Original Article

A Cost Effective Easy Competitive Enzyme-Linked Immunosorbent Assay Suitable for Monitoring Protective Immunity against the Rabies Virus in the Serum of Humans and Dogs

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SUMMARY: The coverage of rabies vaccinations has been reported at 70–80% of dogs in annual reports. However, there are still outbreaks of rabies among humans and dogs in Thailand, thus indicating the necessity of ensuring seroprevalence in vaccinated dogs and efficacy of human immunization. A cost effective easy competitive enzyme-linked immunosorbent assay (CEE-cELISA) was developed here for monitoring protective immunity against the rabies virus in human and dog serum samples using monoclonal antibody clone 1-46-12, which recognizes a conformational epitope of the rabies G protein. The ELISA plate is coated with the whole viral antigen from a commercial vaccine. The serotiter measured by the CEE-cELISA and by the gold standard assay (rapid fluorescent focus inhibition test), detecting the neutralizing antibody, showed a strong correlation, with an R value of 0.958 and 0.931 in humans and dogs, respectively. These correlations were detected in the serum samples from humans and dogs at antibody concentrations up to 100 and 10 IU/ml, respectively. This CEE-cELISA could be an alternative assay for evaluating mass rabies vaccination rapidly at a low cost as well as for detecting antirabies antibodies in the serum of not only humans but also other animal species.

INTRODUCTION

The World Health Organization (WHO) and World Organization for Animal Health have announced their goal to end human deaths from dog-transmitted rabies by the year 2030 (1). In Thailand, rabies still occurs in both humans and animals, as reported annually, and the country is categorized as an endemic area (2). Therefore, Thailand is striving to become rabies-free by 2020, in advance of the worldwide goal of 2030 by campaigning for mass rabies vaccination for both domestic and stray dogs to prevent the spread of this disease from animals to humans.

Rabies prevention involves two main strategies in Thailand: (i) dog vaccination to interrupt virus transmission to humans and (ii) human vaccination as a series of vaccine injections before or after exposure for preventing rabies in humans (3). However, rabies vaccination is not effective in all cases and should be monitored. Failure of postexposure prophylaxis has been reported in several cases (4). In addition, low-, medium-, and high-responder profiles for various vaccines have been demonstrated (5,6). Therefore, quantitation of an antirabies antibody is necessary to determine the immune status and to ensure that a sufficient amount of protective antibody has been produced to prevent and treat rabies infection.

Several tests have been developed to assess the level of a rabies virus–neutralizing antibody, and the rapid fluorescent focus inhibition test (RFFIT) is the “gold standard” for measuring the antirabies antibody concentration in serum samples (7). However, antibody monitoring has generally not been considered because only a few institutions have the RFFIT, which is not only expensive and time-consuming but also cumbersome in terms of the process of sending samples for detection. Therefore, a simple, cheap, and accessible assay for monitoring antibodies is necessary to overcome these problems and the erroneous notion that vaccination is infallible, and monitoring is not necessary.

The aim of this study was to develop and make available a cost-effective and easy competitive enzyme-linked immunosorbent assay (CEE-cELISA) for monitoring of protective immunity, thus ensuring the efficacy of human immunization and seroprevalence in vaccinated dogs.

MATERIALS AND METHODS

Sample collection: Fifty-two serum samples were...
collected from dogs living at Mae Guang Dam. They were caught for field sterilization and for rabies vaccination in collaboration with the local subdistrict municipal department for rabies control. This forest area has many stray dogs that are not reported as having been vaccinated. Regarding sample collection from humans, 364 serum samples were collected from veterinary students at the Faculty of Veterinary Medicine, Chiang Mai University, Thailand; they had either never been vaccinated or had been vaccinated previously. All serum samples were separated from blood samples by centrifugation at 1,300 × g for 10 min and were stored at −20°C until use. This study was conducted with the approval of the ethical committee for human rights related to human experimentation of the Faculty of Medicine, Chiang Mai University, Thailand (MIC-2559-0418).

RFFIT: This test was performed according to the standard procedure recommended by the WHO (8). Human and dog serum samples were analyzed for neutralizing antibody levels by the RFFIT at the National Institute of Infectious Diseases, Japan.

