RESEARCH PROPOSAL

Determination of immunogenicity following anti-rabies vaccination in elephants (*Elephas maximus maximus*) of Pinnawala Elephant Orphanage, Sri Lanka: A proposal

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ABSTRACT

Rabies is a vaccine-preventable fatal viral disease, which affects both domestic and wild mammals. Though the dogs are the main reservoir species of rabies in Sri Lanka, elephants being mammals, are also susceptible to rabies. However, no detailed controlled study has been conducted concerning immunogenicity following anti-rabies vaccination in elephants. Therefore, this research project was designed to determine the pattern of immunogenicity in both routinely vaccinated and zero-positive Sri Lankan elephants following anti-rabies vaccination (ARV). Thirty easily accessible elephants from the Pinnawala elephant orphanage will be used as the routinely vaccinated group of elephants (RVG). This group of elephants will be randomly allocated into two groups with 15 elephants in each (RVG-A and RVG-B) and only elephants in RVG-A will be given a booster of 1-mL of ARV, a year after the initial anti-rabies vaccination. Two previously unvaccinated calves will be selected as the zero-positive control group of the study, and they will be allocated into two groups (ZPCG-A and ZPCG-B). All elephants will be immunized using 1-mL of ARV while the elephant calf in group ZPCG-B will be immunized using 2 mL of ARV as the first dose. A blood sample will be collected before vaccination from all the elephants recruited to the study. Post-vaccination blood samples will be collected on planned dates from the different study groups. Sample analysis of the study will be done by FAVN test at the World Health Organization (WHO) Reference Laboratory for Rabies and Wildlife in Anses-Nancy, France and by ELISA at the Dept. of Physiology, Faculty of Medicine, University of Colombo. Necessary approvals including the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) approval were obtained from the Department of Animal Production and Health and the Dept. of Wildlife Conservation to dispatch samples to the collaborating laboratory for analysis.

Keywords: Anti-rabies vaccination, Elephants, Immunogenicity, ELISA, FAVN.

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INTRODUCTION

Rabies, being a vaccine-preventable fatal viral disease, accounts for tens of thousands of human deaths annually in Asia and Africa. Rabies has been reported in more than 150 countries and territories including Sri Lanka. Though domesticated dogs are largely responsible for up to 99% of human rabies cases, both domestic and wild animals can be affected.[1] Hence, measures to control and minimize the risk of infection in populations of wildlife, stray, and domestic animals are in progress in rabies-endemic countries.[2]

As in other rabies-endemic countries in Asia, dogs are the main reservoir species of rabies in Sri Lanka while mongoose is the main victim of rabies among wild animals. Sri Lanka has had a marked reduction in human deaths due to rabies during 12 years from 1999 to 2010.[3] However, there were 22 human rabies deaths reported in Sri Lanka in 2022.[4] Sri Lanka is the first country in the WHO Southeast Asia Region, to have a documented national strategy for dog-mediated rabies elimination adopting a strategic framework by 2030.[5] Among the methods, vaccination of dogs has been identified as the most important control method.[6] Anti-rabies vaccination gives rise to humoral and cellular immune responses in the vaccinated animal; however, immunity development depends on several factors such as the type of anti-rabies vaccine used, number of vaccinations given, age, gender, health and nutritional status of the vaccine recipient.[7]

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Humoral immune response developed by a single dose of anti-rabies vaccination in a previously unvaccinated dog has been observed to be significantly reduced in 5 months while that in a previously vaccinated dog remains detectable for more than one year.[6,8]

Captive elephants of Sri Lanka, both state-owned (about 200 in total) and privately owned (around 100 at present), manifest an important role in cultural, religious, and state functions, and tourism trade in Sri Lanka.[9] Therefore, every single elephant that is not in the wild, though it is a wild animal, is important to the country for several reasons. Elephants being mammals, they too are susceptible to rabies infection. The first Sri Lankan captive elephant to be rabid in which both brain smear and serum sample were confirmed positive by fluorescent antibody test and serum rapid fluorescent focus inhibition test respectively, was reported in 1997. This incident suggested the necessity to protect our culturally and economically valuable captive elephants from this killer disease.[10] Another case on a rabid elephant has been reported in Sri Lanka in 1998.[11]

