ANIMAL EXPERIMENT PROTOCOL

Determination of immunogenicity and potency of canine anti-rabies vaccines marketed in Sri Lanka: A proposal on an experimental study using rabbits as the laboratory animal model and field study using sample dog population

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ABSTRACT

Anti-rabies vaccines (ARVs) with good immunogenic capacity play an important role in controlling the deadly zoonotic rabies in Sri Lanka. The ARVs currently marketed in the country have not been tested for their efficacy and potency at the local level before being released to the market. This article outlines the proposed project formulated to determine the efficacy and potency of these ARVs in generating immunity against rabies. The authors propose that this project is articulated in three main categories. Part I is the immunogenicity measurement using New Zealand White rabbits as the laboratory animal model. Part II is a field study with a sample dog population, including domestic and free-roaming dogs and Part III is the potency testing of the ARVs using the mouse challenge test. Immunogenicity will be assessed by measuring the humoral immunity development following vaccination, using the methods of Fluorescent Antibody Virus Neutralization (FAVN) and ELISA. In parallel to the humoral immunity assessment, full blood counts of the animals will also be assessed in each of the sampling dates. The project is proposed to be conducted in collaboration with the WHO/ WOAH Reference Laboratory for Rabies in Nancy, France, and the Medical Research Institute, Colombo 08, Sri Lanka.

Keywords: Project proposal, Anti-rabies vaccines, New Zealand White rabbits, Potency, Immunogenicity, FAVN, ELISA.

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Introduction

Rabies is a vaccine-preventable zoonotic disease endemic to Sri Lanka. The application of different control methods enabled the reduction of the total number of confirmed human rabies deaths over the years, which went from 300 in 1970, 100 in 1995, 50 in 2005 to 24 in 2015. Out of these 24 human deaths in 2015, the highest number of deaths were reported from Kurunegala and Colombo districts: 8 from Kurunegala and 4 from Colombo. Based on the most recent records, 20-30 human deaths are being reported annually due to rabies.[1] In Sri Lanka between 2005 and 2014, a total of 8712 animal rabies cases (clinically diagnosed) had been reported where, 6788 (78%) were dog rabies cases, 1197 (13.7%) were livestock, 663 (7.6%) cats, and 64 (0.7%) wild animals.[2] In 2015, among the 607 confirmed animal rabies-positive cases, there were 471 dogs, 104 cats, 9 squirrels, and 8 cows.[3] In the global context rabies is responsible for huge economic losses too.[4] As Sri Lanka is an island, the elimination of this deadly disease is not a difficult task to perform, and thereby the economic burden to the country could be reduced drastically.

Rabies virus has been maintained principally in the dog population in Sri Lanka.[5] Both domestic and stray dogs have been identified as principal reservoirs and the main source of human rabies cases in Sri Lanka.[5] Therefore, the immunization of dogs plays a major role in controlling this zoonotic disease. Based on the recommendations of WHO and WOAH, the antibody level of ≥ 0.5 IU/ mL is considered as the protective threshold or an adequate antibody response following vaccination.[6] There was some pioneering research work conducted to determine the immunogenicity following the vaccination of dogs. One of the studies has shown that

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a single vaccination for puppies is inadequate to maintain an antibody titre ≥ 0.5 IU/mL (the recommended protective threshold) until the annual booster. This was irrespective of whether their mothers were vaccinated or not against rabies.[7] It has also shown that vaccination should be done at the age of 6 weeks.[7,8] The dogs that were given regular vaccinations have an adequate level of humoral immunity which indicate the importance of annual boosters.[9] These findings were used to introduce a new canine anti-rabies vaccination schedule for Sri Lanka in 2013 in which they indicated a booster vaccination in the first year.[10] However, based on those studies, determining the exact time of the booster vaccination in the first year was a bit challenging because necessary scientific data in relation to the pattern of immunogenicity in the first year of vaccination was not available from those studies.

Most of the anti-rabies vaccines (ARVs) are cell culture vaccines containing killed or live attenuated viruses produced in different countries. It is mentioned that the adjuvant added to these vaccines could increase adverse vaccine reactions in pets, as these adjuvants could act as immune modulators.[11,12] Seven different brands of inactivated anti-rabies vaccines which are available in single-dose and/or multidose preparations for animals have been registered under the Veterinary Drug Control Authority of Sri Lanka. In this study, we will be examining seven single-dose preparations from the seven different brands and two different multi dose preparations including the vaccines used in the government rabies control programme.

