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Detection of immunity in sheep following anti-rabies vaccination

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Purpose: Rabies is a fatal but preventable disease with proper pre-exposure anti-rabies vaccination (ARV). Dogs, as household pets and strays, are the reservoir and vector of the disease, and dog bites have been associated with human rabies cases in Sri Lanka over the past few years. However, other susceptible species having frequent contact with humans may be a source of infection. One such species is sheep and immunity following ARV has never been tested in sheep reared in Sri Lanka.
Materials and Methods: We have tested serum samples from sheep reared in the Animal Centre, Medical Research Institute of Sri Lanka for the presence of anti-rabies antibodies following ARV. Sheep serum samples were tested with Bio-Pro Rabies enzyme-linked immunosorbent assay (ELISA) antibody kits used for the first time in Sri Lanka and our results were verified by a seroneutralization method on cells (fluorescent antibody virus neutralization, FAVN test) currently recommended by World Organization for Animal Health and World Health Organization.
Results: Sheep received annual ARV and maintained high neutralizing antibody titers in their serum. No maternal antibodies were detected in lamb around 6 months of age. Agreement between the ELISA and FAVN test, i.e., coefficient concordance was 83.87%.
Conclusion: Annual vaccination in sheep has an effect on maintaining adequate protection against rabies by measurements of anti-rabies antibody response. Lambs need to be vaccinated earlier than 6 months of age to achieve protective levels of neutralizing antibodies in their serum. Introducing this ELISA in Sri Lanka will be a good opportunity to determine the level of anti-rabies antibodies in animal serum samples.

Keywords: Sheep, Rabies vaccination, Enzyme-linked immunosorbent assay, Fluorescent antibody virus neutralization test, Rabies antibodies

Introduction

Rabies, a viral zoonotic disease that affects human and mammals, which cause acute progressive encephalitis. An estimate of 59,000 human deaths occur annually, with over 3.7 million disability-adjusted life years due to rabies [1]. In Sri Lanka, the rabies virus is being maintained in the dog population *(Canis lupus familiaris)* and 20–30 human deaths are reported annually due to rabies [2]. The source of infection in human rabies cases has mostly been household pets and stray dogs [3]. Other susceptible mammalian species exposed to infected dogs can also transmit the rabies virus, one of which is the sheep *(Ovis aries)*, which has close contact with humans.

In Sri Lanka, sheep are reared in small and large-scale farming conditions, and some are reared for laboratory purposes. Between 2005 and 2014, a total of 8,712 animal rabies cases were reported in Sri Lanka. Among them, 6,788 (78%) were dog rabies cases, while 1,197 (13.7%) were livestock, 663 (7.6%) cats, and 64 (0.7%) wild animals. Among the cases reported in livestock, a total of 13 sheep were clinically diagnosed (not laboratory-confirmed) as rabid by Nihal et al. [4].

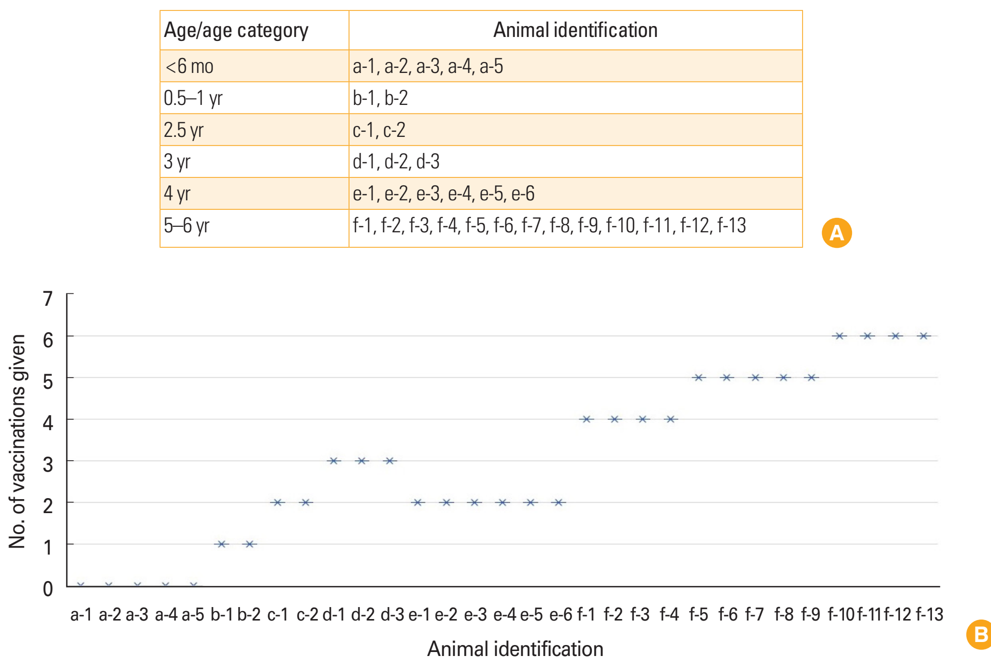
In the Middle East, rabies-endemic countries like Saudi Arabia have reported the identification of rabies among livestock [5]. Kasem et al. [5] tested a total of 199 samples (carcass or head) from rabies suspected animals using Direct Fluorescent Antibody Test (DFAT). Out of the 158 rabies-positive samples, 26 (16.5%) were from sheep indicating the risk of potential zoonosis. Among the East Asian countries, from 2012 to 2018 Mongolia reported a total of 2,359 animal rabies cases. These were confirmed by DFAT, the standard for routine rabies determination. Among those confirmed cases, 861 were from cattle, 268 from goats, and 251 from sheep [6].