Literature Review

A study conducted in 2006 by Isaza R. *et al*., with 16 Asian elephants (*Elephas maximus*) with no known prior anti-rabies vaccination history recommends a two-doses of inactivated rabies virus vaccine of 4 mL (Imrab Bovine Plus, Merial Inc, Athens, Ga) 35 days apart with an annual booster for adequate protection.[12] Another study conducted in 2009 by Miller MA and Olea-Popelka F, with 14 routinely vaccinated African elephants (*Loxodonta africana*) with 2 mL of inactivated rabies virus vaccine (Imrab 3, Merial Inc, Athens, Ga) suggests vaccinating with a frequency of less than annual if antibody titers are routinely monitored.[13] Further, a study using Asian elephants demonstrates that the transfer of maternal immunity mainly occurs prenatally while it persists above the protective levels up to 3 months of age.[14] The absence of a specific schedule for anti-rabies vaccination in elephants is evident as per the previous studies.[12-14]

The horse is known to be an animal similar to the elephant in some anatomical and physiological features. Published literature suggests that a single vaccination on a previously vaccinated horse would provide protection for more than one year while that on a previously unvaccinated horse would not last for so long. Even if the horses are previously vaccinated, they would succumb to rabies if challenged with virulent rabies virus 14 months post-vaccination.[15,16] Though primary studies have been performed with both African and Asian elephants, no detailed controlled study has been conducted in relation to immunogenicity and immune response monitoring. Scientific reports on immunological responses produced by elephants currently vaccinated with available anti-rabies vaccines are almost absent. Therefore, the present study is proposed to determine rabies-neutralizing antibody titres and their persistence in vaccinated captive elephants. Attempts also will be made to compare titres with those of unvaccinated elephants. This project was designed to conduct in collaboration with the Anses-Nancy reference laboratory for rabies and wildlife (OIE/WHO/EU reference laboratory for rabies in France).

Objectives

General objective

This research project will be carried out to determine the immunogenicity and the duration of immunity following anti-rabies vaccination (ARV) in Sri Lankan Elephants, *Elephas maximus maximus*.

Specific (scientific) objectives

To determine the pattern of the humoral response and the apparent duration of immunity against rabies for ARV in routinely vaccinated elephants and unvaccinated elephants by:

- Evaluating the immunogenic capacity (ability of ARVs to produce antibody titers above 0.5 IU/mL which is considered as the protective level).
- Determining the persistence of detectable immunity above the protective level.
- To determine the difference in the pattern of humoral immune response following vaccination in routinely vaccinated elephants and unvaccinated elephants.
- To assess whether immunogenicity measurement by ELISA is comparable to that of FAVN test.
- To determine whether the ARV dose (1-mL) is adequate to produce a protective antibody response in elephants irrespective of the weight, gender and age of the animal.

Additional (non-scientific) objectives

- To develop an anti-rabies vaccination protocol for Sri Lankan elephants.
- To make a recommendation to authorities to minimize cost (by possibly reducing frequency of boostering) for annual vaccination for elephants based on the humoral immune response determined in this study.
- To facilitate and promote rabies antibody testing using semi quantitative ELISA which is cheaper and easier to implement compared to the FAVN reference test (if the ELISA results demonstrate a satisfactory correlation with those of FAVN).

MATERIALS AND METHODS

Study Groups

Routinely vaccinated elephants at pinnawala elephant orphanage

The population of captive elephants in Sri Lanka in the year 2010 was approximately 200 to 250.[17] Pinnawala Elephant Orphanage has 82 elephants: 36 males, and 46 females. As per the records, elephants at Pinnawala have been annually vaccinated with the anti-rabies vaccine (Rabisin® of Boehringer Ingelheim -29 avenue Tony Garnier Lyon, 69007, France) since 1998. The diet formulation, health care, and management practices are similar for all elephants who are approachable in the presence of mahouts. A subgroup

of such elephants from Pinnawala, with annual anti-rabies vaccination, is the best to determine the rabies antibody titres of vaccinated elephants and the duration of such immunity. For elephants in Pinnawala, we have properly maintained vaccination records for ARV.