Rabies antigens: Two kinds of rabies antigen—a whole rabies virus vaccine (PV2061 strain of Pasteur) with protective efficacy of > 2.5 IU/vial (Speeda Rabies Vaccine™ Company) and 1.87 mg/ml rabies virus recombinant glycoprotein (RV-rG protein)—were used for coating the ELISA plate. The RV-rG protein was prepared from the supernatants of Tn5-infected baculovirus with insertion of the rabies G gene from the CVS-26 strain of rabies virus by means of a baculovirus expression vector system (BestBac 2.0, Expression Systems LLC, Davis, CA), according to previous reports (9,10).

Monoclonal antibodies (mAbs): Three mAbs, clone 1-46-12 (0.4 mg/ml), clone 7-1-9 (0.69 mg/ml), and clone 1-30-44 (0.82 mg/ml) were used. The first mAb is a conformational-epitope–specific mAb clone 1-46-12, which neutralizes the rabies virus (CVS, ERA, HEP-Flury and Nishigahara strains) via binding of only a small number (less than 20) of the antibody molecules per virion (11). The second one is a linear-epitope–specific mAb clone 7-1-9, which requires more than 250 IgG molecules for neutralization (CVS, ERA, HEP-Flury strains) (12). The last one is mAb clone 1-30-44, which recognizes an acid-sensitive conformational epitope of the glycoprotein in most of laboratory strains of the rabies virus (13).

The CEE-cELISA design: The ELISA plate was coated first with 100 µl of rabies antigens diluted 1:100 in coating buffer at 4°C overnight. The plate was then washed five times to remove unbound antigen and was treated subsequently with 3% BSA in PBS at 37°C for 1 hour to block nonspecific reactions. Fifty microliters of serial dilutions of serum samples from 1:100 to 1:800 were added into duplicate wells for competitive binding to the antigen, with the same volume of an appropriate mAb (competitive), whereas the OD mAb is the OD of the wells containing the mAb alone.

The serum titer was defined as a reciprocal of the dilution, which showed 30% inhibition of the OD values by competing mAbs (14). The titer was converted to ELISA units per milliliter (EU/ml) by comparing the ratio of the test serum titer with the WHO reference serum titer. The concentration determined by the CEE-cELISA and the RFFIT was expressed in EU/ml and international units per milliliter (IU/ml), respectively.

Statistical analysis: The results from two tests, the CEE-cELISA and RFFIT, were subjected to correlation analysis by means of Spearman’s rank correlation in terms of the R value. Sensitivity and specificity of the CEE-cELISA were calculated via the receiver operating characteristic curve (ROC curve).

Ethical standards: Written informed consent was obtained from each patient. The study protocol was approved by the Faculty of Medicine, Chiang Mai University, Thailand and was in accordance with the Declaration of Helsinki (acceptance numbers MIC-2559-0418).

RESULTS

Binding of mAb clones to rabies antigens: Three clones of mAbs—clones 1-46-12, 1-30-44, and 7-1-9—were tested with two rabies antigens (the whole rabies virus vaccine and rabies glycoprotein) by the indirect ELISA.

The whole rabies virus vaccine showed the best results on binding with mAb clone 1-46-12 in an appropriately accurate absorbance range of 1:100 dilution (Fig. 1A). Although clone 1-30-44 recognizes a conformational epitope similar to that of clone 1-46-12, its binding affinity for the rabies vaccine yielded OD of 0.500, which was only half of that for clone 1-46-12, which yielded OD of 0.865. Clone 7-1-9, which recognizes a linear epitope, manifested no binding to this antigen.

The rabies glycoprotein yielded the best results at a dilution of 1:400 with mAb clone 7-1-9 (Fig. 1B). This antigen is a rabies glycoprotein, which has a linear form and can be detected with an mAb that is specific to the linear epitope. Therefore, this antigen showed no reactivity with clones 1-46-12 and 1-30-44 (which are specific to a conformational epitope) as evidenced by the lowest O.D. background. A higher OD, 1.200, did not improve the reactivity of the two clones but reduced the binding activity of clone 7-1-9. This result was due to high steric hindrance for the rabies glycoprotein antigen, as seen in a chevron curve.

Therefore, the results from testing of the three mAbs revealed that the whole rabies virus vaccine was the most suitable for use with mAb clone 1-46-12, while rabies glycoprotein showed the best reactivity with clone 7-1-9. Clone 1-30-44 was found to have low reactivity with both antigens.