In Sri Lanka, the registration of ARVs were based on the dossiers that were submitted at the time of application for registration by the company handling the vaccine. The efficacy and potency of these vaccines have not been tested before registration in Sri Lanka as no process has been developed so far for this purpose. The ARVs currently available in the market are used to vaccinate any vulnerable species of animals in Sri Lanka, including the zoological gardens and elephant orphanages.

Effectiveness of the ARVs could be assessed by measuring humoral and cell mediated immunity. Among the two available tests, Fluorescent Antibody Virus Neutralization test (FAVN) and Rapid Fluorescent Focus Inhibition Test (RFFIT) for the determination of humoral immunity following vaccination, FAVN test is the method recommended by the World Organization for Animal Health (WOAH). This test, which is an adaptation of the original RFFIT, has high accuracy and specificity in measuring virus-neutralising antibodies.[13-15] An Enzyme-linked immunosorbent assay (ELISA) kit with high specificity and sensitivity is comparable with that of the gold standard FAVN test in measuring humoral immunity following anti-rabies vaccination. ELISA measures binding antibodies to viral antigens.[16-18] RFFIT is a well-established method at the rabies research lab of Medical Research Institute (MRI), Colombo where we conducted our pioneering work.[7-10,19] As per the European Pharmacopoeia protocols, the serological potency assay could be used to assess the potency of vaccines as an alternative method to the *in vivo* potency tests.[15,20]

Literature Review

Use of a suitable laboratory animal model

As dog vaccination plays an important role in controlling and eliminating rabies from Sri Lanka, it is necessary to select ARVs with different brands and presentations with good immunogenic capacity. Also, a method should be established to check these ARVs' immunogenicity. This kind of work should be conducted under specific controlled conditions by maximally minimizing variations which could affect the measurement of humoral immunity following vaccination. Despite the dog being the best animal model for this study as the target species, due to variations such as genetics, species, age, nutrition, parasitic infections, environments etc it is best to consider a suitable animal model to concentrate on immunity development without these variations.[21] For this study it is necessary to have animals with zero antibodies against rabies either by maternal transfer or by previous ARV injection or by previous exposure to rabies virus as this would affect the immunogenicity of ARVs in the planned study due to the presence of memory cells. On the other hand, in Sri Lanka, there are no animal facilities where dogs are bred for laboratory purposes, and animal welfare issues may also negatively impact the whole research. Moreover, national and local authorities in Sri Lanka have invariably targeted the stray dog population during vaccination programmes although there is no 100% coverage and also majority of owned dogs have also been vaccinated against rabies. As such, recruiting dogs that are not having antibodies against rabies is not practically feasible to achieve a large sample size to test all the vaccine brands.

Most of the available ARV brands are recommended for any vulnerable species of animals. Therefore, a suitable laboratory animal species of close genetic similarity (which minimizes genetic variation among the animals used in the study) with zero antibody titers could be used considering our requirements while maintaining similar environmental and feeding conditions to all animals thus minimizing the variability. As per specific objectives of the project we focus only on the gender base variability in this experimental study. Rabbits have been suggested as a laboratory animal model for the development of immunoglobulins for post-exposure treatment in recent times.[22-24] Also, rabbits have been used for testing the efficacy of animal and human vaccines and in certain instances as a novel animal model.[25-29] On the other hand, rabbits are a vulnerable group of animals for rabies as indicated in the literature.[30,31] It is also mentioned that rabbits played a significant role in relation to study of rabies and the development of a rabies vaccine in 1881 by Roux, Chamber l and Thuillier who were the members of Louis Pasteur's team. Further, the rabies vaccine they developed subsequently protected experimentally challenged rabbits and dogs. It is indicated that the same rabies vaccine was used successfully to treat Joseph Meister, who received 14 bites from a rabid dog.[32] Therefore, it was decided to select New Zealand White rabbits purchased from MRI. Use of this strain of rabbits will

not affect the outcome of the study. Even for cell culture-based ARV development for human use, cell lines of different animal tissues such as purified chick embryos, and monkey kidneys are used. Also in the post-exposure treatment, equine rabies immunoglobulin produced by hyperimmunization of equines is used. In Ethiopia, sheep brain-derived Fermi-type rabies vaccine is still being manufactured and used in patients.[33-37] The determination of humoral immunity in serum samples of dogs and of humans by RFFIT and FAVN test uses cell lines of baby hamster kidneys (BHK21) with specific reference serum samples as a positive control.