Unvaccinated (zero-positive) elephants at pinnawala elephant orphanage

Since most captive elephants in government institutions have been exposed to anti-rabies vaccines, it is impossible to find a group of unvaccinated elephants against Rabies within government institutions. Out of captive elephants in Sri Lanka, approximately 112 elephants are privately owned.[17] However, no study has been performed to detect whether these privately owned elephants have been properly immunized. Thus, it is rather impossible to find a group of unvaccinated elephants among privately owned elephants for the study as a control group. Therefore, it is intended to have two recently born calves as the zero-positive control group in view of adding more members to the group later.

Anti-rabies vaccine Rabisin® for vaccination of elephants

No vaccine has so far been specifically developed to be used in elephants.[18] As per the clinical records at Pinnawala Elephant Orphanage, Rabisin® (Boehringer Ingelheim -29 avenue Tony Garnier Lyon, 69007, France) has been used for vaccination of elephants for more than 20 years with no sign of hypersensitivity reaction. Therefore, Rabisin® (1-mL) has been observed to be practically appropriate for elephants, as far as Pinnawala is concerned through experience. In addition, it is important to use a vaccine that is already currently in use in Sri Lanka, so the results of this study will be directly transposable to the field conditions of the country.

Determination of the level of immunity following anti-rabies vaccination using ELISA and FAVN test

Rapid fluorescent focus inhibition test (RFFIT) and fluorescent antibody virus neutralization (FAVN) test are the methods recommended by the OIE and the WHO for the determination of neutralizing antibody titres (humoral immunity) against anti-rabies vaccination.[19] Neither the RFFIT nor the FAVN test is considered as a screening test. Though both the RFFIT and FAVN tests use live viruses to challenge the sensitive cells (BHK-21 or MNA cells) and finally measure the neutralizing antibody titers (quantitative assay) in serum samples, both are expensive and time-consuming.[19] The FAVN test, being the OIE/WHO recommended gold standard method, is more sensitive than the RFFIT for the detection of rabies virus-neutralizing antibodies around the threshold of positivity. It is also known that the FAVN test is easier to perform compared to the RFFIT.[20]

Currently, a cheaper, semi-quantitative ELISA test, such as the BioPro RABIES ELISA Ab kit (O.K. SERVIS BioPro, s.r.o. Boretick 2668/1 19300 Praha 9-Horni Pocernice Czech Republic), is already established at the Dept. of Physiology, Faculty of Medicine, University of Colombo in Sri Lanka. This semi-quantitative blocking ELISA test could be used to assess serum antibody titres in elephants, and the results could be compared with those of the FAVN test. If semi-quantitative ELISA shows that it could be reliably used on elephants for estimating rabies protective antibody titres, such testing on all elephants in Sri Lanka could be performed at a lower cost and could include all captive elephants which are dispersed all over the country. Inaccessibility to sophisticated laboratories also would be unnecessary if semi-quantitative ELISA could be reliably used for this purpose.

Both FAVN test and semi-quantitative ELISA in estimating rabies antibody titres on all subject elephants will be conducted. In addition to estimating the titre levels, such an attempt would provide data to compare the two testing procedures for possibly validating the use of the ELISA in Sri Lanka on elephant serum samples.

