

USE OF LATEX AGGLUTINATION TEST TO DETERMINE RABIES ANTIBODIES IN PRODUCTION OF RABIES ANTISERA IN HORSES

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Abstract. A therapeutic anti-rabies immunoglobulin for human use has been produced mainly in horses. The presently available seroneutralization test, the rapid fluorescent focus inhibition test (RFFIT), is laborious and rather difficult to carry out in horse farms. This study was undertaken to develop a simple latex agglutination test (LAT) for determining rabies antibodies in horse sera. LAT was validated by testing a total of 468 horse serum samples characterized by RFFIT. Of these, 253 of 260 samples with antibody titers of less than 100 IU/ml had agglutination score of 1+, whereas 174 of 208 samples with antibody titers equal to or greater than 100 IU/ml had agglutination scores of 2-4+. Results of LAT correlated with those of RFFIT ($r = 0.87$, $p < 0.0001$). LAT has the advantages of being rapid, simple to perform, easy to interpret, and applicable as an on-site testing tool for the estimation of rabies antibodies in horses.

Key words: rabies antibodies, LAT, RFFIT, horse sera

INTRODUCTION

Rabies remains a public health problem in Asia. In most countries where human rabies is reported, the public health sector bears the majority of the costs related by providing services for bite treatment and the delivery of post exposure prophylaxis (PEP) to protect the victims of a suspect rabid bite (Kasempimolporn *et al*, 2008). Human or equine immunoglobulins, administered to severely rabies-

exposed patients at the onset of PEP represent a safety net for the patient until vaccine-induced endogenous antibodies are formed (WHO, 1997).

The Queen Saovabha Memorial Institute (QSMI) is the sole manufacturer of equine rabies immunoglobulin (ERIG) in Bangkok, Thailand. Purified ERIG is processed from plasma of horses, which are immunized with rabies vaccine as previously described (Luekrajang *et al*, 1996) and plasma is collected via plasmapheresis (Feige *et al*, 2003). The horse farm of QSMI is located at Hua Hin, some 200 km south of Bangkok. The serum neutralizing test is used as the bioassay method of choice for measuring the acceptability of horse antisera. The measurement of anti-

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body levels in plasma and the production of ERIG are carried out at a manufacturing plant in Bangkok.

Difficulty has been encountered in producing high titer antisera in horses as variations in immune response between horses may occur. It is desirable for the veterinarians responsible for the production of horse antisera to evaluate the levels of antibodies of the horses simply and quickly and to determine whether additional (booster) doses of vaccine are required. Immunized horses are not routinely monitored for seroconversion due to lack of laboratory facilities. The veterinarians at the horse farm usually submit blood samples for serological testing to the laboratory at QSMI and exsanguination of the horses is performed after the test blood samples show acceptable titers.

The procedures favored by most laboratories for rabies antibody determination are a tissue culture neutralization test using live rabies virus employing an immunofluorescent-antibody staining technique, such as the rapid fluorescent focus inhibition test (RFFIT) (Smith *et al*, 1973), and fluorescent antibody virus neutralization test (Chiquet *et al*, 1998). These methods are the reference techniques for the World Health Organization (WHO, 2005) and the World Organization for Animal Health (OIE, 2004). The assays require relatively sophisticated laboratory facilities including the production of rabies virus and cell cultures, which are complicated, time consuming and costly. To overcome this disadvantage, an enzyme-linked immunosorbent assay (ELISA) has been described to quantify the level of rabies antibodies (Nicholson and Prestage, 1982; Kavaklova *et al*, 1984; Grassi *et al*, 1989). ELISA is less time consuming and less expensive than RFFIT, but even so it still can-

not be carried out by the veterinarian at QSMI horse farm as facilities or resources to perform such tests are not available.

Recently, a new commercial immunoenzymatic test that utilizes protein A linked with horseradish peroxidase for detecting and quantifying rabies antibodies in the sera of humans and domestic and wild carnivores has been introduced (Servat *et al*, 2007). Such a test kit may not be suitable for all animal sera, particularly those of herbivores (Mebatsion *et al*, 1989). The utility of a latex agglutination test (LAT) for the determination of rabies antibodies after human vaccination has been reported (Perrin *et al*, 1988; Madhusudana and Saraswati, 2003). The LAT is simple to perform and needs minimum laboratory equipment. In this study, we report the results obtained with horse sera tested with LAT and RFFIT tests.