Using rabbit as the laboratory animal model provides us with an advantage over rat and mouse models to collect approximately 6.0 mL of blood sample from each animal for immunity, and selected haematological measurements. Therefore, all of this literature-based evidence justifies the selection of rabbits as a suitable laboratory animal model for this experimental study.

Use of ELISA with FAVN test

Although laboratory facilities for RFFIT are available at the MRI, it is time-consuming and neither RFFIT nor FAVN test are considered as a screening test. These two tests use live viruses to challenge the cells in the BHK21 cell lines and finally measure the neutralizing antibody titres in serum samples. FAVN test being the WOAH/WHO recommended gold standard method, it is easier to perform compared to RFFIT. Therefore, this project will be conducted in collaboration with the NANCY laboratory for rabies and wildlife which is an EU/WOAH/WHO reference laboratory for rabies in France (Establishment of laboratory facilities for FAVN test and its validation in Sri Lanka needs time). While conducting FAVN test, if the virus/virus particles binding assay, ELISA could be conducted, which is considered as a screening test, less time consuming and easy to perform, the immunogenicity of vaccines by these two methods could be compared at the same time. That will enable us to establish ELISA method too in Sri Lanka as the cost for the determination of humoral immunity by ELISA is quite inexpensive and less time-consuming compared to RFFIT or FAVN test.[16-18,38-51] In occasions where a person is being bitten by a dog, there may be a need to check the humoral immunity of the animal. This will help determine whether the affected person will require post-exposure treatment or not. If the ELISA method is well-established, it could be used as a screening test in these situations.

Field study in a sample dog population

As per the advice of the international collaborators, a confirmation study should be performed in dogs, which is the target species for the vaccine. A representative sampling of field dogs who are seronegative for rabies before vaccination will be considered in order to validate our findings on a pattern of immunity development with the laboratory rabbit model. To select the aforementioned sample of dogs, there are no facilities that specifically breed dogs for laboratory purposes in the country, nor are there dogs kept without vaccination in animal shelters in Sri LankaTherefore, the interior area/s of the Municipal Councils in several districts and their suburbs which are not covered by the government rabies control programme will be selected for the recruitment of free-roaming and domestic dogs to the planned field study with necessary approvals from government authorities.

The major questions to be addressed by conducting this research are:

- Whether ARVs of different brands available in the Sri Lankan market possess a good immunogenic capacity and potency. There have not been any studies in the country that assessed and compared all the available ARVs in the market in relation to their immunogenicity and potency.
- ELISA is an accurate method to determine humoral immunity following vaccination as a screening test compared to FAVN test? (To collect evidence from the Sri Lankan context)
- There is any correlation in the immunogenicity based on the gender and blood cell measurements (an exploratory component of the study)?
- Whether the findings in the experimental animal model are confirmed in a sample dog population under field conditions?
- Possible comparison of humoral immunity with cellular immunity to vaccination. The protection against rabies is provided by both humoral and cellular immunity following vaccination. Currently, facilities for the measurement of cellular immunity following vaccination against rabies are not available in the country. Discussions held with an immunologist at the medical research institute regarding the establishment of a suitable method to measure cellular immunity following rabies vaccination were not successful. On the other hand, freshly collected blood samples are required to isolate the blood mononuclear cells to perform the cellular immunity detection methods available in the international collaborating institute in France. Therefore, we will be looking for the possibility of conducting a study in the international collaborating laboratory using laboratory-bred dogs using vaccines available in the Sri Lankan market with the approval of the Department of Animal Production and Health.

Objectives

General objective

This research project will be carried out to determine the immunogenicity and potency of canine anti-rabies vaccines (ARVs) available in the Sri Lankan market using rabbits as the laboratory animal model and to evaluate the immunogenicity of selected vaccines in a sample dog population under field conditions.

Specific (Scientific) objectives

I. To determine the pattern of immunogenicity and potency of ARVs available in the market, using rabbits as the model, by:

- Evaluating the immunogenic capacity (the ability of ARVs to produce antibody titres above 0.5 IU/mL which is considered as the protective level)
- Determining how long antibody titres are maintained above the suggested protective level during a one-year period with a single ARV of different brands.
- Determining the potency of ARVs marketed in Sri Lanka according to the European Pharmacopoeia monograph.