A study conducted in 2020 by Benavides et al. [7] demonstrated the correlation between seroprevalence and the number of local livestock rabies mortalities. The relation between the level of rabies virus neutralizing antibodies (RVNA) in unvaccinated livestock and the widespread abortive infection, where seropositive animals did not die from rabies, suggested the importance of serological status in livestock.

To the best of our knowledge, no studies have been performed in Sri Lanka to investigate anti-rabies vaccination (ARV) in sheep and the levels of protection following vaccination.

A total of 31 sheep (Madras Red Cross breed from Tamil Nadu, India) are currently reared in the Animal Centre of the Medical Research Institute (MRI) in Colombo, Sri Lanka for blood collection, which is used for culture media preparation. This particular set of vaccinated sheep has been used to study levels of protection following ARV given the accessibility, unique identification of animals, and reliable data collection related to their vaccination history. These sheep are reared under a semi-intensive management system where they can be exposed to other susceptible mammals such as mongooses, civets, bats, and occasionally stray dogs. Therefore, these sheep have been vaccinated annually against rabies by the Colombo Municipal Council (CMC). As these sheep are frequently handled by veterinary surgeons and support staff in the Animal Centre of the MRI, as well as the staff of CMC, there is a requirement in terms of public health to determine the level of humoral immunity in these animals vaccinated against rabies for several years.

The objective of this study was to determine the level of humoral immunity (anti-rabies antibody titers) in the serum of the sheep and to analyze the results with regard to the vaccination history and the age of the animals.



Age/age category	Animal identification
<6 mo	a-1, a-2, a-3, a-4, a-5
0.5–1 yr	b-1, b-2
2.5 yr	c-1, c-2
3 yr	d-1, d-2, d-3
4 yr	e-1, e-2, e-3, e-4, e-5, e-6
5–6 yr	f-1, f-2, f-3, f-4, f-5, f-6, f-7, f-8, f-9, f-10, f-11, f-12, f-13

Fig. 1. (A) Animal identification for each of the age/age category. (B) Number of anti-rabies vaccines given to each of the animals.

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Materials and Methods

Animals and vaccination status

In all, 31 Madras Red Cross sheep reared at the MRI were included in this study. These sheep have been vaccinated annually against rabies since 2014 (vaccination records were available since 2014). Details such as age/age category, vaccination history, number of vaccinations (Fig. 1), and the brand of vaccine used are given in Table 1. The last ARV was given in 2019 and the blood samples were collected in 2020, approximately 1 year after the last booster vaccination. Each year, all sheep were vaccinated subcutaneously with the same brand of vaccine used by CMC. The vaccine brands used were Raksharab, rabies killed vaccine, and a vaccine that was withdrawn from the registration in 2017. Non-vaccinated lambs under 6 months of age were included in the study to detect the possible presence of maternal antibodies in their serum. All sheep reared at the MRI were included in the study group with no control group.

