International Journal of Infectious Diseases (2007) 11, 441—445

http://intl.elsevierhealth.com/journals/ijid

Development and evaluation of a competitive ELISA for estimation of rabies neutralizing antibodies after post-exposure rabies vaccination in humans

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Received 21 February 2006; received in revised form 2 September 2006; accepted 11 September 2006
Corresponding Editor: Jane N. Zuckerman, London, UK

KEYWORDS
Rabies;
C-ELISA;
Post-exposure vaccination

Summary
Objectives: Currently three tests are approved for the estimation of neutralizing antibodies after rabies vaccination: the mouse neutralization test (MNT), the rapid fluorescent focus inhibition test (RFFIT), and the fluorescent antibody virus neutralization (FAVN) test. Performance of these tests requires a lot of expertise and is generally carried out in reference laboratories and, hence, they are not available to many people. The aim of the present study was to develop and evaluate a competitive ELISA (C-ELISA) for estimation of neutralizing antibodies in order to make this testing more widely available.
Methods: The C-ELISA was designed based on competition between a murine neutralizing monoclonal antibody (Mab) and the antibodies in serum of vaccinated people. The test was initially standardized using known negative and known positive serum samples for determining the optimal dilution of the Mab as well as the cut-off value (%) for ascertaining the level of inhibition. Nine hundred and ninety serum samples were tested from 250 people who had been administered purified chick embryo cell vaccine (PCECV). Serum samples were collected on days 0, 14, 30 and 90 post-vaccination, and were tested by C-ELISA.
Results: All the serum samples that were positive by RFFIT were also positive by C-ELISA. The titers obtained with C-ELISA were marginally higher than the RFFIT titers, but a significant correlation was noted between the two tests ($r = 0.897$). None of the negative controls were detected to be positive for rabies antibodies by either of these tests. Therefore the C-ELISA was found to be 100% specific and sensitive in comparison to RFFIT. Further, the initial rise and fall of antibody titers on different days post-vaccination was comparable for both tests.

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doi:10.1016/j.ijid.2006.09.013

Conclusions: The C-ELISA described herein can be used to quantify rabies neutralizing antibody levels after vaccination. This test is simple and can be conveniently used under field conditions for monitoring seroconversion after post-exposure rabies vaccination. Moreover it does not require handling of infectious virus by the end user.

Introduction

Rabies is a fatal zoonotic viral infection of the central nervous system that is transmitted by the bite of an infected animal. According to a World Health Organization (WHO) estimate, 50 000 human deaths due to rabies are reported worldwide every year, the majority of them being in the developing countries of Asia and Africa.[1] Amongst these 50 000 cases, India alone accounts for 20 000.[2] More than 95% of cases of human rabies in India are due to dog bites. The disease is also re-emerging as an important public health problem in North America where several cases of human rabies have occurred due to exposure to bats.[3] Recently some cases of human rabies due to exposure to bats have also been reported from Brazil.[4]

In India more than 4 million people receive post-exposure vaccination annually. In January 2005, use of the nerve tissue vaccine was discontinued and presently the vaccines used are purified chick embryo cell rabies vaccine (PCECV), purified vero cell rabies vaccine (PVRV) and purified duck embryo vaccine (PDEV). All these vaccines protect the individual by producing neutralizing antibodies to rabies glycoprotein (G). WHO has recommended a titer of 0.5 IU/ml of serum as an accepted level of seroconversion.[5]