II. To determine the humoral immune response to vaccination in a sample dog population under field conditions.
III. To assess whether immunogenicity measurement by ELISA is comparable to that of the FAVN test.
IV. To explore the possibility of measuring cellular immunity following vaccination of a group of laboratory-bred dogs at the international collaborating institute using the anti-rabies vaccines (same batch/lot) purchased for the study.

Additional (non-scientific) objectives

- To explore the possibility of establishing a procedure to check the immunogenicity of ARVs prior to registration in Sri Lanka.
- To establish FAVN test and ELISA methods for the determination of immunogenicity following ARV in Sri Lanka for future research work.
- To make recommendations to government authorities in relation to purchasing ARVs.

Parameters that will be used to meet the objectives

The immunogenic capacity/humoral immune response of the vaccines will be evaluated by measuring the antibody titres in the serum. This will be done by using ELISA and FAVN. In the ELISA, the results will be semi-quantitative where we can detect the presence or absence of antibodies in the serum sample. From the ELISA, the results will be as negative for antibodies (Ab -) or positive for antibodies (Ab +), or positive for antibodies with antibody level equal to or lesser than 0.5 IU/mL (Ab $\leq$ 0.5 IU/mL). From the FAVN test antibody levels in the serum can be quantified and determine the concentration by the units of IU/mL. By assessing how the vaccines can induce the production of antibody titre $\geq$ 0.5 IU/mL, which is the protective threshold against rabies, the immunogenic capacity of the ARVs will be assessed.
By performing both ELISA and FAVN on the same set of samples, we can assess the comparability of both tests.
The ELISA and FAVN will be done for the samples collected in both the laboratory animal part and field dog part of the study.
Potency will be performed using the mouse challenge test. The final potency values of the vaccines will be presented as IU per minimum effective dose.

Material And Methods

PART 1: Experimental study with rabbits

The study is conducted with the ethical approvals from the ethics review committee, Faculty of Medicine, University of Colombo (EC-16-050), and the ethics review committee of Medical Research Institute, Colombo (ERC no 34/2017)

Animal model

An order is placed with the Animal House of Medical Research Institute (MRI), Colombo 08, to commence breeding of animals. Approximately 3-4 months old healthy New Zealand

Table 1: Vaccines that are used

No	*Vaccine Brand*	*Vaccine type*	*Country of Origin*	*Name of the manufacturer*	*Date of Manufacturing*	*Pack Size (mL)*	*Local Agent*
01.	Canvac R inj.,	Inactivated Rabies vaccine – virus Rabiei	Czech Republic	Dyntec. Spol. S.R.O	Not mentioned	1, 10	Farmchemie Pvt Ltd
02.	Raksharab	Rabies Antigen CVS Strain Killed	India	Indian Immunologicals Ltd.	1-mL (June 2019) 10 mL (March 2019	1, 10	Analytical Instrument Pvt Ltd.
03.	Nobivac RL	Inactivated combined vaccine against rabies and leptospirosis caused by bacteria *L. canicola* and *L. icterohaemorrhagiae*.	Netherlands	Intervet International B.V.	Aug - 2018	1	Brown & Company PLC
04.	Nobivac Rabies	Nobivac Rabies Inactivated vaccine	Netherlands	Intervet International B.V.	Nov - 2018	1	Brown & Company PLC
05.	Defensor 3	Rabies vaccine Killed virus	USA	PFIZER Animal Health, USA	Not mentioned	1	Unical Ceylon Limited
06.	Rabies killed vaccine	Flury LEP strain of rabies	South Korea	Komipharm International	Dec- 2018	1	Medica Lanka Holdings Pvt Ltd
07.	Rabisin	Rabies virus glycoproteins	France	Merial Animal Health	Not mentioned	1	CIC Vetcare Pvt Ltd

White rabbits weighing 1.5 to 2.5 Kg are purchased from MRI. The rabbits are purchased in equal numbers of male and female (N = 108, males- 54, females - 54). Purchasing is done in batches of 20 animals at a time. An experimental study with rabbits is conducted in collaboration with the MRI Animal House according to accepted ethical guidelines for the use of animals in research. The research student who is a veterinary graduate, has been trained on animal handling, welfare and blood drawing prior to the commencement of the project by the Head/Animal House (Co-Investigator of the project).

