The Pharma Innovation Journal 2024; 13(3): 230-237

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
TPI 2024; 13(3): 230-237

www.thepharmajournal.com
Received: 22-12-2023
Accepted: 24-01-2024

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# Post vaccinal immune response of dogs to prophylactic Antirabies vaccination: A cross-sectional study from Kerala

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**Abstract**
Rabies is one of the most fatal zoonotic diseases that have tormented humans and is still a significant public health problem in many parts of the globe. Dogs are the reservoir of rabies in most rabies-endemic countries around the world. Animal vaccination is a key factor for rabies prevention and control of virus transmission. In Kerala, even though prophylactic vaccinations are being done regularly in dogs, seroconversion studies are not being undertaken and the effects of these vaccinations are not being assessed. Hence the present study was conducted with the objectives to assess the seroconversion following prophylactic anti-rabies vaccination in dogs using Indirect ELISA test and to validate Indirect ELISA in assessing post vaccinal seroconversion using RFFIT as the gold standard.

The study was taken up as a cross-sectional study on dogs above 3 months of age. A total of 116 samples were made part of the study. Out of the 116 samples studied 77 (66.4%) of animals were protected and 39 (33.6%) were unprotected. Among the vaccinated animals, 77 (71.3%) of the study subjects had protective titre. The study demonstrated that a single dose of rabies vaccine did not elicit adequate antibody levels in majority of dogs sufficient to give protection beyond one year. The protective efficacy of vaccine can be influenced by various associated factors *viz*. age, sex, breed, vaccine brand, months since vaccination, number of vaccinations received etc

When animals were given only one vaccine in the first year of life, data indicates that it is not sufficient for the maintenance of antibody titres until the time of annual booster vaccination. The regularly vaccinated group which received more than two vaccine doses, protective titre was seen maintained beyond one year: whereas those animals which had received a single vaccination couldn't maintain their titre for one year.

In the multivariable analyses of the present study, the history of 2 or more vaccination was the only factor significantly associated with the proportion of binding antibody titres ≥0.5 EU/ml More studies with larger sample sizes are recommended to assess the statistical significance of each associated factors *viz*. age, sex, breed, vaccine brand, months since vaccination, number of vaccinations received etc on the immune response following antirabies vaccination. The study findings indicate a recommendation for booster dose after primary dose, annual boosters and vaccination campaigns which are necessary to maintain adequate protection levels and herd immunity. Moreover, we can infer that a quantitative ELISA may be a complementary tool for sero-monitoring immune responses of dogs and cats after rabies vaccination.

**Keywords:** Immune response, Antirabies vaccination, ELISA, RFFIT

## 1. Introduction
Rabies is one of the most fatal zoonotic diseases and is still a significant public health problem in many parts of the globe. As per WHO estimates, India accounts for 36% of the global and 65% of the South East Asian human rabies deaths. Rabies is an acute, viral encephalomyelitis caused by a Lyssavirus belonging to the family Rhabdoviridae.

Dogs are the reservoir of rabies in most rabies-endemic countries around the world. Animal vaccination is a key factor for rabies prevention and control of virus transmission. WHO recommends that countries, where rabies is endemic, should carry out a preventive vaccination program for dogs [WHO,2022]. For successful disease control, it is important that vaccinated animals keep up a protective level of antirabies antibodies. Continuous sero-monitoring following vaccination is one of the major aspects of vaccination strategy. The World Health organization prescribed a 70%–80% epizootiological baseline for maintaining herd immunity in a community. Rabies can be prevented through vaccination, public awareness, responsible ownership, sustained collaboration among stakeholders and reduction in stray dog population.