Animal welfare considerations

As per the special request of the head of the animal center, the study was conducted with the approval of the Director (2020) of the MRI, as there was a public health concern. Ani-

Table 1. RVNA titers of the sheep in different age/age categories, and number and brands of ARVs given with the year of vaccination

Age/age category	No. of animals	Animal identification	RVNA titer[b] (IU/mL)	Year of vaccination and brand of vaccines given (brand is indicated within brackets)[a]					
				2014	2015	2016	2017	2018	2019
<6 mo	5	a-1	0.04						
		a-2	0.04						
		a-3	0.04	×	×	×	×	×	×
		a-4	0.04						
		a-5	0.07						
0.5–1 yr	2	b-1	0.04	×	×	×	×	×	√ (V 3)
		b-2	0.04						
2.5 yr	2	c-1	23.93	×	×	×	×	√ (V 3)	√ (V 3)
		c-2	2.62						
3 yr	3	d-1	0.10					√	
		d-2	0.50	×	×	×	×	In February	√ (V 3)
		d-3	0.66					In November (V 3)	
4 yr	6	e-1	0.66	×	×	×	√ (V 2)	√ (V 3)	×
		e-2	7.92	×	×	×	×	√ (V 3)	√ (V 3)
		e-3	3.46						
		e-4	0.13	×	×	×	√ (V 2)	×	√ (V 3)
		e-5	1.15						
		e-6	13.77						
5–6 yr	13	f-1	0.04						
		f-2	0.04						
		f-3	0.10	√ (V 1)[c]	√ (V 1)	√ (V 2)	√ (V 2)	×	×
		f-4	0.04						
		f-5	1.15						
		f-6	13.77						
		f-7	2.62	√ (V 1)	√ (V 1)	√ (V 2)	√ (V 2)	×	√ (V 3)
		f-8	3.46						
		f-9	2.62						
		f-10	2.62						
		f-11	0.87						
		f-12	3.46	√ (V 1)	√ (V 1)	√ (V 2)	√ (V 2)	√ (V 3)	√ (V 3)
		f-13	2.62						

RVNA, rabies virus neutralizing antibody; ARVs, anti-rabies vaccines; ×, not vaccinated; √, vaccinated.
[a]ARVs are not identified by their brand names due to ethical reasons, and a code is used (e.g., V 1, 2, and 3). [b]RVNA titer was determined by fluorescent antibody virus neutralization test. [c]V 1 is no longer in use as it was withdrawn in from registration by the local marketing agent in 2017.

mal handling was done by well-trained staff. Sheep were reared in a semi-intensive management system with an enclosed open area for free roaming. Samples for the analysis were collected in parallel to the routine blood collection which was performed for regular health monitoring.

Animal identification

Each animal had an ear tag number, which facilitated the identification of the sheep by animal care staff and sample collectors. Following blood collection, each serum sample was given a code number from 1 to 31, used for identification of the samples. For blinding purposes, neither sample collectors nor laboratory staff were informed of animal sample numbers, their respective ear tags, and relevant animal details until the completion of the study. The vaccination history and other relevant animal information were extracted with respect to each sample number from the records maintained at the Animal Centre of the MRI. In this paper, a different numbering system (e.g., a-1, a-2, b-1, b-2, etc.) that corresponded to the 1 to 31 serum identification numbers, was used to identify each animal in chronological order (Fig. 1A).

Blood sampling and sample processing

Approximately 4 mL of blood was collected from the jugular vein of the animals into plain tubes. Blood samples were left at room temperature for about 30 minutes and centrifuged at 3,700 rpm for 15 minutes. Serum was extracted carefully from each sample and stored at -20°C until analysis. Each serum sample was analyzed for the presence of anti-rabies antibodies using the Bio-Pro Rabies enzyme-linked immunosorbent assay (ELISA) antibody kit (Bio-Pro, Prague, Czech Republic), and rabies neutralizing antibody level was determined by the fluorescent antibody virus neutralization (FAVN) test. At the time of serum collection, each serum sample was aliquoted into two sterile cryovials. One set of serum samples was sent to the EU/WOAH/WHO Reference Laboratory for Rabies and Wildlife, ANSES-Nancy, France to perform the FAVN test and the Bio-Pro Rabies ELISA test. The second set of serum samples was analyzed at the Research laboratory, Department of Physiology, Faculty of Medicine, Colombo, Sri Lanka using the Bio-Pro Rabies ELISA test.