Monitoring antibody titers are required in certain circumstances, i.e., elderly people and the malnourished and immunocompromised, and also when clinical trials are conducted for new vaccines or new vaccination schedules. Currently three tests are approved by WHO for determining the levels of rabies neutralizing antibodies: the mouse neutralization test (MNT),[6] the rapid fluorescent focus inhibition test (RFFIT)[7] and the fluorescent antibody virus neutralization (FAVN) test.[8] These tests are time consuming, require expertise, are expensive and are generally carried out in reference laboratories and, hence, are not widely available. Therefore there is a need to develop and standardize simple techniques such as ELISA for the measurement of antibodies. ELISA tests developed earlier by Perrin et al. do not actually measure neutralizing antibodies.[9] However, it is possible to measure the levels of neutralizing antibodies by using the principle of competitive ELISA, where the antibodies in the test serum are allowed to compete with biotin/enzyme-labeled neutralizing monoclonal antibodies with a known titer. Based on this principle Sugiyama et al. have developed a competitive ELISA (C-ELISA) as a simple, rapid and inexpensive alternative to the virus neutralization test and have used this test to determine the overall immune status of rabies-vaccinated domestic dogs in Japan.[10] However, this principle has not been used widely for measuring humoral immunity in people receiving post-exposure rabies vaccination. In this study we have developed a C-ELISA for estimating the neutralizing antibody titer in vaccinated people and evaluated our results with RFFIT, which is an approved test for estimating rabies-neutralizing antibodies.

Materials and methods

Serum samples

In this assay, serial serum samples (n = 990) obtained from 250 individuals who had received a course of post-exposure vaccination with PCECV were evaluated. Their antibody titers on day 0 (n = 250), day 14 (n = 250), day 30 (n = 250) and day 90 (n = 240), were evaluated. These samples were obtained from the antirabic treatment center, Kempegowda Institute of Medical Sciences (KIMS), Bangalore. Negative controls consisted of 50 serum samples obtained from age and gender-matched people who had not received any rabies vaccine. In addition, a panel of 50 positive serum samples, previously tested positive by RFFIT, was used to validate the results of the C-ELISA.

Monoclonal antibodies (Mabs)

In a recently completed study, we produced and characterized Mabs to rabies virus (CVS 11 strain) in our laboratory by using established procedures.[11] We were able to generate 50 hybridomas to G protein of which 11 Mabs showed a high virus neutralizing titer for CVS in RFFIT. We chose one of these Mabs (2C5E8) in designing the C-ELISA. This Mab had a neutralizing titer of 1:10 000 000 and belonged to isotype of IgG, subtype of IgG2a. The Mab recognized a conformational epitope as revealed by a native polyacrylamide gel electrophoresis. This Mab was biotinylated using an established procedure described earlier.[12] The optimal dilution of the Mab to be used in the competition assay as well as the percentage inhibition to be used as a cut-off value was determined by using a panel of known high positive (n = 50), low positive (n = 50) and negative sera (n = 50).

Preparation of antigen for C-ELISA

The CVS strain of rabies virus was grown in BHK-21 cells. Cell-culture supernatants containing high titers (10^7 FFD_{50} (50% fluorescent focus forming dose)) of rabies virus were clarified by low-speed centrifugation at 500 *g* for 30 min, to remove cellular debris. After clarification and inactivation with beta-propiolactone (BPL, 1:4000) the virus was partially purified by ultracentrifugation at 70 000 *g* and 4 °C for 2 h using an ultracentrifuge (Sorvall Discovery, 100S). The pelleted virus was resuspended in phosphate-buffered saline (PBS) to a final volume of 1/100 of the original and stored at −70 °C until required.

Rapid fluorescent focus inhibition test (RFFIT)

This was performed as per the WHO advocated procedure with some modifications. Instead of tissue culture chambers

we used 96-well flat-bottomed tissue culture plates and the cell line used was BHK-21 (CL 13). The virus used was the CVS strain adapted to grow in BHK-21 cells and the dose used was 100 FFD_{50}. The highest dilution of serum showing 50% inhibition of fluorescent foci in the infected cells was taken as the titer of the serum, which was converted to international units (IU/ml) by comparison to an in-house reference sera calibrated against the 2nd international reference serum with a unitage of 30 IU/ml (obtained from the National Institute of Biologicals, UK).