Methodology

Prospective study with elephants at pinnawala elephant orphanage

According to the accepted ethical guidelines for the use of animals for the research, 30 easily accessible elephants from Pinnawala elephant orphanage will be used as the routinely vaccinated group (RVG) of elephants. Exactly one year after the initial vaccination of all the elephants with 1-mL of anti-rabies vaccine (Rabisin®), this group of elephants will be randomly allocated into two groups (RVG-A and RVG-B) of 15 elephants each. RVG-A will comprise elephants, which will be vaccinated with a second dose of ARV (1-mL) one year after the first dose while RVG-B will comprise elephants that do not receive a booster vaccination one year after the first dose. Two previously unvaccinated calves will be selected as the zero-positive control group (ZPCG) of elephants for the study. These two calves will be allocated into two groups (ZPCG-A and ZPCG-B) of one calf in each. We plan to recruit more animals to this group later depending on availability.

Sample size calculation

Sample size was calculated using the following equation.

$$n = \frac{z^2 \times \hat{p}(1-\hat{p})}{\varepsilon^2}$$

where;
z - z score of confidence level
ε - margin of error
n – Sample size
p̂ - population proportion.

For this calculation $\hat{p}$ has been taken as a guess estimate of 30% and the margin of error as 20% since no study has been performed before in Sri Lanka with serum antibody levels of elephants.

$$n = 1.96^2 \times 0.3\ (1- 0.3)/\ 0.2^2 = 20.2$$

Though the calculated sample size was 20.2, it was decided to include additional numbers depending on the availability at the study site (Pinnawala elephant orphanage). Thirty manageable and cooperative elephants will be identified for the RVG while two cooperative unvaccinated calves will be used as the ZPCG. All the elephants in RVG and ZPCG are in the same study setting.

Vaccination of elephants

All the elephants in RVG and ZPCG-A will be immunized using 1-mL of ARV (Boehringer Ingelheim -29 avenue Tony Garnier Lyon, 69007, France) while the calf in ZPCG-B will be immunized using 2-mL of ARV (two doses of the same vaccine mixed in a single injection) as the first dose of the study with 18-gauge needle intramuscularly into the area of biceps femoris muscles which is the routine/usual practice. To exactly determine the effect of the dose of vaccine for immunogenicity of elephants one calf will be immunized with a single dose of ARV while the other will be with two doses of ARV in the same syringe as two references suggested use of both single dose of the vaccine and double dose of vaccine.[12,13]

A blood sample (D=0) will be collected before initial vaccination of all recruited elephants. The elephants of RVG-B will get the second vaccination and the elephants of ZPCG-A and ZPCG-B will get both the second (first booster/RV1) and the third (second booster/RV2) vaccinations when the antibody titer level reduces below 0.5 IU/mL threshold. It is assumed that the antibody titer level in RVG-B would reduce within 2 years from the first vaccination when they were in group RVG. Antibody titer levels of ZPCG-A and ZPCG-B may reduce within 2 months after the first vaccination and within 2 years after the second vaccination.[12,13] Vaccination of elephants will be performed only by the veterinarian of the Pinnawala elephant orphanage with minimal stress to the elephants.

Anti-rabies vaccine

The anti-rabies vaccine (ARV) used for the study will be purchased as per department regulations considering the date of manufacture and reliable quality of the vaccine (Table 1).

Assurance for the maintenance of the cold chain will be obtained from the local agent company that handles the vaccine as it is difficult for direct purchases from the sole agent's main stores in Sri Lanka under prevailing government regulations. All vaccine vials will be purchased from the same lot/batch having a shelf-life of 2 to 3 years. ARVs will be stored at 4°C in the veterinary section of the Pinnawala Elephant Orphanage.

Blood collection from elephants

Schedule for blood collection and vaccination of elephants in different groups are indicated in the following tables (Tables 2-4).