Recruitment of rabbits to different groups

Twenty animals at a time purchased from the MRI are randomly allocated to different groups identified by numerical values from 1 to 9 (Based on different brands of vaccines, it is not the order of brands listed in the given Table 1 in this proposal). To minimize the variations (session effects) among groups, two/three animals are allocated per group at a time. This process of recruitment is continued until the required sample size per group is met. As any of the investigators don't have conflicts of interest over purchasing of vaccines or other economic benefits by conducting this study, other than the scientific benefits, we are not biased in the allocation of animals into groups and group numbering.

Sample size calculation for the experimental study

Sample size is calculated to determine a significant difference in antibody titres following different ARV brands. According to the literature, the peak antibody response following ARV vaccination is recorded 3-6 weeks. The findings of Minke *et al*., (2009) report a mean antibody level (SD) 1.07 (0.84) and 0.11 (0.06) 6 weeks after immunization in dogs given two brands of ARV.[52] These values are used in the calculation of sample size. Sample size to determine the difference between two means is given by,

$N = [(1/q1+1/q2) S2 (Z\alpha + Z\beta)2] /E2 (50)$

Where,

N= Calculated sample size

q1 and q2 = Proportions of subjects in the two groups

S = Standard deviation

E = Effect size

Zα = Z value corresponding to the α error

Zß= Z value corresponding to the ß error

The difference between means reported in Minke *et al* (2009) gives a standardised effect size of 1.14. Using this and an α error of 5% and a ß error of 20% (power of 80%) and assuming equal proportions in the groups, the sample size required per group is calculated as 12 per group. Therefore, the total number of rabbits included in this experimental study are 108 considering the minimal number of animals that should be in a group (n=12 per group; 6 males and 6 females) to obtain significant statistical comparisons. These animals are divided into 9 groups (equivalent to the number of different ARV brands; single/ multi-dose, available in the market). Each group having 6 males and 6 females enables us to determine immunogenicity following ARV according to their gender. Determination of the immunogenicity based on the gender of the laboratory rabbits will give us the opportunity to investigate possible gender variations that can occur with respect to immunogenicity. Individual cages where animals are kept during the project will be marked to identify animals in each group.

Vaccination of rabbits

All animals are immunized using ARV (1mL) subcutaneously into the area where loose skin is available over the neck by the research student who is a qualified veterinary surgeon. A blood sample (D0) is collected before vaccination. The vaccine volume/ dose of 1 mL is recommended by all vaccine manufacturers as the minimum immunizing dosecapable of inducing effective immunity. All the ARV brands used in this study are recommended to be administered to vulnerable species, including dogs and rabbits. Therefore, both animal categories will be vaccinated with 1 mL of ARV.

Vaccines

As per the information given below, only seven brands of ARVs in the market in 2019 which have been used in this study. Details of ARVs are given in Table I. Considering the date of manufacture, vaccines have been ordered/purchased directly as per university regulations. Assurances for the maintenance of cold chain have been 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 university regulations. Single-dose vials of all vaccine brands and multi-dose preparations of 2 brands including the brand used in the government rabies control programme have been selected. Testing all multi-dose vaccine brands is not feasible due to practical and economical constraints. All vaccine vials of a single brand have been selected from the same batch/lot.

A specific identification code is assigned to these ARVs after purchasing them in order to maintain confidentiality and to avoid bias that may arise due to brand names. The same number will be assigned to groups of animals too. This may help to distribute the possible variations in vaccines such as different manufacturers, different dates of manufacture, expiry dates, batch variation etc. equally among the animals. This will also reduce possible errors in the results. ARVs are stored at 4°C in the Dept. of Physiology, Faculty of Medicine, Colombo.

Blood collection from rabbits

Approximately 6.0 mL of blood was collected around 9.00 a.m. from the middle ear artery of each animal under aseptic conditions. Blood collection is done after applying an anesthetic cream locally to reduce the pain to the rabbits. Samples are collected on D0 before vaccination and on D7, D14, D30, D90, D180, and D360 after vaccination to determine the immunogenicity following vaccination. It also helps to determine the maintenance (pattern) of immunity over a one-year time period at different time points. This will enable us to achieve the specific objectives considering animal welfare as well. As per the international collaborators, in small

animals' the highest antibody titre could be achieved earlier than 1 month and therefore, D7 and D14 blood samples have been collected following vaccination. Immediately after collection, blood samples are divided into two containers; approximately 4.0 mL of blood is separated into a plain tube for serum separation and 2.0 mL of blood is separated into an EDTA container for blood cell parameter measurements. Serum separated from the 4.0 mL of blood is stored at -20°C until processing for humoral immunity.