ELISA test

Analysis of serum samples

The Bio-Pro Rabies ELISA Antibody kits and the reagents were purchased from Bio-Pro (Prague, Czech Republic). This test is a blocking ELISA test that detects rabies virus antibodies, as previously described in Wasniewski and Cliquet [8]. The positive and negative controls and a panel of previously calibrated control sera (CS1, CS2, and CS3) were supplied by the manufacturer. The sheep serum samples were titrated, strictly following the protocol provided by the manufacturer. Briefly, serum samples were processed as positive, negative, and control sera and were diluted 1:2 with the sample diluent provided with the kit. A volume of 100 µL of this dilution was transferred to the microplate. After overnight incubation at 4°C–8°C, while gently shaking on an orbital shaker, the microplates were washed and then 100 µL of the biotinylated anti-rabies antibodies were distributed to each well. The microplates were incubated for 30 minutes at 37°C while gently shaking and then washed. After this step, 100 µL of streptavidin peroxidase conjugate was added to each well, followed by incubation for 30 minutes at 37°C while gently shaking. The presence of the antigen-biotinylated antibody complexes was revealed by adding 100 µL of TMB chromogen solution to each well. The microplates were incubated in the dark for 15–20 minutes at room temperature with gentle shaking, and the enzymatic reaction was then stopped by adding a solution of 0.5 M H_2SO_4. Each microplate was read at 450 nm using an ELISA reader (Multiskan FC version 1.01.16; Thermo Fisher Scientific, Waltham, MA, USA).

Calculation of percentage of blocking, quality control, and validation

The percentage of blocking (%PB) was calculated for each sample based on the manufacturer's instructions using the optical density (OD) value generated by the ELISA reader for each sample and control sera. The validation criteria described by the manufacturer had to be met in order to validate the results obtained for the samples, and results of the CS1, CS2, and CS3 confirmed that the test was working in optimal conditions.

The $\%PB = [(OD_{NC} - OD_{Sample})/(OD_{NC} - OD_{PC})] \times 100$ where OD_{NC} was the OD of the negative control, OD_{Sample} was the OD of the sample, and OD_{PC} was the OD of the positive control. According to the manufacturer's specifications, serum samples with a %PB equal to or higher than 70% were considered to have anti-rabies antibody levels equal to or higher than 0.5 IU/mL. This 0.5 IU/mL threshold is recommended by WOAH/WHO and is recognized as the protecting threshold of antibodies against rabies. The positivity threshold recommended by the manufacturer, allowing detection of seroconversion

after vaccination was set at 40% (%PB).

FAVN test

RVNA titers were determined by the FAVN test, according to the procedure described previously by Cliquet et al. [9]. The positive WOAH standard serum adjusted to 0.5 IU/mL was used as a positive control by Wasniewski et al. [10]. The antibody titers of the serum samples were expressed in international units per milliliter (IU/mL) by comparing the results with those of the positive control. The threshold of positivity was set at 0.5 IU/mL.

Agreement between ELISA and FAVN tests

The agreement between the ELISA test done in the Sri Lankan laboratory and the FAVN test was assessed by calculating the coefficient of concordance, which was expressed as a percentage. It was the ratio of the samples determined to be true positive (%PB equal to or higher than 70% in ELISA) plus true negative (%PB value below 40% in ELISA) divided by the total number of tested samples.

Results

Virus neutralizing antibody titers obtained with the FAVN test

RVNA titers for animals in each age/age category according to the number of ARV received and the brand of vaccine used are summarized in Table 1. No maternal neutralizing antibodies were detected in the samples of lambs around 6 months of age, the titers being close to 0 IU/mL (4 animals with a titer equal to 0.04 IU/mL and one lamb with a titer equal to 0.07 IU/mL). The two sheep (b-1 and b-2) that had received their first vaccination approximately 1 year before the sample collection did not maintain protective levels of antibodies in their serum as the titers were negative (0.04 IU/mL). The two sheep in the age category of 2.5 years that received two ARVs in 2018 and 2019, showed protective levels of RVNA in their serum with high titers (2.62 IU/mL and 23.93 IU/mL, respectively). In the 3-year-old age category, one of the three sheep had a negative titer (0.10 IU/mL), while the other two were positive with titers around the cut-off of positivity. All three sheep in this age category received the same brand of vaccine for all three vaccinations in the respective years.

In the 4-year age category, there was a difference in the vaccination patterns between the individual sheep. Among the sheep that received their most recent booster vaccination in 2019 (identified as e-2, e-3, e-4, e-5, and e-6 in Table 1), only one sheep (e-4) had an antibody level <0.5 IU/mL and all the others had antibody levels ≥0.5 IU/mL. Surprisingly, the sheep that received the most recent booster in 2018 (e-1) had a protective level of antibodies with a titer equal to 0.66 IU/mL, even though it was close to the cut-off value.

In the 5–6 age category, i.e., the group of the 13 oldest sheep reared in the Animal Centre of the MRI, four sheep had neutralizing antibody titers below 0.5 IU/mL. The vaccination history of these four sheep (f-1, f-2, f-3, and f-4) demonstrated that all of them received four vaccines in total, administered in 2014, 2015, 2016, and 2017. They did not receive any boosters afterwards.