Blood (6–8 mL) will be collected from the auricular vein around 8.30 am to 10.00 am from each elephant recruited to the study under aseptic conditions after applying surgical spirit and anesthetic gel, on D0 before vaccination in both RVG and ZPCG (both A and B groups). Post-vaccination blood samples of RVG will be collected on D30, D120, D180, D365 and at two-month intervals thereafter. RVG-A will be given the second vaccination at D365 while the revaccination for RVG-B, ZPCG-A, and ZPCG-B will be done when the antibody titres reduce below the 0.5 IU/mL threshold assessed by ELISA. Two blood samples 7 days apart will be collected one week after the drop in the threshold in RVG-B, ZPCG-A, and ZPCG-B. Post-vaccination blood samples of ZPCG-A and

Table 1: Details of the anti-rabies vaccine used

Vaccine brand	*Vaccine type*	*Country of origin*	*Name of the manufacturer*	*Pack size*	*Local agent*
Rabisin	Rabies virus glycoproteins	France	Boehinger Ingelheim animal health	Single dose (1 mL)	CIC Vetcare Pvt Ltd

Table 2: Schedule for blood collection and vaccination of elephants in RVG-A

Group	*Y-1*	*D0*	*D30*	*D120*	*D180*	*D365*	*RV1+60 = D425*	*D365+120 = D485*	*D545*	*D605*	*D665*	*D725 (=2Y)*
RVG-A (Adults)	V	BS + V (1-mL)	BS	BS	BS	BS +RV1	BS (bimonthly)					Last BS +RV2

**BS= Blood Sampling, V= Vaccination, RV1= Revaccination (booster 1), RV2= Revaccination (booster 2), Y=Year*

Table 3: Schedule for blood collection and vaccination of elephants in RVG-B

Group	*Y-1*	*D0*	*D30*	*D120*	*D180*	*D365*	*Bimonthly till <0.5 IU/mL*	*D.. =RV1*	*RV1+D7*	*RV1+D14*
RVG-B (Adults)	V	BS + V (1-mL)	BS	BS	BS	BS	BS	BS	BS	BS

**BS= Blood Sampling, V= Vaccination, RV1= Revaccination (booster 1), Y=Year*

Table 4: Schedule for blood collection and vaccination of elephants in ZPCG-A and ZPCG-B

Group	*D0*	*Monthly till <0.5 IU/mL*	*D.. =RV1*	*RV1+D7*	*RV1+D14*	*Six-months Intervals till <0.5 IU/mL*	*D…=RV2*
ZPCG-A (Calves)	BS + V (1-mL)	BS	BS	BS	BS	BS	BS
ZPCG-B (Calves)	BS + V (2x1-mL)	BS	BS	BS	BS	BS	BS

**BS= Blood Sampling, V= Vaccination, RV1= Revaccination (booster 1), RV2= Revaccination (booster 2)*

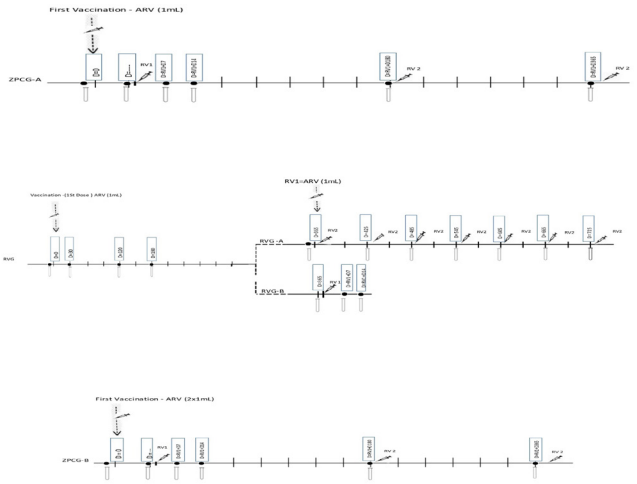

Figure 1: Schematic diagram indicating the study groups, identified days of blood collection, and the points of vaccination. A; Routinely vaccinated group (RVG). RVG-A elephants will be vaccinated with a 2nd dose of ARV (1-mL). RVG-B elephants will not receive a 2nd dose of ARV. B; Zero-positive control group-A (ZPCG-A), ZPCG-A elephants will be vaccinated using 1-mL of ARV. C; ZPCG-B elephants will be vaccinated using 2 mL of ARV