Laboratory rabbits are housed at the MRI under standard laboratory conditions confirming ethical guidelines on the use of animals in research. The welfare diary is maintained by research students during experimental procedures with the help of the Head/animal house who is a co-investigator of this project. Stress caused to rabbits during research is minimized by proper handling during blood collection and immunization. A local anesthetic cream is used before blood collection from the ear artery to minimize slight pain induced during blood collection.

PART II – Field study with a sample of dogs

Recruitment of a sample of dogs under field conditions

As indicated under Section 3.3, 162 free-roaming and domestic dogs (120 + 35% contingency margin to obtain the required number of seronegative dogs; approximately n = 40 in each group; It is necessary to have a minimum of 30 animals for statistical comparisons and as per European Pharmacopoeia. We will not have a separate group of 10 dogs as the control group in this study considering ethical and welfare issues that may arise when dogs are kept without an ARV in this rabies-endemic country) are recruited from the interior areas of several districts in the country. These areas have not been covered by the rabies control programme. The sample collection and vaccination are performed after obtaining permission from the required government authorities. Percentage of free-roaming animals that are included in the field study will be 40% of the total (10% of the total for each group) in order to have more animals in the follow-up visits.

Procedure of recruitment of dogs and blood sampling

The dogs that are recruited include puppies (6 weeks to 3 months; n = 14 in each group), juveniles (>3 months to 1 year, n=14 in each group), and young adults (>1 to 2 years; n = 14 in each group) based on information obtained from the owners of domestic dogs. Assumption of the age of free-roaming animals is based on body size/growth. Announcements are made by the officers of the Municipal Council Authorities/ research student in the chosen areas using the method adopted by the rabies control programme indicating the date of the vaccination programme and requesting owners to bring unvaccinated animals in the given age ranges, prior to arrangement of vaccine centers for the recruitment of dogs. Domestic dogs are recruited after obtaining the written consent of the owners. Information of owners such as name, contact number, and address are noted down to send messages for the follow-up blood sample collection on identified time points (This information will not be released to a third party and will be kept under lock and key in order to maintain confidentiality). Dogs that roamed freely are caught and restrained by trained dog catchers for vaccination and blood sample collection. Required details of dogs such as age, the presence of special identification marks, tattoo number allocated to the dog during the study are noted down by the research student. The domestic and free-roaming dogs are divided into 4 groups representing all three age groups in each group. They are injected 1-mL of one of the four ARVs selected subcutaneously into the area with loose skin on the back. The brand of ARV used by the government rabies control programme for the past 10 years and three other brands which produce 1st, 2nd and 3rd highest potency levels compared to other vaccines at the end of the second year of experimental study have been selected for the field study with dogs (If the antibody response to the vaccine used in the rabies control programme falls within the indicated method of selection, then the vaccine in the next position based on the potency are selected). Same numbers are assigned to four selected brands of ARVs as in the experimental study with laboratory rabbits. For the recruited dogs only one dose of ARV is given without a booster until D360 as the government rabies control programme is not in a position to give a booster to animals prior to the annual booster due to logistic problems. A minimum number of blood samples collected (6 mL) is two either from the Cephalic or Saphenous vein of the recruited dogs: first one on D0 before vaccination and the second one after vaccination on D30. If the animals are available on subsequent days; D90, D180, and D360 samples are also collected to study the pattern of immunity maintenance until D360 (As per the behavior of dogs, they very rarely change the place of stay/area of roaming). Blood collected are separated as indicated under an experimental study with rabbits (Section 5.6) and subjected to the same procedure of serum separation. Analysis of D0 samples prior to vaccination will facilitate selection of seronegative dogs. Seropositive dogs on D0 will be excluded from final analysis. Vaccinated animals are identified by a special dog collar/tattoo number for easy recognition of animals on D30 and thereafter. In addition, a photograph of each animal recruited to this study is taken. D360 blood samples are collected from available dogs prior to annual booster vaccination. After vaccination and blood sample collection free free-roaming dogs are released to the same place from where the animals are caught.