Comparison between FAVN tests and ELISA results

ELISA test interpretation and %PB that resulted from both laboratories and respective FAVN test results are presented in Table 2 and Fig. 2. We observed few discrepancies in the ELISA test results between laboratories and between the FAVN test results. Sheep numbers d-1 and f-3 had a low antibody titer of 0.10 IU/mL, despite being interpreted as Ab+ ≥0.5 IU/mL and Ab+, respectively with ELISA tests done in both laboratories. For sample f-4, the Sri Lankan laboratory obtained results as Ab+ and the collaborating laboratory obtained Ab- with ELISA, while the antibody titer was 0.04 IU/mL by FAVN test. Four serum samples (numbered e-2, e-4, f-6, and f-8) were found to be Ab+ ≥0.5 IU/mL for the ELISA test performed at the Sri Lankan laboratory, while the collaborating laboratory obtained results as Ab+. However, when considering the %PB for three of these samples (e-2, f-6, and f-8), the levels obtained by the collaborating laboratory were close to 70% (64.61%, 68.54%, and 68.93%, respectively). These 3 sheep had RVNA titers >0.5 IU/mL with the FAVN test, except sample e-4 which was found to be negative (0.13 IU/mL). The discrepancies found between the ELISA tests therefore could not be considered a major issue as the results were close to threshold levels. For sample f-11, the ELISA tests from both laboratories were interpreted as Ab+, while the antibody titer was 0.87 IU/mL (close to the cut-off value), and the %PB values obtained by both the Sri Lankan and collaborating laboratories for this sample were close to 70%, i.e., 62.63% and 67.83%, respectively.

Analytical performances of ELISA carried out in Sri Lanka

Out of 31 samples tested by the FAVN test, 10 were negative (results found below 0.10 IU/mL), three were between 0.10

Table 2. Comparison of ELISA results (interpretation and %PB) and the respective FAVN test results for each animal

Age/age category	Animal identification	ELISA results; research laboratory, Sri Lanka		ELISA results; reference laboratory, France		RVNA titer (IU/mL) by FAVN test
		Interpretation	%PB[a]	Interpretation	%PB	
<6 mo	a-1	Ab–	34.49	Ab–	5.57	0.04
	a-2	Ab–	34.59	Ab–	6.71	0.04
	a-3	Ab–	26.13	Ab–	26.84	0.04
	a-4	Ab–	16.12	Ab–	17.41	0.04
	a-5	Ab–	9.19	Ab–	15.74	0.07
0.5–1 yr	b-1	Ab–	12.44	Ab–	32.82	0.04
	b-2	Ab–	8.38	Ab–	12.58	0.04
2.5 yr	c-1	Ab+ ≥0.5[b]	99.19	Ab+ ≥0.5	84.86	23.93
	c-2	Ab+ ≥0.5	94.39	Ab+ ≥0.5	70.07	2.62
3 yr	d-1[c]	Ab+ ≥0.5	82.79	Ab+ ≥0.5	79.20	0.10
	d-2	Ab+ ≥0.5	93.77	Ab+ ≥0.5	92.74	0.50
	d-3	Ab+ ≥0.5	96.69	Ab+ ≥0.5	99.93	0.66
4 yr	e-1	Ab+ ≥0.5	88.16	Ab+ ≥0.5	90.16	0.66
	e-2[d]	Ab+ ≥0.5	96.05	Ab+	64.61	7.92
	e-3	Ab+ ≥0.5	99.21	Ab+ ≥0.5	88.47	3.46
	e-4[d]	Ab+ ≥0.5	79.33	Ab+	58.27	0.13
	e-5	Ab+ ≥0.5	97.36	Ab+ ≥0.5	95.89	1.15
	e-6	Ab+ ≥0.5	94.93	Ab+ ≥0.5	92.51	13.77
5–6 yr	f-1	Ab–	17.79	Ab–	21.03	0.04
	f-2	Ab–	18.42	Ab–	31.00	0.04
	f-3[c]	Ab+	52.88	Ab+	53.19	0.10
	f-4[d]	Ab+	44.05	Ab–	32.43	0.04
	f-5	Ab+ ≥0.5	79.35	Ab+ ≥0.5	78.32	1.15
	f-6[d]	Ab+ ≥0.5	88.08	Ab+	68.54	13.77
	f-7	Ab+ ≥0.5	97.18	Ab+ ≥0.5	84.77	2.62
	f-8[d]	Ab+ ≥0.5	81.18	Ab+	68.93	3.46
	f-9	Ab+ ≥0.5	97.12	Ab+ ≥0.5	98.45	2.62
	f-10	Ab+ ≥0.5	89.96	Ab+ ≥0.5	77.32	2.62
	f-11	Ab+	62.63	Ab+	67.83	0.87
	f-12	Ab+ ≥0.5	102.26	Ab+ ≥0.5	101.50	3.46
	f-13	Ab+ ≥0.5	95.77	Ab+ ≥0.5	76.32	2.62