ZPCG-B will be collected monthly after the first vaccination and at six-month intervals after the second vaccination (The schematic diagram, Figure 1 facilitates the understanding of the protocol). It is assumed that the antibody titer level in RVG-B would reduce within 2 years from the first vaccination when they were in group RVG. Antibody titer levels of ZPCG-A and ZPCG-B may reduce within 2 months after the first vaccination and within 2 years after the second vaccination.[12,13] Immediately after the collection of blood, 6-8 mL of blood will be separated into a plain tube for serum separation. The serum collected will be stored at -20°C until processed for antibody testing. Collection of blood will be performed only by the veterinarian of the Pinnawala Elephant Orphanage with minimal stress to the elephants.

Serum Sample Analysis

Determination of humoral immunity (Measurement of antibodies present in serum samples)

The humoral immunity determination will be done both by florescent antibody virus neutralization (FAVN) test and enzyme linked immunosorbent assay (ELISA). Semi-quantitative ELISA assay will be performed at the Dept. of Physiology, Faculty of Medicine, University of Colombo. The FAVN test will be performed at the OIE/WHO/EU Reference laboratory for rabies and wildlife in Anses-Nancy, France. The minimum number of samples analyzed by the FAVN test and ELISA will be 216 (30X5+30+15X2+2X2+2). The maximum number of samples that may be possible to analyze will be 374 (30 X 5+ 30 X 6 +15 X2+ 2 X 3 + 2 X 4).

Determination of virus-neutralizing antibody titers

The FAVN test measures the ability of neutralizing antibodies that may be present in the sample to neutralize and block the rabies virus replication before infecting sensitive cells used in the test.[20] These antibodies are called rabies virus-neutralizing antibodies (RVNA). When performing the test, the serum is subjected to serial dilutions to make a smaller number of antibodies available in the serum sample. Then, the serum dilutions are mixed with a standard amount of live fixed rabies virus and incubated. Subsequently, the binding of RVNA present in the sample to the virus takes place. Then a fixed concentration of sensitive tissue culture cells is added and incubated with the virus and test sample. Thereafter, the rabies virus particles that have not been neutralized by the antibodies infect the sensitive cells. Around 48 hours later, after fixation of the cell monolayer, infected wells in the 96-well plate are examined by using an inverted fluorescent microscope. An "all or nothing" reading is performed. If the fluorescent microscope examination shows a large number of infected cells that indicates the serum sample has a very low level of rabies neutralizing antibodies. The endpoint titer is calculated based on the number of virus-infected wells containing various dilutions of the sample on the plate. Based on the results of the OIE reference positive control, added in each FAVN test; the sample results can be reported as a standardized concentration represented in international units (IU) per mL of serum (e.g. 0.5 IU/mL which corresponds to the international threshold of protection against the rabies virus).[20]

Determination of humoral immunity by ELISA using BioPro Rabies kits

The BioPro ELISA kit is known to produce better results with high sensitivity compared to the PLATELIA™ RABIES II ELISA kit. BioPro ELISA kit has been reported to have a better correlation with the FAVN test as well.[20] It is an in vitro diagnostic blocking ELISA test that detects rabies virus antibodies.[19,21] Although the BioPro ELISA kit has been recommended for serum sample analysis of domestic and wild animals, the kit producing/marketing company in the

Czech Republic and the rabies experts in the OIE/WHO/EU reference laboratory for rabies recommend the use of this kit for serum sample analysis in other rabies vulnerable animals too. Currently, this kit is being used in another project involving New Zealand white laboratory rabbits with positive results.[22]

Transportation of samples to collaborating OIE/WHO/EU Reference Laboratory in Anses-Nancy, France

International collaborating laboratory will organize transport facilities to transport samples for humoral immunity assessment by FAVN under suitable conditions once in 6 or 12 months depending on the feasibility. Until the time of shipment, samples will be stored at -20°C at the Genetics Unit, Faculty of Medicine, University of Colombo. All the samples transported to the collaborating laboratory will be stored under specific conditions until analysis.