As indicated in the justification, the background information of dogs selected for the field study such as nutritional status, health status, parasitic infections, genetics etc cannot be controlled when conducting the study. Investigators are well aware of these confounding factors which are beyond the control of the investigators, and these will be considered when expressing results of immunogenicity and making recommendations following anti-rabies vaccination based on the field study.

Sample Analysis of experimental and field studies

Determination of blood cell parameters

Blood have been analyzed manually or (using an automated hematology analyzer depending on the availability of funds) to determine blood cell counts (erythrocyte, leukocyte and platelet counts), packed cell volume (PCV), hemoglobin concentration [Hb] and their derived parameters such as mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC).

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

This has been done by the FAVN test and ELISA at the WOAH/WHO/EU Reference laboratory in Nancy, France. Minimum number of samples analyzed by FAVN test and ELISA are 1164 (7 samples per animal X 120 rabbits + minimum 2 samples per animal X 162 dogs). The maximum number of samples that may be possible to analyze are 1650 (840 from rabbits + 5 samples per animal X 162 dogs).

Determination of virus-neutralizing antibody titres

FAVN test measures the ability of antibodies that may be present in the sample to neutralize and block rabies virus from infecting cells used in the test. These antibodies are identified as rabies virus-neutralizing antibodies (RVNA). When performing the test, a serum is subjected to serial dilutions to make less 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 tissue culture cells are added and incubated with virus and test samples. Thereafter, the virus that has not been neutralized by the antibodies in the sample infect the cells. Thereafter, infected wells in the 96 well plate are examined by an inverted fluorescent microscope. If a fluorescent microscope examination shows a large number of infected cells that indicates the serum sample has a very low level of antibodies.

The endpoint titre is calculated based on the number of virus-infected wells containing various dilutions of the sample on the plate. The FAVN test results can be reported as a standardized concentration represented in international units (IU) per mL of serum (e.g. 0.5 IU/mL).

Determination of humoral immunity by ELISA using BioPro RABIES kits (O.K. SESVIS BioPro, s.r.o., Prague, Czech Republic)

The BioPro ELISA kit is known to produce better results with high sensitivity and specificity 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. It is an in vitro diagnostic blocking ELISA test that detects rabies virus antibodies.[16,17]

Those anti-rabies antibody titers of animals using ELISA (IU/mL) are statistically correlated with those obtained using the FAVN test of the correspondent sample in this study.

Determination of Cellular Immunity

As fresh blood samples are required for cellular immunity measurement, it is not possible to send samples to the international collaborating laboratory. Therefore, cellular immunity will be determined on the blood samples collected from laboratory-bred dogs in the international collaborating institute before and after vaccination of dogs using the vaccines purchased for this project. (We have already obtained permission from the Department of Animal Production and Health to send the required number of doses of each vaccine brand for this purpose). For that, canine peripheral blood mononuclear cells (PBMCs) will be extracted from whole blood. This will be done by density-gradient centrifugation. Dogs will be given one dose of the vaccine. Blood samples will be collected on day 7 and 42 post-vaccination to detect circulating plasma cells and memory B cells. For the detection of rabies antigen specific ELIspot assay will be used. Cellular immunity is assessed by monitoring of IFNg secreting cells and cytokines profile in PBMCs supernatant using ELIspot assay and Luminex technology respectively.

Part III: Determination of potency of ARVs used in the study

Potency testing of ARVs

This has been carried out according to the method described in European Pharmacopoeia at the international collaborating laboratory. For this purpose, 20 mL from each vaccine brand have been transported.

Transportation of samples to collaborating WOAH/WHO/EU Reference Laboratory in Nancy, France

International collaborating laboratory has organised transport facilities to transport samples for humoral and possibly cellular immunity assessment under suitable conditions once in 6 months. Until the time of shipment, samples are stored at -20°C. Vaccine vials are transported in 4°C. Research student have visited the collaborating institute once or twice a year depending on the availability of adequate funds to visit the laboratory for analysis of samples. All the samples transported to the collaborating laboratory are stored under specific conditions until analysis. Approval of the Director General of Dept. of Animal Production and Health has been obtained for the transportation of samples collected from rabbits and dogs to an international reference laboratory.

Validation and quality control of methods

Rabies conjugate and a working reference serum are provided by the collaborating laboratory, and it has been used for quality control of the assays conducted for the measurement of immunity. Quality control sera are used in the blood cell parameter measurement as well.