ELISA, enzyme-linked immunosorbent assay; %PB, percentage of blocking; FAVN, fluorescent antibody virus neutralization; RVNA, rabies virus neutralizing antibody; OD, optical density; Ab, antibodies.
[a]%PB=[(ODNC–ODSample)/(ODNC–ODPC)]×100 where, ODNC was the OD of the negative control, ODSample was the OD of the sample, ODPC was the OD of the positive control. [b]When the semiquantitative measurement of the presence of Ab is performed by using ELISA and if the titer is equal to or higher than 0.5 IU/mL level, it is indicated as Ab+ ≥0.5. Units of antibody titer measurement is IU/mL. [c]Show discrepancies in ELISA and FAVN test results. [d]Shows differences in semiquantitative measurement of ELISA results between two laboratories.

and 0.13 IU/mL, while 18 were positive, with titers + ≥0.5 IU/mL. Out of 13 samples with negative FAVN test antibody titers, four were found to be positive with the ELISA test, of which three corresponded to sera tested by the FAVN test between 0.10 and 0.13 IU/mL. Of the 18 samples that had serum antibody levels ≥0.5 IU/mL based on the FAVN test, one sample was tested as Ab+ with ELISA, with a %PB of 62.63%. The nine true negative samples and 17 true positive samples resulted in 83.87% coefficient of concordance between FAVN test results and those found by ELISA in Sri Lanka.

Discussion

Pre-exposure to ARV has been identified as the key element in preventing the spread of the rabies virus among animals and humans in rabies-endemic countries. Detection of anti-rabies antibodies following vaccination is a major factor for assessing the efficacy of vaccination in animals and humans

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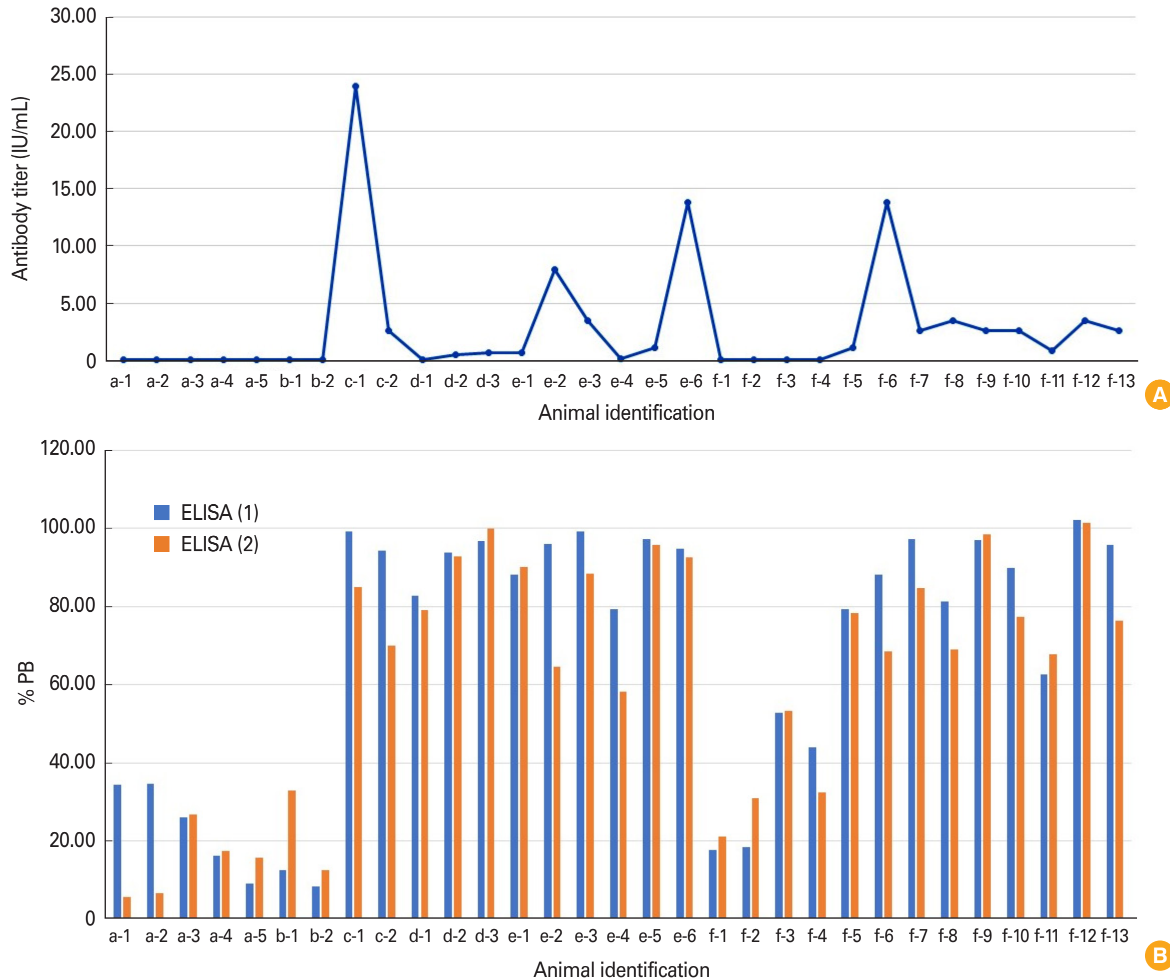