C-ELISA

The polystyrene wells of ELISA strips (Nunc, Denmark) were coated with 1 μg of antigen in 0.05 M carbonate buffer pH 9.6 (0.5% of sodium carbonate and 0.4% of sodium bicarbonate), at 4 °C overnight. The plates were then washed three times with PBS containing 0.5% Tween 20 (PBST) to remove unbound antigen. Subsequently they were treated with 5% skimmed milk powder in PBS (150 μl/ well) at 37 °C for 2 h to block the nonspecific reactions. Serial two-fold dilutions of sera (100 μl) were placed in duplicate in the antigen-coated wells and then incubated at 37 °C for 30 min. After washing the plates three times with PBST an appropriate dilution (1:800) of biotinylated anti-G Mab (100 μl) was added and further incubated for 1 h. In order to determine the actual OD value obtained in the absence of competition, five wells received only 100 μl of PBS in lieu of patient's serum sample followed by the same dilution of biotinylated Mab. After five washes, streptavidin-peroxidase (Bangalore Genei, 1:5000) was added to each well and incubated as above. After a final five washes, the substrate OPD (orthophenylenediamine) solution containing 0.01% H_2O_2 was added and plates incubated in the dark for 30 min at room temperature. The development of color was stopped with 4 N H_2SO_4. The resulting optical density at 490 nm (OD_{490}) was measured on an ELISA reader (Lab systems, India). The percentage inhibition was calculated using the formula $[1 - (OD_{ser}/OD_{Mab})] \times 100$, where OD_{ser} is the mean of OD of serum + Mab wells and OD_{Mab} is the mean of OD of wells containing Mab alone. The serum titer was defined as the reciprocal of the highest dilution that showed 30% or more of inhibition of OD value of the competing Mab. The titers were converted to IU/ml by comparison to the titers observed with an in-house reference serum (as described earlier).

Statistical analysis

The results obtained with the two tests were analyzed by Pearson's product movement correlation test and the *r*-value determined.

Results

Standardization of C-ELISA

Initially, the C-ELISA was standardized for optimal conditions using a 1:100 dilution of negative, high positive and low positive sera, based on the results of RFFIT done earlier. The results are shown in Figure 1. The optimal dilution of the biotinylated neutralizing Mab to be used in the C-ELISA was determined to be 1:800. This was arrived at as more than 80% inhibition was observed with strong positive sera and less than 10% inhibition with known negative sera. The cut-off percentage inhibition was taken as 30% based on the inhibition values obtained with low positive sera.

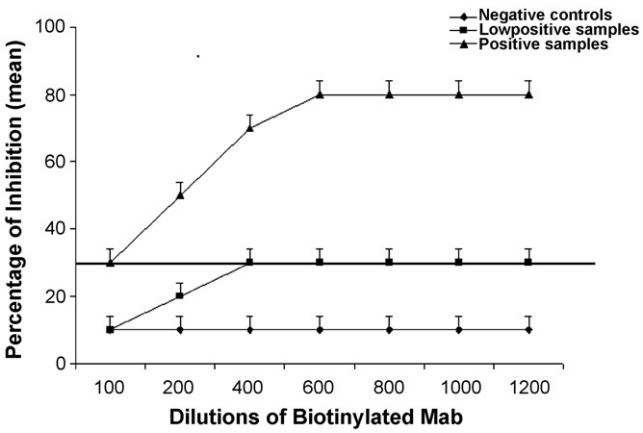

Figure 1 Standardization of C-ELISA. Different dilutions of Mab were tested with a 1:100 dilution of high-titered positive sera ($n = 50$ (▲)), low-titered positive sera ($n = 50$ (■)) and known negative sera ($n = 50$ (◆)). The mean percentage inhibition obtained with each set of serum against different dilutions of Mab is depicted. The horizontal line represents the percentage cut-off value, which is 30%.

Comparison of RFFIT and C-ELISA

To evaluate the C-ELISA, 990 serum samples obtained from individuals who had received a post-exposure immunization with PCECV were assayed in both the RFFIT and the C-ELISA. The serum samples were tested in duplicate at different dilutions (1:500 to 1:8000) and the geometric mean titer (GMT) of the reciprocal of the highest dilution showing 30% inhibition was calculated. The antibody titers obtained by C-ELISA as compared to those obtained by RFFIT are shown in Figure 2 and Table 1. The distribution of all the samples tested (both positive and negative) and their percentage inhibition of OD values are shown in Figure 3. Though the antibody titers obtained by C-ELISA were marginally higher than those obtained by RFFIT, there was a good correlation between the two (Figure 4; $r = 0.897$). It can be also observed that there is a good correlation between the two tests on the different days following vaccination (Figure 2). The antibody titers reached a peak in the C-ELISA and the RFFIT on day 30