It is important to determine the level of antirabies antibodies in animals to know the efficacy of control measures. (Nale *et al.*, 2021) [10] The World Organisation for Animal Health (WOAH/OIE) and World Health Organisation (WHO) and Food and Agricultural Organisation (FAO) recommend canine rabies control as a strategy for eliminating dog-mediated human rabies. The National Rabies Control program works in tandem with the global goal 'zero by 30′ initiated by the tripartite to achieve zero human rabies deaths by the year 2030. As per WHO criteria, a serum titre of 0.5 IU/ml and above of anti-rabies antibodies is considered adequate protection against rabies. (Nale *et al*, 2021) [10]. According to the World Health Organization (WHO) guidelines, a booster vaccine dose should be given if the rabies antibody titre falls below 0.5 IU/ml. Further, the effectiveness of the anti-rabies vaccination programs also needs to be evaluated through monitoring of antibodies elicited following vaccination

The probability of success of rabies vaccinations of dogs depends on the type of vaccine used, the number of rabies vaccinations, the breed size of the dog, age at vaccination, and number of days after vaccination when the antibody titers are tested (Berndtsson *et al.*, 2011) [8]. Immune response to vaccination is quantified by using different techniques such as Virus neutralization test (VNT), Rapid fluorescent focus inhibition test (RFFIT), Fluorescent antibody virus neutralisation test (FAVN), and ELISA (Ondrejkova *et al.*, 2002; Kostense *et al.*, 2012) [13, 6]. Among these RFFIT has high sensitivity and is extensively used (Singathia *et al.*, 2012) [15].

In Kerala, even though prophylactic vaccinations are being done regularly in dogs, seroconversion studies are not being undertaken and the effects of these vaccinations are not being assessed. Hence the present study was conducted with the objectives of assessing the seroconversion following prophylactic anti-rabies vaccination in dogs using the Indirect ELISA test and to validate Indirect ELISA in assessing post-vaccinal seroconversion using RFFIT as the gold standard.

## 2. Materials and Methods

The study was taken up as a cross-sectional study on dogs above 3 months of age who were vaccinated against rabies using different commercially available vaccines. Blood Samples were collected from different Veterinary institutions of Thiruvananthapuram district. Serum samples collected from dogs after the Mass Dog Vaccination program 2022 (MDV) of different districts of Kerala were also made part of the study. The study was conducted during the period June 2022 to June 2023. A total of 116 samples were made part of the study. The immune response was assessed using an Indirect Enzyme-Linked Immunosorbent Assay (INDIRECT ELISA) at the Immunodiagnostic lab of State Institute For Animal Diseases (SIAD) Palode, and the results obtained were compared with the results of RFFIT (Gold standard). Data regarding details of the dogs' age, gender, breed, vaccination history and last date of vaccination were collected at the time of blood collection using a structured questionnaire.

The blood from dogs was collected aseptically from cephalic or saphenous vein puncture into BD vacutainer® and blood was allowed to clot for 2 to 4 h at room temperature. The serum was separated by centrifugation at 3000 rpm ($4^0$ C) for7 min and stored at -$20^0$ C until further use.

### 2.1 Indirect Enzyme-Linked Immunosorbent Assay (Indirect ELISA)

All the samples were subjected to Quantitative Indirect ELISA using a Platelia II (Biorad, France) and was performed as per the manufacturer's instructions. The quantitative results are expressed as ELISA Units per ml (EU/ml) which are equivalent to International Units per ml (IU/ml) as measured by virus neutralisation tests. Seropositivty and seronegativity of the tested serum were determined using a threshold titer value of 0.5 EU/ml. The results obtained were compared with results obtained using RFFIT for the samples.