Fig. 2. (A) Rabies virus neutralizing antibody titer (IU/mL) by fluorescent antibody virus neutralization test. (B) Comparison of enzyme-linked immunosorbent assay (ELISA) results (percentage of blocking, %PB) from the research laboratory, Sri Lanka: ELISA (1) and reference laboratory, France: ELISA (2). $\%PB=[(OD_{NC}-OD_{Sample})/(OD_{NC}-OD_{PC})]\times 100$ where OD_{NC} was the optical density (OD) of the negative control, OD_{Sample} was the OD of the sample, and OD_{PC} was the OD of the positive control. %PB ≥70% were considered to have anti-rabies antibody levels ≥0.5 IU/mL. The positivity threshold allowing detection of seroconversion after vaccination was set at 40% of %PB.

[11-13]. As an indicator of adequate antibody response and protection following vaccination, WOAH and WHO currently recommend a rabies-neutralizing antibody level of ≥0.5 IU/mL [14].

The two WHO/WOAH currently recommended neutralizing antibody detection methods are the FAVN test [9] and the Rapid Fluorescent Focus Inhibition Test [15]. Because of the need for specific laboratory facilities and highly trained staff, as well as time requirements for these tests, alternative methods, i.e., ELISA, have also been recognized in recent years as acceptable tests to detect binding antibodies [16-18].

To the best of our knowledge, this is the first study done to determine the immunity following ARV in sheep and also the first to assess the performance of the Bio-Pro Rabies ELISA kit on sheep samples, and also the initial step in using this ELISA kit under Sri Lankan laboratory conditions. The Bio-Pro Rabies ELISA has been demonstrated to have high diagnostic sensitivity and specificity and high reproducibility, and it is a good alternative to the virus neutralization test for testing serum samples from pets and wildlife. However, ELISAs are currently not applicable to the international movement of domestic carnivores; they are only dedicated to the follow-up of ARV in domestic carnivores and wildlife for research purposes [14]. This ELISA kit was initially developed to check the efficacy of oral rabies vaccination campaigns in Europe by determining the level of humoral immunity in foxes sampled in vaccinated areas [19]. Later on, it was validated on dog and cat samples [8], fox and raccoon dog samples [20,21], and also more recently on wild boar samples [22].

In the Animal Centre, MRI sheep are reared mainly for the

purpose of blood collection. This blood is used for culture media preparation in different research laboratories. Despite these sheep being vaccinated against rabies, the level of protection they have has never been investigated. Therefore, we conducted this study to determine the level of neutralizing antibodies in these vaccinated sheep.

We included five lambs that have not been vaccinated, with the aim of possibly detecting maternal antibodies in their serum. The lambs, which were around 6 months of age, did not seem to maintain adequate levels of protection against rabies, which suggested that their maternal antibodies did not last up to this age. A recent study by Tizard [23] has suggested that in rabies-endemic countries, lambs need to be vaccinated against rabies after 3 months of age and that annual boost vaccination is needed. A study using keyhole limpet hemocyanin, an immunogenic protein antigen xenogeneic to the mammalian immune system, to determine the effective age of developing immunological competency in sheep, has shown that administering vaccines around 2 months of age can be advantageous for a strong immune response and successful vaccination [24]. The ELISA results found to be negative for these five animals suggest that the ELISA test is accurate for distinguishing naïve from vaccinated sheep, even though the number of tested animals is too low to determine the specificity of the test.