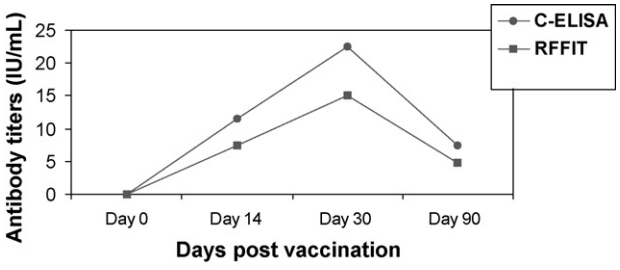

Figure 2 Antibody titers (GMT) obtained with C-ELISA (◆) and RFFIT (■) on different days post-vaccination.

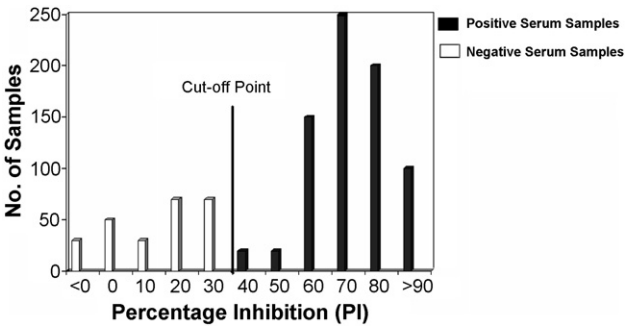

Figure 3 Distribution of percentage inhibition (PI) values for 740 positive serum samples and 250 negative serum samples obtained by the C-ELISA. The threshold cut-off value of a PI of 30 was determined on the basis of OD values of low positive serum samples (mean PI + 3 standard deviations).

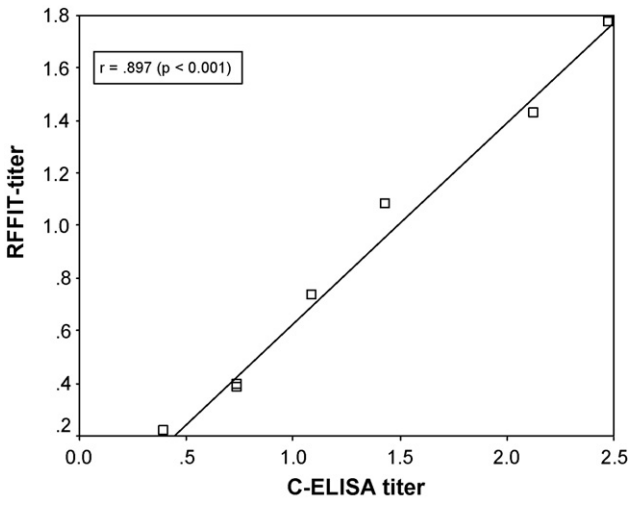

Figure 4 Scatter plot showing correlation between GMT of C-ELISA and RFFIT determined using SPSS software (Statistical Package for Social Sciences). The *r*-value was found to be 0.897.

post-vaccination. The two assays gave similar patterns of initial rise by day 14 and subsequent fall by day 90. As all the samples tested positive by C-ELISA were also positive by RFFIT, the specificity/sensitivity index was found to be 1.

Discussion

Rabies is a fatal disease once symptoms develop. However it is a preventable disease if state of the art modern prophylactic techniques are instituted soon after exposure. Vaccination with a potent rabies vaccine plays a crucial role. The presence of adequate titers of neutralizing antibodies in serum indicates protective immunity.