### 3.2 RFFIT (Gold Standard)

Rapid Fluorescence Focus Inhibition Test (RFFIT) was conducted to estimate rabies antibody level in the sera of the dogs. This was performed as per the WHO-advocated procedure with some modifications advised by the Department of Neurovirology, National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore, a WHO Collaborating Centre for Reference and Research on Rabies. Instead of tissue culture chambers, 96-well flat-bottomed tissue culture plates were used, and the cell line used was Baby Hamster Kidney (BHK−21). The virus used was a CVS strain adapted to grow in BHK-21 cells, and the dose used was 100 FFD50. The highest dilution of serum showing 50% inhibition of fluorescent foci in the infected cells was taken as the titre of the serum, which was converted to international units (IU/ml) by comparison to an in-house reference sera calibrated against the reference serum. Rabies neutralising antibody titre of 0.5 IU/ml is defined 'protective' by WHO and WOAH

### 3.3. Data management and analysis

Data was entered in Microsoft Excel and analysis done using IBM SPSS Statistics for Windows, Version 27.0 software. Categorical variables were expressed as proportions and quantitative variables were expressed as mean and standard deviation. To test the significance of association between relevant variables, Chi-square test and Independent- samples T test was used. P value less than 0.05 was considered significant. The strength of association was expressed as Odds ratio with 95% confidence interval. For validation, sensitivity, specificity, positive and negative predictive values were estimated.

## 4. Results

**4.1. Baseline information:** In the study out of 116 serum samples, 72 (62.1%) were from male and 44 (37.9%) was female animals. The number of non-descript breeds were 64 (55.2%) and descript breeds were 52 (44.8%). The mean (SD) age of the animals studied were 26.43 (23.36) months. Median value for age was 24 months with range between three and 120 months. The baseline characteristics of the study subjects are given in Table 1.

### 4.2. Protection status in study subjects

On performing Indirect ELISA, 67 (57.8%) of the animals were protected and 49 (42.2%) were unprotected. With RFFIT, 77 (66.4%) animals were protected and 39 (33.6%) unprotected. Eight serum samples in the study were collected from non-vaccinated animals. None of the non-vaccinated animals were found protected. Out of the 108 vaccinated animals 67(62.04%)were found to be protected by ELISA and 77 (71.3%) were found protected on RFFIT. Mean (SD) antibody titre using ELISA was 1.76 (1.47)EU/ml with median 0.5, ranging from 0 to 4 EU/ml. Mean (SD) antibody titre using RFFIT was 1.47(1.10)IU/ml with median 0.7, ranging from 0.23 to 3.75 EU/ml. Table 2 shows protection status of study subjects.

**Table 1:** Baseline characteristics of study subjects

| Characteristic | Category | Number | Percentage |
|---|---|---|---|
| **Sex** | Male | 72 | 62.1 |
| | Female | 44 | 37.9 |
| Age in months | 3 -6 | 27 | 23.5 |
| | 6-24 | 45 | 39.1 |
| | 24-60 | 38 | 33.0 |
| | 60-120 | 6 | 5.2 |
| Breed | Non-descript | 64 | 55.2 |
| | Labrador | 26 | 22.4 |
| | Spitz | 11 | 9.5 |
| | Dachshund | 4 | 3.4 |
| | Dobermann | 1 | 0.9 |
| | German shepherd | 4 | 3.4 |
| | Golden retriever | 1 | 0.9 |
| | Beagle | 1 | 0.9 |
| | Rottweiler | 2 | 1.7 |
| | Street dogs | 2 | 1.7 |
| Vaccination status | Regularly vaccinated | 64 | 55.2 |
| | Single dose Vaccination | 44 | 37.9 |
| | Non-Vaccinated | 8 | 6.9 |

**Table 2:** Protection status of study subjects

| Test method | Protected | | Unprotected | |
|---|---|---|---|---|
| | N (%) | Mean antibody titre (SD) | N (%) | Mean antibody titre (SD) |
| Indirect ELISA | 67 (57.8%) | 1.76 (1.47) | 49 (42.2%) | 0.18 (0.06) |
| RFFIT | 77 (66.4%) | 1.47 (1.10) | 39 (33.6%) | 0.23 (0.00) |