Validation and quality control of methods

Back titration of the challenge rabies virus, OIE positive reference serum, and negative serum will be used by the collaborating laboratory during the FAVN test for quality control of the assays conducted for the measurement of humoral immunity. During ELISA assays, quality control solutions provided with the assay kits will be used.

Approval for the study

Ethics approval was obtained for the planned study from the Ethics review committee of the Faculty of Medicine, University of Colombo (EC-22-025). Approvals from the Department of Wildlife Conservation and the Department of Animal Production and Health including the approval from the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (E 003464) were obtained to dispatch serum samples to collaborating laboratory in Anses-Nancy, France for FAVN analysis.

Addressing ethical concerns

The samples collected will not be used for purposes other than to achieve listed specific objectives. Furthermore, investigators having personal protective equipment worn and the elephants being properly restrained will ensure protection during vaccination and blood sample collection on scheduled dates. Laboratory staff too will take all precautionary measures (lab coats, gloves, face masks) during the processing of samples for determination of immunity level. Mahouts will be provided aprons, boots, gloves, and face masks for their protection. Data on animals and vaccine details will be kept with the research student and principal investigator. Anti-inflammatory drugs will be used only if reactions are developed following vaccination which is very unlikely as per the experience of investigators with veterinary qualifications. If any adverse reactions occur, they will be reported to the responsible authorities. We don't have any conflicts of interest as we use vaccines provided by the elephant orphanage for two main groups of elephants (RVG-A and RVG-B). Therefore, we are not biased during the allocation of animals to groups and the vaccine used in different groups.

Statistical analysis

Statistical analysis will be conducted using SPSS software. Mean antibody levels in different groups (RVG-A, RVG-B, and ZPCG) will be described using standard descriptive methods. A comparison of antibody levels between the two genders will be conducted using pooled t-tests. Changes in antibody levels within the group over time will be analyzed using paired samples T-test or relevant non-parametric test. The association between the results of the FAVN test and ELISA testing will be analyzed using Kappa statistics and also by calculating the sensitivity and specificity of ELISA in relation to the FAVN test.

Disposal of samples used in the research

If there are any remaining serum samples, these will be disposed according to accepted procedures after 6 months once the project is completed.

DISCUSSION

The absence of any information on a detailed controlled study on elephants following anti-rabies vaccination led us to propose this study in order to determine the pattern of the humoral response and the apparent duration of immunity against rabies for ARV in routinely vaccinated elephants and unvaccinated elephants by evaluating the immunogenic capacity (ability of ARVs to produce antibody titers above 0.5 IU/mL which is the proposed threshold level) and by determining the persistence of detectable immunity above the threshold level. Other specific objectives are to determine the difference in the pattern of humoral immune response following vaccination in routinely vaccinated elephants and unvaccinated elephants, to assess whether immunogenicity measurement by ELISA is comparable to that of FAVN test and to determine whether the ARV dose (1-mL) is adequate to produce a protective antibody response in elephants irrespective of the weight, gender and age of the animal.

Elephant being an endangered species, the proposed study with this species is a sensitive research area. Therefore, ethics and other relevant government Department approvals were obtained prior to initiation of the project. International collaborating laboratory being the OIE/WHO/EU reference laboratory for rabies and wildlife in France, it has the mandate to perform serological tests related to rabies.

If both the humoral and cellular immunity measurements could have been done in the project, the scientific value is enormous. Unavailability of necessary facilities and limitations in funding led us to limit the immunity assessment to antibody titre measurement. Despite the limitations, the findings of the study will be useful not only for achieving the identified specific objectives of the study but also to develop an anti-rabies vaccination protocol for Sri Lankan elephants,

to make a recommendation to relevant authorities to minimize cost (by possibly reducing frequency of boostering) for annual vaccination for elephants based on the humoral immune response determined in this study, and also to find out whether the data from ELISA and FAVN test demonstrate a satisfactory correlation between the two.

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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.