The RFFIT, FAVN test and MNT are neutralization-based tests and have been used for detecting and measuring neutralizing antibodies to rabies virus. However, these tests are tedious and require use of live virus and animal/cell culture, making them unsuitable for determining the protective status following post-exposure prophylaxis under field conditions. Of these, the more recently developed neutralization test —FAVN — is relatively economical but still has the same limitations.[13]

To overcome this disadvantage, ELISAs have been developed for detecting antibodies to rabies virus.[7,14–16] The conventional ELISA developed earlier does not measure the neutralizing antibodies alone and antibodies to other epitopes are also measured. Thus, results of ELISA may not actually indicate the protective status of the vaccinated individual. This problem was overcome in the present study by developing a competitive ELISA using a neutralizing G Mab (2C5E8) that recognized a conformational epitope on the rabies G protein, allowing the serum samples to compete with this Mab for the G antigen of the concentrated virus coated onto solid phase. In a direct C-ELISA for measuring antibody levels, a known amount of specific indicator antibody (Mabs conjugated with an enzyme) is mixed with the serum under test and allowed to compete for a limited amount of antigen bound to a solid matrix. When the enzyme substrate is added to the system (in the presence of a chromogen), a decrease in color intensity indicates competition and therefore the presence of antibody in the test serum. The decrease in color intensity is proportional to the level of antibody unless there is complete inhibition of the labeled antibody. C-ELISA has been used earlier for measuring the immune status of animals to diseases such as rinderpest virus,[17] morbillivirus[18] and bluetongue virus,[19] and for ascertaining immune status in human leishmaniasis.[20]

The initial standardization of the assay was done using checkerboard titration of neutralizing Mab with known positive and negative serum samples and the cut-off value (percentage inhibition of OD value) was determined. In this study, 990 serum samples obtained from patients who were immunized with rabies vaccine (PCECV) were tested. Though the titers obtained with C-ELISA were marginally higher than RFFIT titers (Figure 2) there was an excellent correlation between the two tests ($r = 0.897$). In addition, the antibody titers observed on different days post-vaccination showed an identical pattern of initial rise and subsequent fall. The test is both specific and sensitive and takes about 4 hours to complete. The actual test procedure is not complicated as compared to conventional ELISA. Because of the use of Mabs

Table 1 Comparison of GMT of neutralizing antibody (IU/ml) obtained with RFFIT and C-ELISA

Day of sampling	RFFIT titer (GMT) IU/ml	C-ELISA titer (GMT) IU/ml
Day 0 ($n = 250$)	ND	ND
Day 14 ($n = 250$)	7.5	11.25
Day 30 ($n = 250$)	15	22.5
Day 90 ($n = 240$)	4.84	7.5

GMT, geometric mean titer; RFFIT, rapid fluorescent focus inhibition test; ND, not detected.

to G protein for competition, it is not necessary to use a highly purified G protein for initial capturing of neutralizing antibodies. Concentrated, inactivated and partially purified virus can be used. Results can be obtained within 4 hours compared to RFFIT, which takes 48—72 hours, and MNT, which takes 14 days.

The earlier study of Sugiyama et al. evaluated C-ELISA using several Mabs against G protein but found a poor correlation with the neutralization test when they tested immunized dog sera.[10] This finding is in contrast to our results in the current study, where we have found a very good correlation between the tests when human samples were tested. However, they found a good correlation ($r = 0.9$) when Mabs against N protein were used in the C-ELISA. It is probable that because of the low antigenicity of the veterinary vaccine and administration of only one or two doses of vaccine, dog sera may not have sufficient levels of antibodies reactive to G protein. This is not the case with human sera as all our subjects had received five doses of highly potent rabies vaccine.

One limitation of this assay is that the titers obtained are not expressed as conventional IU/ml as is done with MNT, RFFIT or the FAVN test. However, it may be possible to include the presently used international standard of rabies immune globulin (RIG) or more practically an in-house reference serum (calibrated against the international standard) in this C-ELISA and titers can be converted to IU/ml. However, more studies are required to address this issue.

In summary, the C-ELISA is a convenient and practical assay for detecting and measuring neutralizing antibodies to rabies virus. The reagents necessary for the test can be prepared in a reference laboratory and the actual test can be performed in peripheral laboratories. Its development provides a rapid, simple, safe, and economical means for large serological surveys, both in man and animals, which is essential for determining the efficacy of vaccination after pre- or post-exposure therapy.

Conflict of interest: No conflict of interest to declare.

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