Standardization of the CEE-cELISA: The two best
pairs antigen–mAb, i.e., whole rabies virus vaccine and mAb clone 1-46-12 as well as rabies glycoprotein and mAb clone 7-1-9, were then tested at a low (0.6 IU/ml) and high (30.5 IU/ml) concentration of the antirabies antibody in human serum to find the best pair antigen–mAb and conditions for setting up the CEE-cELISA for monitoring of an antirabies antibody in serum samples. The serum titer was defined as being reciprocal to the dilution level that caused 30% inhibition of OD values by competing mAbs.

The pair “whole rabies virus vaccine antigen and mAb clone 1-46-12” was tested with a serum sample that had a high concentration of an antirabies antibody and showed strong inhibition (91%) and a gradual decrease to 75%, 53%, and 43% when dilution of the serum sample increased (Fig. 2A). Although, the highest dilution, 1:800, did not cause 30% inhibition, we could still calculate the concentration of the antibody from the graph. The serum sample that had a low concentration of the antirabies antibody (1:100 dilution) produced 30% inhibition and a gradual decrease to 0% or no inhibition when the dilution was increased.

The pair “rabies glycoprotein and mAb clone 7-1-9” showed a different result. The serum sample that had a low concentration of the antirabies antibody (at the lowest dilution of serum, 1:100) manifested a very low percentage of inhibition: 4%, 5%, 3%, and −2% (Fig. 2B). These percentages of inhibition did not allow us to determine the dilution of serum samples that can cover 30% inhibition by means of the graph. Similarly, no difference was seen in serum samples that contained a high concentration of the antirabies antibody because no dilutions reached 30% inhibition.

Therefore, the pair “whole rabies virus vaccine antigen and mAb clone 1-46-12” was suitable for determining the levels of an antirabies antibody by the CEE-cELISA because this pair was able to detect both the low and high concentrations of the antirabies antibody in the serum samples.

**Monitoring of an antibody to the rabies vaccine in serum samples by the CEE-cELISA:** Human and dog serum samples were analyzed for the levels of an antirabies antibody by the CEE-cELISA as compared with the RFFIT.

The CEE-cELISA was performed on 364 human serum samples from veterinary students, and the antirabies
antibody levels were 15.95 ± 24.04 EU/ml (mean ± SD) and 22.14 ± 70.68 IU/ml according to the CEE-cELISA and RFFIT, respectively. Moreover, the CEE-cELISA showed a strong correlation (R = 0.962) with the RFFIT for all serum samples (Fig. 3A). It was noticed that serum samples contained antibodies between 152.73 and 1107.48 IU/ml or above 100 IU/ml according to the RFFIT, but the CEE-cELISA could detect antirabies antibodies only at up to ~100 EU/ml. Therefore, serum samples were reanalyzed by dividing them into two groups: one with a concentration less than 100 IU/ml and the other with a concentration above 100 IU/ml. Serum that contained antibodies at less than 100 IU/ml still showed a strong correlation with the RFFIT (R value of 0.958; Fig. 3B). As expected, the R value dropped to 0.257 when the antibody level was higher than 100 IU/ml and showed no correlation between the CEE-cELISA and RFFIT (Fig. 3C).

Fifty-two dog serum samples contained an antirabies antibody level of 3.04 ± 3.85 EU/ml and 5.61 ± 10.91 IU/ml according to the CEE-cELISA and RFFIT, respectively, and only 60% of the dogs had a protective amount of the antibody (> 0.5 IU/ml). The comparison between the CEE-cELISA and RFFIT showed a strong correlation (R = 0.947) for all serum samples (Fig. 4A). Moreover, the CEE-cELISA was found to have 100% specificity and 96% sensitivity. Similarly, in humans, serum samples contained antibodies at 10.43–51.06 IU/ml or above 10 IU/ml according to the RFFIT, but the CEE-cELISA could detect antibodies only at up to ~10 EU/ml. Serum samples that contained antibodies at up to 10 IU/ml showed a good correlation (R value of 0.931; Fig. 4B). The R value dropped to 0.192 when the antibody level was higher than 10 IU/ml and showed no correlation between the CEE-cELISA and RFFIT (Fig. 4C). Thus, this CEE-cELISA was suitable for detecting the antibody in dog serum; this level was no higher than 10 IU/ml.

