by the ethics committee of the National Institute of Hygiene and Epidemiology (NIHE), Hanoi, Vietnam. All sample collection and experimental procedures complied with the institute guidelines for human blood collection and use. A total of 214 human serum samples collected in 2013 (117 males, 97 females, mean age: 35.1 years) were used to evaluate the RAPINA test. Of the 214 serum samples, 187 were collected from professional dog butchers working at slaughterhouses in Hanoi (102 males, 85 females, mean age: 36.1 years), seven from rabies laboratory-confirmed patients and 20 from laboratory workers at NIHE (Hanoi) and the Pasteur Institute (Ho Chi Minh). Among the 214 human sera collected, 62 and 152 samples were obtained from vaccinated and unvaccinated people, respectively (Table 1). All samples were heat inactivated at 56°C for 30 minutes and kept at

Table 1. Characteristics of samples used for evaluation of RAPINA

Sample origin	Vaccination history		Total
	Vaccinated	Unvaccinated	
Professional dog butchers	52	135	187
Rabies confirmed patients	0	7	7
Laboratory workers	10	10	20
Total	62	152	214

minus 30°C until used.

Information regarding vaccination history was obtained through a structured interview questionnaire on the type of vaccine (cell culture or nerve tissue derived vaccines), schedule of vaccination, number of doses administered and the duration from receiving the last dose of vaccine to the time of the interview and serum sample collection.

### Laboratory testing

The evaluation of RAPINA was implemented by comparison with rapid focus fluorescence inhibition test (RFFIT), approved as a standard method for determination of neutralizing antibody levels against rabies by the World Health Organization (WHO) [1].

### Rapid Focus Fluorescence Inhibition Test

The RFFIT test was performed according to the Standard Procedure of WHO Laboratory Techniques in Rabies, 1996 [1, 8]. Briefly, 2 IU/ml standard serum and test sera were diluted three fold in an 8-well Lab-Tek TC Chamber Slide, then an equal volume of challenge virus, CVS11 (ATCC VR-959), was added to all serum dilutions. The chamber slides were then incubated at 35°C, 5% CO<sub>2</sub> for 90 minutes. After incubation, mouse neuroblastoma cell suspension containing  $1 \times 10^5$  cells/0.2 ml was added to each chamber. The chamber slides were further incubated for 20 hours and fixed with cold acetone. The fluorescence antibody staining was performed using fluorescence isothiocyanate conjugated with anti-rabies nucleoprotein monoclonal antibody (Fujirebio Diagnostics, Inc., Malvern, PA). Twenty microscopic fields were observed at 160 magnification for each dilution serum. The 50% end-point dilution of test sera and standard serum were obtained by the Spearman—Kärber formula. The antibody titer in the test serum (in IU/ml) was calculated by comparing the 50% end-point dilution of test serum with that of 2 IU standard serum. The 50% end-point dilution of 2 IU standard serum ranged from  $10^{-2.34}$ – $10^{-2.39}$  (equal to dilution from 1:218 to 1:245).

### Rapid Neutralizing Antibody Detection Test

RAPINA is an in vitro diagnostic immunochromato-

graphic test allowing detection of 0.5 corresponding international unit per milliliter (IU/ml) antibodies in human and animal sera or plasma [6, 7]. The test was performed according to the guidelines of the manufacturer. In brief, 60  $\mu$ l of heat-inactivated serum was mixed with an equal volume of inactivated CVS11 virus contained in the test kit. The virus, serum mixture was then incubated at 37°C for 30 minutes. After incubation, 100  $\mu$ l of the virus, serum mixture was added to the hole of the card test, and the results were obtained exactly 15 minutes later. The serum was considered to have  $\geq 0.5$  IU/ml of neutralization antibodies against rabies virus if only one band appeared at the control line. The serum was considered to contain neutralization antibodies against rabies virus under 0.5 IU/ml if two bands at both the control and test lines were observed (Fig. 1).

The results would be considered invalid or discordant if an invalid RAPINA test result was shown (Fig. 1) or a difference of NAb levels in test serum was obtained from

RFFIT and RAPINA tests. All samples showing invalid or discordant results were repeated at the rabies laboratory (NIHE) and concurrently sent to the microbiology laboratory of Oita University, Japan for confirmation. If the test results obtained from these two laboratories were still different, the samples were sent to the WHO reference rabies laboratory for confirmation. In that case, the final test result would be the one reported by the WHO reference laboratory.

The technical criteria of the RAPINA test such as sensitivity, specificity, false positive, false negative rates, positive prediction and negative prediction values were compared with RFFIT by the formula of the National Association of Testing Authorities (NATA), Australia [9].

#### Data analysis

SPSS software, version 16.0 was used for data input and analysis.