Statistical Analysis

Statistical analysis is conducted using SPSS software. Mean antibody levels in the 9 different experimental groups with rabbits are described using standard descriptive methods. A

comparison of antibody levels between the two genders are conducted using one-way ANOVA test. Changes in antibody levels within the group over time are analyzed using paired samples T test or relevant non-parametric test.

Association between the results of FAVN test and ELISA testing are analyzed using Kappa statistics and also by calculating the sensitivity and specificity of ELISA in relation to FAVN test. Correlations between Antibody levels and hematological parameters are analyzed using Pearson's correlation coefficients.

Similar comparisons are made among 4 groups of field dogs and also for comparison of cellular immunity and potency of vaccines.

Disposal of samples and animals used in the research.

Rabbits have been issued to interested parties to be used as pets upon completion of the project. If there are remaining serum samples, these are disposed of according to accepted procedures after 6 months once the project is completed.

Discussion

The current study aims to determine the humoral immunity development or immunogenicity following anti-rabies vaccination (ARV) by means of measuring the rabies virus neutralizing antibody titers (RVNA) in the serum. Determination of immunogenicity is first done using a laboratory animal model, New Zealand White (NZW) rabbits. In the experiment using the laboratory rabbit model, all the ARVs available in the Sri Lankan market will be used. The levels of antibody titers are determined prior to the vaccination and following vaccination over a one-year period at different time points. The results of this part of the study will explain how the humoral immunity/RVNA titers will vary over a one-year period following a single dose of vaccination. The reason for using the laboratory animal model, NZW rabbits, for this part of the study, is to minimize the variations that can occur due to genetics, species, nutrition, parasitic infection, environment, etc. This will help us to get the final results with minimal variations. During the randomization of rabbits into different groups, we consider gender as the only difference among the animals. Rabbits were allocated to each ARV to have an equal number of males and females per vaccine. So, the final results are analyzed 1) based on the type of ARV and 2) based on the gender of the animals to detect the presence of gender variation with respect to humoral immunity development.

The study is currently ongoing and in the laboratory animal part of the study, 108 laboratory rabbits were used. Rabbits have been randomly allocated for the 9 different ARVs to have 12 rabbits per vaccine (6 males and 6 females). Serum staples of the rabbits are analyzed using both ELISA and FAVN to determine the RVNA levels at each time point.

For the second part of the study, the field study with a sample dog population, four vaccines have been selected from the potency testing results. Potency testing is mentioned in the third part of the study. This potency analysis is performed at the collaborating institute, WOAH/WHO/EU Reference Laboratory in Nancy, France using the mouse challenge test. The potency analysis was completed and based on the results four vaccines were selected for the field study. Also, the potency test results of all the ARVs were published in an international journal.[53]

Dogs are recruited under three age categories. Blood samples are collected before the vaccination and 30 days after the vaccination. Serum samples are analyzed using both FAVN and ELISA to determine the antibody levels. Since dogs are the main reservoir of rabies virus and the main cause of human rabies cases,[5] detecting the humoral immunity development against vaccination in field dogs is a crucial part of the study. There are no facilities to have a large sample of dogs under controlled conditions to assess the immunogenicity of all the ARVs in the Sri Lankan market. Therefore, we performed the first part of the study using laboratory animal models to test all the available vaccines and then moved to the field study to test four selected vaccines. The field study can be taken as a replication of the real field situation in the country, and we can have a better idea about how the Sri Lankan dogs respond to the anti-rabies vaccinations. To assess how immunity development can vary among different age groups we are having three age categories for each of the vaccines. As mentioned under the specific objective, the immunogenic of the laboratory animal model, the NZW rabbits, and the dogs under field conditions will be evaluated separately at the end of the sample analysis. These results will also be evaluated with respect to each of the vaccines. The antibody assessment results from ELISA will also be compared with that of the FAVN, and we would be able to establish ELISA in the local setup as a test to measure the immunity following anti-rabies vaccination. At the end of the study, we will be able to give recommendations to the regulatory bodies on the purchasing and registration of ARVs in Sri Lanka.

We don't have any conflicts of interest as we are purchasing all these vaccines using the money that will be requested from the funding organization. Therefore, we are not biased during the allocation of animals to groups and the vaccine used in different groups.

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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.