MATERIALS AND METHODS

Test specimens

A total of 468 horse immune serum samples were evaluated in this study. Each test serum was collected at various days after immunization with rabies vaccine. These specimens were assayed by RFFIT (Smith *et al*, 1973) at QSMI and were examined by LAT by examiners without previous knowledge of the results. Thirty non-immune horse sera were included as controls.

RFFIT

Serum samples were heated at 56°C for 30 minutes before testing in order to inactivate complement. Dilutions of test serum in cell culture medium were mixed with a constant amount of rabies virus in a 96-well microplate. Serum and virus were then incubated at 37°C for 90 minutes. A concentration of 5×10^5 BHK-21/13S

cells/ml was added to each well, and incubated at 37°C for another 24 hours. The cells were then rinsed, fixed in acetone, and stained with fluorescein-conjugated rabbit anti-rabies virus globulin (TRC®, Thai Red Cross Society, Thailand). A serum is considered to have antibody when 50% or more of the cells are protected from the virus. The titer of antibody in the test serum was obtained by comparison with reference serum standard (The State Serum Institute, Copenhagen, Denmark). Titers were expressed as international units per ml (IU/ml). Samples with antibody levels <100, 100-200 and >200 IU/ml were grouped and subjected to LAT examination.

LAT

Sensitized polystyrene blue latex beads (diameter, 0.8 µm; Sigma, St Louis, MO) were prepared as previously described (Perrin *et al*, 1988). One ml of 10% latex beads were washed 3 times with 5 ml of 0.05 M bicarbonate buffer, pH 9.6. Beads were centrifuged at 5,000g for 30 minutes. The latex beads were suspended in 3 ml of bicarbonate buffer together with 1.75 ml of a 1: 10 dilution of rabies virus vaccine (Verorab®, Merieux, France). The mixture was shaken at 37°C for 3 hours and at 4°C overnight. The beads were suspended in 5 ml of carbonate buffer containing 5% trehalose and 0.3% bovine serum albumin. The beads were incubated at 37°C for 30 minutes and then washed twice with phosphate-buffered saline (PBS), pH 7.4. The sensitized latex beads were resuspended at a 3% concentration in PBS and kept at 4°C until used.

The assay was performed by spreading 25 µl aliquot of sensitized latex beads onto a double-concave glass slide, adding 25 µl aliquot of the serum to be tested, and mixing for 5 to 15 minutes at room tem-

Table 1
Comparison between antibody determination in horse immune sera using LAT and RFFIT.

LAT	Neutralizing antibody titer (IU/ml)		
	<100	100-200	>200
1+	253	34	0
2+	7	92	2
3-4+	0	8	72

perature. A positive reaction for antibodies to rabies virus was agglutination visible in bright light, indicated by the formation of fine granular particles. The degree of agglutination was scored as 4+, 3+, 2+ and 1+ depending on size of agglutinated particles. A negative reaction was no agglutination, giving the serum and latex suspension with a milky, smooth consistency. Positive and negative control sera were tested with each assay.

RESULTS

The performance of a LAT developed in our laboratory was compared to that of the standard serological technique. Horse sera which contain rabies antibodies are capable of agglutinating rabies virus antigen-sensitized beads. The agglutination was visible with immune sera which had been characterized previously by RFFIT and no agglutination was observed with non-immune sera (data not shown), confirming the specificity of agglutination assay. Among 468 test immune sera, 260 contained virus neutralizing antibody titers of less than 100 IU/ml, 134 had titers between 100-200 IU/ml and the remaining samples had a titer greater than 200 IU/ml

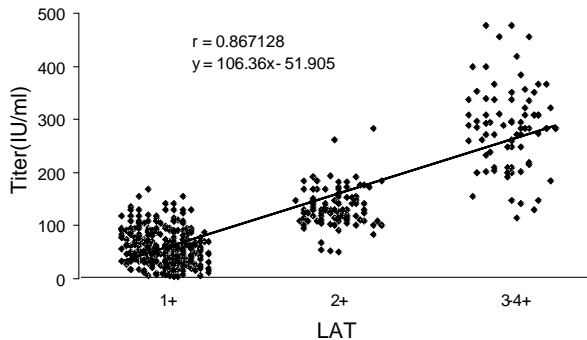


Fig 1—Correlation between LAT results and the virus-neutralizing antibody titers. Antibody titers were obtained using RFFIT.