**Table 3:** Factors associated with protection status of study subjects

| Factor | Categories | Protected N (%) | Unprotected N (%) | P value* | OR (95% CI) |
|---|---|---|---|---|---|
| Sex | Male | 48 (66.7) | 24 (33.3) | 0.933 | 1.034 (0.468-2.286) |
| | Female | 29 (65.9) | 15 (34.1) | | |
| Age in months | ≤12 | 28 (63.6) | 16 (36.4) | 0.662 | 0.839 (0.381-1.848) |
| | 12-120 | 49 (67.6) | 23 (32.4) | | |
| Breed | Non-Descript | 41 (64.1) | 23 (35.9) | 0.558 | 0.792 (0.363-1.727) |
| | Descript | 36 (69.2) | 16 (30.8) | | |
| Vaccination status | Regularly Vaccinated | 54 (84.4) | 10 (15.6) | <0.001 | NA |
| | Single dose Vaccination | 23 (52.3) | 21 (47.7) | | |
| | Non-Vaccinated | 0 | 8 (100) | | |
| Months since Vaccination | ≥ 12 months | 12 (54.5) | 10 (45.5) | 0.192 | 0.535 (0.208-1.379) |
| | <12 months | 65 (69.1) | 29 (30.9) | | |

Chi-square test

**Table 4:** Comparison of mean of factors associated with protection status

| Factor | Protection status | Mean (SD) | P value* |
|---|---|---|---|
| Age | Protected | 27.39 (25.1) | 0.541 |
| | Unprotected | 24.56 (19.5) | |
| Months since vaccination | Protected | 3.97 (6.9) | 0.244 |
| | Unprotected | 5.71 (6.05) | |

* Independent-samples T test

**Table 5:** Comparison of antibody titre across groups

| Factor | Categories | RFFIT TITRE | ELISA TITRE |
|---|---|---|---|
| | | Mean (SD) | Mean (SD) |
| Months since Vaccination | ≤ 1 | 1.16(1.01) | 1.45((1.56) |
| | >1 | 0.90(1.0) | 0.99(1.14) |

### 4.4 Validation of ELISA with RFFIT (Gold standard)

**Table 6:** Two way table comparing Elisa with Rffit (Gold Standard)

| | RFFIT positive (gold standard) | RFFIT negative (gold standard) |
|---|---|---|
| Indirect ELISA positive (test positive) | 67 | 0 |
| Indirect ELISA negative (test negative) | 10 | 39 |
| | 77 | 39 |

**Table 7:** Validation of ELISA with RFFIT (gold standard)

| Test | Sensitivity | Specificity | Positive predictive value | Negative predictive value |
|---|---|---|---|---|
| Indirect elisa | 87.01 | 100 | 100 | 79.5 |

### 4.3. Factors associated with protective antibody levels.

Out of the 72 samples from males, 48(66.7%) and out of the 44 serum samples from females 29 (65.9%) were protected. No statistical significance could be noted in the difference in the protection status of male and female animals. Forty-four samples were from animals aged 12 months and below, and 72 samples were from the above 12-month age group. 67.6% of animals above 12 months of age were found protected whereas 63.6% of animals below 12 months of age were also found protected. Though the level of protection was higher in the above 12 months age group the difference was not statistically significant. Out of the 64 nondescript animals studied 41 (64.1%) were protected whereas out of the 52 descript breeds in the study, 36(69.2%) were protected. The difference in protection status of Descript and Non-descript breeds was found not statistically significant. Out of the 116 samples,64 (55.2%) were regularly vaccinated animals (2 or more vaccinations), 44(37.9%) were vaccinated once and 8(6.9%) were unvaccinated. Fifty-four (84.4%) of the regularly vaccinated animals were protected whereas 23 (52.3.%) of animals vaccinated once were also detected as protected. The difference in the protection status of regularly vaccinated and one-time vaccinated animals was found statistically significant. Serum samples of 22 animals were assessed for antibody response one year post vaccination. Twelve (54.5%) of them were found protected and in the protected group 91% had received two or more vaccinations, and of the one-time vaccinated animals, only 9% retained the protective titre (immunity) till one year. Out of the 94 Serum samples that were assessed for antibody titre within one-year post-vaccination 69% were found protected. The mean age of protected group was found to be more than 27 months and of the unprotected group was 24 months. The mean of months since vaccination in the protected group was 3.97 and that of the unprotected group was 5.71. The mean antibody titre was 1.16 within one month of vaccination, beyond one month of vaccination the mean antibody titre was found to be 0.90 by RFFIT and 1.45 and 0.99 within one month and beyond one month respectively by Indirect ELISA. Out of the 77 animals detected as protected by RFFIT, 67 could be found protected by Indirect ELISA and all the 39 animals detected negative by RFFIT could be detected as negative itself by Indirect ELISA also. Thereby the sensitivity of Indirect ELISA in comparison to RFFIT was found to be 87.01% and the specificity was found to be 100%. The Positive Predictive Value (PPV) was 100 and the Negative Predictive Value (NPV) was 79.5.