The two sheep between 0.5 and 1 year old did not exhibit protective levels of anti-rabies antibodies in their serum. We are not aware of other studies done regarding the maintenance of immunity in sheep following ARV vaccination for a total period of 1 year. In one study, sheep that have been vaccinated orally with transgenic maize expressing rabies virus glycoprotein seem to have higher RVNA titers than the negative group at 120 days after primary vaccination [25]. This is the only study that determined antibody levels for a long period after primary vaccination. As a result, is advisable to opt for annual booster vaccination following the initial primary vaccination as recommended by Tizard [23]. In the vaccination history, we observed that 3-year-old sheep had received a total of three ARVs, while 4-year-old sheep had received only two. Similar discrepancies in the number of ARVs received by the sheep in the 5–6-year age category were noted.

In previous studies, where the FAVN test and Bio-Pro Rabies ELISA test results were compared, higher agreements between the two tests were observed compared to this study; a percentage concordance of 86% was determined for cat and dog samples [8], 95% for red foxes and raccoon dogs [20], and 73% for wild boar samples [22]. In this study, we obtained a percentage of concordance of 83.87% with sheep samples. This suggests, even with a low number of samples investigated, that ELISA is reliable for testing sheep samples.

Despite having a specificity of up to 100% with the ELISA test, the previous studies also revealed few discrepancies for sera found to be negative with the FAVN test and positive with Bio-Pro ELISA. These discrepancies could be explained by the antibody type detected by the ELISAs, which depends on the coating antigen, i.e., whole virus, glycoprotein, or nucleoprotein. This means that the ELISA kits do not specifically target neutralizing antibodies [26]. When considering specifically the Bio-Pro Rabies ELISA kit, the antigen coated on the microplate is a crude rabies glycoprotein. Therefore, some traces of other rabies virus proteins could be coated on the microplate without affecting the test performance [8]. Since this coated glycoprotein in the ELISA microplate is not fully purified, the test might also detect other antibodies directed against proteins of the rabies virus together with the neutralizing antibodies. This could explain the results of sample numbers d-1 and f-3 where ELISA test results from both laboratories were interpreted as Ab+, while the RVNA titer was low.

The results give an overall idea of the level of protection of vaccinated sheep kept in experimental conditions, as well as the areas that need to be improved. These include the need for annual vaccination of sheep and vaccinating lambs at a young age. The curve of rabies antibodies after primary vaccination consists of rapid production of specific antibodies after the first vaccination corresponding to the seroconversion, followed by a regular decrease, then a new rise after a booster to obtain a higher level, and then a new decrease but with a higher level than that previously achieved [27]. The serum samples in this study were collected approximately one year after the vaccination, probably too long after the seroconversion of the animals. This could explain the negative results obtained by the FAVN test, despite the sheep having been vaccinated annually. However, these results did not show that the animal is not protected against rabies because it can be protected even if the level of rabies antibody is below 0.5 IU/mL after 1 year [28].

Since this was the first time the Bio-Pro Rabies ELISA kit was used under Sri Lankan laboratory conditions, we also tried to determine the coefficient of concordance by verifying the sample results with the collaborating laboratory. Even though the sample size was insufficient, the coefficient of concordance was 83.87% which suggested good agreement

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between the tests. These results are a positive point for the use of this ELISA kit under Sri Lankan laboratory conditions. For example, this kit could be useful to determine the immunological status of vaccinated dogs in Sri Lanka.

In conclusion, the results of this study suggest the importance of annual booster vaccination in sheep for the maintenance of an adequate level of rabies-neutralizing antibodies. In addition, it might be preferable to vaccinate lambs at an earlier age than 6 months to achieve a good level of protection against rabies. As this was the first time this Bio-Pro Rabies ELISA kit was used in Sri Lankan laboratory conditions and on sheep samples, we obtained satisfactory results compared to the recommended methods (FAVN test and Bio-Pro Rabies ELISA) performed in the collaborating reference laboratory in France. Introducing the use of this ELISA in Sri Lanka seems to be a good opportunity to determine the level of anti-rabies antibodies in animal serum samples and thereby to assess the efficacy of vaccination.

This study needs to be extended with a larger number of animal samples with pre-planned vaccination schedules to obtain strong evidence on vaccination efficacy and immunogenicity in sheep following ARV.

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