(Table 1). The agglutination observed were graded from 1+ to 4+. A score of 1+ corresponded to titers less than 100 IU/ml, 2+ corresponded to titers equivalent 100-200 IU/ml, and 3+ to 4+ corresponded to titers greater than 200 IU/ml. The LAT and RFFIT outcomes correlated significantly, with a correlation coefficient of 0.87, $p < 0.0001$ (Fig 1). Determination of rabies antibodies in well-characterized seropositive serum samples by LAT was positive over a wide range of serum titers, ranging from <100 to >100 IU/ml. Interpretation of positive LAT serum samples became easier at titers >200 IU/ml.

DISCUSSION

Horses are found to be suitable for the economical large-volume production of therapeutic ERIG for human use. ERIG is produced by immunizing horses with a cell culture-derived rabies vaccine. Plasmapheresis in immunized horses is performed and crude plasma is submitted to the manufacturing plant if acceptable serologic titer is demonstrated. Antibody titer of less than 100 IU/ml is considered too low for use. However, we have found that

there are variations in antibody levels between individual horses. The immune response variation among horses may be due to differences in horse immunogenic background, nutritional status and general health (Redwan *et al*, 2009). For this reason, on-site testing for demonstration of adequate seroconversion following rabies vaccination is necessary. The presently available test, such as RFFIT, is laborious and time consuming.

Agglutination reactions observed with particular antigens can be extended to a wide variety of soluble antigens by attaching them to the surface of latex beads. The utility of this procedure has been demonstrated for detection of several viral antibodies, such as rabies virus (Perrin *et al*, 1988), HIV (Quinn *et al*, 1988), varicella virus (Steinberg and Gershon, 1991) and avian influenza virus (Horie *et al*, 2009). The LAT developed in this study is a simple procedure that is easy and rapid to perform. We used commercial cell-culture rabies vaccine of human use as a coating antigen. The antigen can be prepared easily, as it is not necessary to purify the viral proteins or virions. This test is proposed for determining the antibody status of immunized horses and for optimizing immunization schedules in an effort to reduce a waste of low-titer raw plasma.

We compared LAT with standard RFFIT. Using RFFIT, all sera had titers ranging from 3 to 480 IU/ml (data not shown). When the samples were grouped according to their titers and tested by LAT, there is good correlation in degree (magnitude) of positivity, although it is not known whether results of LAT represent the detection of neutralizing antibodies. However, in this study discrepant results were obtained for 41 (9%) serum samples, with some samples showing high antibody

titers exhibiting low degree of agglutination, and vice versa. These discrepant results may be due to a different degree of accuracy and/or diversity among the two test systems. In addition, these may exist components in serum that affect the specific agglutination of sensitized latex.

In previous studies, LAT developed for detecting rabies antibodies in human sera showed 4+ agglutination with serum samples having a titer greater than 8 IU/ml (Perrin *et al*, 1988; Madhusudana and Saraswati, 2003), while 2-4+ agglutination reactions in this study occurred widely in tested cases with titers greater than 100 IU/ml. One explanation for such discrepancies could reside with the antigen coated on the latex beads. For human sera purified whole virus or purified rabies glycoprotein was used, whereas we used commercial human rabies vaccine preparation. In addition, different sources of serum might also be a cause.

An accurate definition of antibody titers should be expressed in terms of the neutralizing titration. LAT is not proposed for determining antibody titration but rather for the rapid screening of rabies antibodies and its sensitivity seems to be adequate. If the results of LAT are directly correlated with RFFIT and if the degree of agglutination correlates with the level of rabies neutralizing antibody, then the presence of moderate agglutination of LAT rabies antibodies should equate with acceptable neutralizing antibody titers. Sera with agglutination scores greater than 2+ were considered as corresponding to neutralizing antibody higher than 100 IU/ml.

The simplicity of LAT permits its performance under field conditions with a capacity for testing many sera in the same run. The test can be performed at a horse farm for routine use if sensitized beads are

supplied from a central laboratory. Reagents were stable for 2-3 months at 4°C, after which they lost stability (data not shown). LAT gives a faster result (within minutes) than RFFIT (2 days) or other laboratory tests which may not be available on a daily basis. LAT does not require the use of live virus and so does not require virus containment facilities or any sophisticated equipment. Although LAT is subjective in interpretation and performing the assay requires some degree of skill, inter-reader variability between skilled persons was low. When a panel of samples were tested by two skilled persons, the LAT results showed a relatively high degree of agreement (95%) (data not shown).

In summary, data presented show that LAT can be used for in-process control to continuously monitor antibody response of horses at various stages of rabies immunization. It is reasonably robust and can be used on-site in horse farms.

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