## Discussions

Out of the 116 samples studied77 (66.4%) of animals were protected and 39 (33.6%) were unprotected. In the study subjects, 108 were vaccinated and out of the 108 vaccinated animals 67 (62.04%) were found to be protected by ELISA and 77 (71.3%) were found protected by RFFIT. A preliminary study by Swapna S A and Ramkumar V in Kerala in 2019 on 10 dogs revealed that 70% of dogs showed minimum required protective titre (≥0.5 IU/ml) at one-month post-vaccination. Tandon *et al*., 2018 [18] reported a protection status in 79% of vaccinated animals in a study in Jammu Kashmir on 180 samples by Indirect ELISA. A Similar study in Jabalpur by Dubey *et al*., 2022 [1] on 146 samples seropositivity was found as 23% using Quantitative Indirect ELISA. In a study by Nale *et al.*, 2021 [10] in Mumbai, out of 120 serum samples, 47 (39.2%) serum samples, showed an antibody titre equal to or above the cut-off value of 0.5 IU/ml by Indirect ELISA. Out of the 260 vaccinated dog samples studied in Bangalore in 2023, 71% were found protected by RFFIT and 87% were found protected by Indirect ELISA. Except for studies in Bangalore and Jammu better protection is detected among the study subjects in Kerala compared to the similar studies elsewhere in the country.

The Mean antibody titre of the study subjects using ELISA was 1.76 EU/ml and the Mean antibody titre using RFFIT was 1.47IU/ml which is well above the threshold titre of 0.5IU/ml. According to Tresa *et al.*, 2016 [18] in a study in Kerala the Intramuscular route of vaccine administration in 20 dogs resulted in seroconversion by the 28th day with a mean titre of 1.75±0.28 IU/ml. The geometric mean titer (GMT) for all dogs sampled was 1.50 IU/ml as reported by Wallace *et al*.,2017 in his astudy in United states.

The protection level was higher among males (66.7%) compared to females (65.9%) in our study. Even though the difference is not statistically significant it is in agreement with the reports by earlier researchers like Tandon *et al.*, 2018 [18] and Dubey *et al*., 2022 [1]. Berndtsson *et al.*, 2011 [8]. The lower antibody titre in females may be due to the breeding season of bitches followed by immune suppression.

Seroconversion level was less among dogs below one year of age (63.6%) compared to dogs above one year of age (67.6%). Similar observation was made in the works of Berndtsson *et al.*, 2011[8], in Sweden and Tandon *et al.*, 2018 [17] in Jammu, India and Wera *et al.*, 2022 [21] in Indonesia. The higher protection status among the more than one-year age group may be due to the repeated vaccinations or booster vaccinations received by older dogs.

Protection status was higher among the descript breeds of dogs (69. 2%) than in the nondescript category (64.1%). Dubey *et al*., 2022 [1] has reported in his study in Jabalpur that none of the nondescript dogs has protective antibody titres of 0.5IU/ml. The protective titres achieved by the descript breeds were also higher compared to the non-descript dogs. Non-descriptive dogs are mainly reared by poor or lower- medium income class people who can't afford proper nutrition for the dogs and the dogs may not be dewormed regularly which may be a reason for discrepancy in protection status.