## RESULTS

Of 214 sera tested, 33 and 178 sera were found to have NAb  $\geq 0.5$  IU/ml and  $< 0.5$  IU/ml, respectively by both RAPINA and RFFIT. Three serum samples were positive (Nab  $\geq 0.5$  IU/ml) by RAPINA, but were determined to have NAb  $< 0.5$  IU/ml when tested by RFFIT (Table 2). Of the three samples with discordant results between RAPINA and RFFIT methods, two samples were obtained from people who were vaccinated with Fuenzalida vaccine (suckling mouse brain derived rabies vaccine) more than six years before testing NAb, while the remaining sample was collected from an unvaccinated person (Table 3). The three samples with discordant results were retested at the rabies laboratory, NIHE, Vietnam and concurrently sent to Oita Medical University for confirmation. The results obtained from the two laboratories were the same as, and consistent with, the first test results of NIHE laboratory.

### <EXAMPLE>

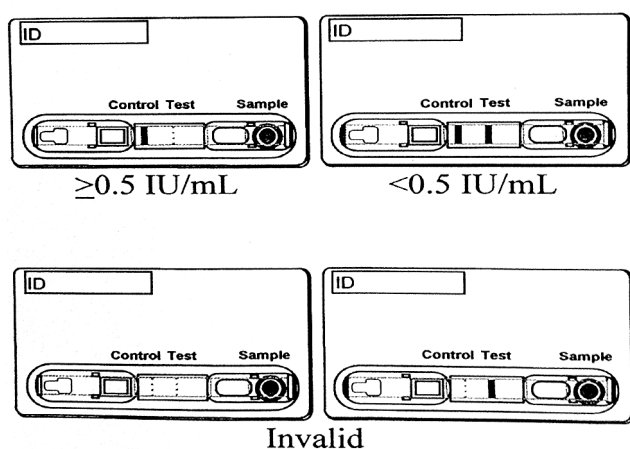


Fig. 1. Interpretation of RAPINA test

Table 2. The comparison of RAPINA with RFFIT

RFFIT	RAPINA		Total
	Antibody titer $\geq 0.5$ IU/ml	Antibody titer $< 0.5$ IU/ml	
Antibody titer $\geq 0.5$ IU/ml	33	0	33
Antibody titer $< 0.5$ IU/ml	03	178	181
Total	36	178	214

Sensitivity =  $33/33 \times 100 = 100\%$

Specificity =  $178/(178 + 3) \times 100 = 98.34\%$

False negative rate = 0%

False positive rate =  $3/(178 + 3) \times 100 = 1.66\%$

Positive predictive value =  $33/(33 + 3) \times 100 = 91.7\%$

Negative predictive value =  $178/178 \times 100 = 100\%$

Concordance rate =  $(178 + 33) \times 100/214 = 98.6\%$

Table 3. The discordant data of neutralization antibody levels against rabies virus obtained by RAPINA and RFFIT

Sample origin	History of vaccination	Results	
		RFFIT (IU/ml)	RAPINA
Professional dogs butchers			
Subject 1	Vaccinated with Fuenzalida* vaccine	0.18	Pos
Subject 2	Unvaccinated person	0	Pos
Subject 3	Vaccinated with Fuenzalida vaccine	0	Pos
Rabies patients			
Subject 4	Unvaccinated person	0.19	Neg
Subject 5	Unvaccinated person	0.39	Neg

\*Fuenzalida – a suckling mouse brain derived rabies vaccine.

Pos: Positive (NAb  $\geq$  0.5 IU/ml)

Neg: Negative (NAb < 0.5 IU/ml)

Of the 52 samples collected from vaccinated professional dog butchers, only eleven samples (21.15%) had NAb level  $\geq$  0.5 IU/ml and 45 serum samples had NAb level < 0.5 IU/ml by both methods. Among these 45 serum samples, three samples had NAb from 0.10 to < 0.5 IU/ml, and 38 people were negative for NAb against rabies virus (NAb < 0.1 IU/ml) when tested by RFFIT. None of the professional dog butchers had ever received PreP against rabies, but they had received either complete or incomplete courses of Fuenzalida vaccine more than six years before participating in the study.

Five out of seven sera collected from rabies-confirmed patients had NAb  $\geq$  0.5 IU/ml, but two samples (subjects 4 and 5) had NAb < 0.5 IU/ml by both methods and showed NAb equal to 0.19 and 0.39 IU/ml by RFFIT (Table 3). However, if 0.5 IU/ml was used as the detection cut-off value, the concordance of RAPINA test was 100% (7/7) in comparison with RFFIT. None of the rabies-confirmed patients had received a dose of anti-rabies vaccine previously.

Among 20 laboratory workers, ten had NAb  $\geq$  0.5 IU/ml and the ten others were negative for NAb. All vaccinated laboratory workers had sufficient protective NAb.

Generally, when compared with RFFIT, the sensitivity, specificity, false negative rate, false positive rate and concordance of RAPINA were 100%, 98.34%, 0%, 1.66% and 98.6%, respectively. The positive predictive value and negative predictive value were calculated as 91.7% and 100%, respectively when RAPINA test was used (Table 2).