According to ROC statistical analysis, the lowest concentration of an antirabies antibody that could be detected by the CEE-cELISA was 0.725 IU/ml with 100% specificity and 96% sensitivity (Fig. 5A). Reproducibility was calculated from the coefficient of variation (CV) for each serum sample; the assay was repeated twice on
different days of the experiment. The CV of each sample was in the acceptable range, as presented in Fig. 5B. A comparison of antirabies antibody concentrations, when the CEE-cELISA showed a concentration of 1.1 EU/ml corresponded to the RFFIT result at 0.5 IU/ml, which is the protective level (Spearman’s rank correlation calculator). Although the RFFIT and CEE-cELISA assays detect different antibodies, overall, the mAbs used in this CEE-cELISA could detect neutralizing antibodies, just as the RFFIT can. Therefore, the results of the CEE-cELISA highly correlated with the RFFIT results.
DISCUSSION

In Thailand, the pre-exposure prophylaxis schedule against rabies has been proposed for children or pet owners and tourists (15). Each year, the country spends over 30 million US dollars on a rabies vaccine and treatment, but rabies still occurs among animals and humans (16). Therefore, the monitoring of protective immunity after the vaccination of humans and dogs is important for establishing complete rabies prevention programs in Thailand.

The CEE-cELISA was developed in this study from mAb clone 1-46-12, which recognizes (i) a conformational epitope of rabies G protein and (ii) a rabies virus vaccine as antigens for ensuring the efficacy of human immunization and seroprevalence in vaccinated dogs. This CEE-cELISA was also found to be cost effective and easy to use because it involves an ELISA plate coated with the whole virus from a commercial vaccine as an antigen. The whole rabies virus vaccine as an antigen is advantageous because of its low cost (> 50-fold cheaper than rabies glycoprotein), commercial availability, and widespread use as a rabies vaccine in humans.

The serotiters measured by the CEE-cELISA and RFFIT (both detecting a neutralizing antibody) revealed a strong correlation: the R value of 0.958 and 0.931 in humans and dogs, respectively. These correlations were calculated for serum samples of humans and dogs containing a neutralizing antibody at up to 100 IU/ml or 10 IU/ml, respectively. This limitation of detection might be due to the prozone phenomenon or excess serum antibody that binds to the limited amount of the antigen coating the plate. Diluting the serum to reduce excessive antibody concentration or increasing the amount of the antigen coating the plate did not overcome this limitation. The correlations were almost the same as the results of previous reports, which have shown a correlation of 0.96 in a comparison between fluorescent antibody virus neutralization and cELISA using a rabies recombinant glycoprotein expressed in the BHK-21 cell line as an antigen (17). The correlation was observed in the sera below 8.00 IU/ml (17).

Dogs are a major reservoir of rabies in Thailand, whereas humans, cats, and other domestic and farm animals become accidental hosts. The Bureau of Epidemiology of Thailand has reported that rabies positivity prevalence among dogs, cows, and cats is 89.5%, 6.3%, and 3.5%, respectively (18). At least three commercial indirect ELISA kits are available for the detection of human antirabies virus antibodies (19). However, the antirabies ELISA kit might not be suitable for evaluating antibodies in an epidemiological study because of the currently high cost and difficulty with ordering. In addition, a commercial indirect ELISA requires specific reagents for each species, and this situation is not suitable for Thailand, where domestic and livestock animals live together with humans, and rabies can spread among them. Furthermore, there are no commercial ELISA kits available for individual species.

Both human and animal rabies cases have now re-emerged and are increasing in number thus approaching a crisis in Thailand. Children are exposed most frequently to rabies, and pre-exposure prophylaxis should be introduced for them in highly endemic regions to reduce the cost of postexposure prophylaxis, duration of treatment, and risk of rabies infection after a dog bite. For dogs, introduction of an oral rabies vaccine would be beneficial not only for vaccinating stray dogs (which are difficult to capture) but also for overcoming the fear of parenteral injection. The CEE-cELISA in this study would be a useful and suitable tool because it offers a simple method and low cost for mass antibody monitoring.

In conclusion, the CEE-cELISA may be an alternative assay for evaluating mass rabies vaccination rapidly (in a few hours) at a low cost (3- to 4-fold cheaper than RFFIT). Moreover, it does not require a live virus, cell culture, and sophisticated facilities as the RFFIT does. The proposed method can detect antirabies antibodies in the serum of not only humans but also other animal species in the same test. Offering a cheap and accessible test might be effective at persuading high-risk groups or people with individual exposure to the virus to adopt the antirabies antibody measurement as necessary to ensure that rabies vaccination is effective in preventing and treating rabies.

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Conflict of interest None to declare.

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