## DISCUSSION

Several methods for quantifying or semi-quantifying neutralization antibodies against rabies virus have been developed, including the mouse neutralization test (MNT), rapid focus fluorescence inhibition test (RFFIT), fluores-

cence antibody virus neutralization (FAVN) and enzyme-linked immune sorbent assay (ELISA) [1, 8, 10]. The RFFIT and FAVN are two standard methods endorsed by WHO and the World Organization for Animal Health (OIE) to detect neutralization antibody (NAb) levels against rabies virus in human and animal sera, respectively [1, 8, 10]. However, both methods require a high level of skill, expensive equipment and high-level biosafety facilities, in addition to be time consuming. These characteristics make inappropriate to apply these methods in the field.

Recently, a novel method based on the principles of chromatography has been developed to determine NAb levels against rabies in a very short time [6, 7, 11]. Several prototype kits have been developed and evaluated, but, to date, most of these have been evaluated using dog sera [6, 11]. The first version of RAPINA was developed and evaluated by Shiota et al. in 2009. In this first version, monoclonal antibody (mAb #4-12; IgG2a) against an epitope in antigenic site II of rabies virus glycoprotein was used as both the labeled and captured antibody in the test line. The sensitivity and specificity were 88.7% and 91.9%, respectively, when compared with RFFIT [6]. In 2012, the RAPINA test was further improved by implementing different monoclonal antibody mAb AD-8 and mAb # 4-12 for the label and capture antibody, respectively. The use of two different monoclonal antibodies led to an increase in the sensitivity and specificity of RAPINA's second version [7]. In the present study, the sensitivity, specificity and concordance rates of this second version of RAPINA were 100%, 98.34% and 98.6% respectively in comparison with RFFIT, similar to the findings of the previous study using vaccinated and unvaccinated dog and human sera collected in Japan, Thailand and Sri Lanka to assess the RAPINA test [7].

When applying RAPINA for determination of NAb in rabies patients, five out of seven patients in this study were

positive for NAb (NAb  $\geq$  0.5 IU/ml). The limitations of serological diagnosis of rabies in humans have been reported previously [1]. However, it is still a useful way to distinguish rabies from other encephalitic diseases, particularly in the case of unvaccinated patients who do not have a clear history of rabies exposure and are in hospitals where laboratory diagnosis using methods such as RT-PCR as well as real time RT-PCR, are not available. In such cases, RAPINA can be used, and repeated sampling together with testing should be implemented if the first sample is negative.

False positive RAPINA test results were found in the sera of both vaccinated and unvaccinated people for unknown reasons. However, the technical criteria of the second version of RAPINA test were much better than those of the first version. In order to use this test kit in the field and apply the test results obtained by RAPINA in decision as to whether to give people booster or post-exposure prophylaxis, it is necessary to increase the specificity of the test to 100% and minimize the false positive result rate.

This study not only assessed the technical criteria of RAPINA test compared with RFFIT, but also provided the seroconversion rate of vaccinated professional dog butchers. Of 52 professional dog butchers who were said to have been vaccinated with complete or incomplete courses of Fuenzalida vaccine more than six years earlier, only eleven (21, 15%) had sufficient NAb against rabies. This may be the result of inadequate courses of vaccine or a reduction of NAb levels after vaccination. The reduction of NAb levels was also reported by Zanetti and colleagues, who showed that after 180 days post vaccination, seroconversion could not be found in 60% of people who were vaccinated with four doses of suckling mouse-derived vaccine on day 0-0-7-21 [12]. Recently, atypical rabies transmission from animals to humans associated with the butchering and processing of dog meat has been reported in several parts of the world [4, 13–15]. In Vietnam, approximately five million dogs are butchered for meat annually [16], and it has been reported that 2% of dogs in slaughterhouses in Northern Vietnam were infected with the rabies virus [14]. This suggests that professional dog butchers are potentially at high risk of rabies infection through the dog butchering process. It is highly recommended that professional dog butchers undergo education, information and communication (EIC) regarding rabies prevention. Moreover, PreP should be practiced and NAb should be checked every six months in order to prevent rabies in this target population.

It is known that human deaths have occurred from laboratory infection with both laboratory and wild-type rabies viruses [2, 17]. Therefore, WHO has recommended

that laboratory workers who handle the rabies virus or materials which are suspected to be contaminated with the rabies virus should receive PreP and have their NAb level checked every six months. If the NAb level is under 0.5 IU/ml, one booster dose of vaccine should be given [1]. In this study, only 10 of 20 laboratory workers had received rabies vaccinations and acquired adequate protective NAb levels, suggesting that strict enforcement of national bio-safety regulations should be followed. In such a situation, rapid and user-friendly tests such as RAPINA will be useful for the evaluation of protective NAb levels.

In conclusion, RAPINA is a quick test allowing detection of 0.5 corresponding international unit per milliliter (IU/ml) antibodies in human sera. The sensitivity, specificity, false negative rate, false positive rate and concordance of RAPINA in comparison with RFFIT were 100%, 98.34%, 0%, 1.66% and 98.6%, respectively. With its remarkable sensitivity, specificity and easy performance, RAPINA test is expected to be useful for rapid determination of NAb in the field.

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