None of the non-vaccinated animals showed protection status and there was no influence of maternal antibodies noted in the puppies of non-vaccinated group at 3 months of age. Regularly vaccinated animals that received 2 or more vaccinations had a

higher protection level (84.4%) compared to those animals which received only a single vaccination (52.3%). The difference in the protection status of regularly vaccinated and one-time vaccinated animals was found statistically significant. Handous *et al*., 2023 [4] in Tunisia, Berndtsson *et al*., 2011[8] in Sweden, Wera *et al.,* 2022 [21] in Indonesia, Pimburage, *et al.*, 2017 [14] in Sreelanka Yakobson *et al*., 2016 [23] in Israel Dubey *et al*., 2022 [1] in Jabalpur, India all reported similar observations and recommended booster vaccinations for effective seroconversion. In the multivariable analyses, the history of 2 or more vaccinations was the only factor significantly associated with the proportion of binding antibody titres ≥0.5 EU/ml. Being of age more than 12 months increased the odds of antibody titres being >0.5 EU/ml, however not on a significant level according to our defined significance level.

Most animal vaccines promise a protective titre for one year and hence prophylactic anti rabies vaccination in cats and dogs is advised annually following the primary vaccination and a booster at the age of 3 to 4 months. In the present study, 69.1% of animals who were within one year of vaccination were protected, whereas only 54.5% of animals beyond one year since vaccination showed a protected status. Out of the 54% of animals that were found protected when assessed after one year since vaccination, 90% had received more than two vaccinations. Of the one-time vaccinated animals, only 9% retained the protective titre (immunity) beyond one year. Earlier studies by Handous *et al*., 2023 [4] in Tunisia, Berndtsson *et al.,* 2011[8] in Sweden, Wera *et al.,* 2022 [21] in Indonesia Wera *et al*., 2022 [21] reported that 46.8% of the study subjects maintained the protective titre till 12 months post-vaccination whereas only 14.7% were found to maintain the protective titre beyond one year.

By assessing the mean of months since vaccination in the protected and unprotected groups it could be inferred that the protective titre is maintained for up to 4 months and by the sixth month the titre will start waning below the cut-off value. The mean antibody titre was 1.16 within one month of vaccination, beyond one month of vaccination the mean antibody titre was found to be 0.90 by RFFIT. According to the observations of this study, the peak of antibody titre is achieved in 4 weeks and remains stable for up to four months and wanes thereafter.

The absence of protective levels of rabies virus-neutralising antibody titres in vaccinated dogs does not necessarily indicate that they are susceptible to rabies infection if challenged but there is inadequate information regarding the outcome in dogs that merely seroconvert and do not reach protective levels. Kennedy *et al*., 2007 [5] discussed that the dog's total immunity does not reduce but only shifts from a more dominant IgM to a more IgG-based immunity. Additionally, the role of cellular immunity and antibodies other than neutralising antibodies that contribute towards immunity from rabies requires further investigation.

The diagnostic test evaluation of OIE for validation of Platelia Rabies II ELISA by an external evaluation study at AFSSA, Nancy, France revealed a diagnostic sensitivity of 88.6% and specificity of 99.2%. The comparative studies between Platelia Rabies II ELISA and the reference methods shows that the majority of discrepant results are found in the 'borderline samples' with titres just above or below the cut-off of values of 0.5 EU/ml. In our study the sensitivity of Indirect ELISA in comparison to RFFIT was found to be 87.01% and the specificity was found to be 100% which is a comparable to the results of OIE validation test. The variations in RFFIT and ELISA titres of the current study was also noted in the borderline samples with titres just around the cut off value 0.5 IU/ml. in their Assessment of Immune Responses to rabies vaccination in Free-Ranging Dogs in Bengaluru, India using ELISA demonstrated that the sensitivity and specificity of the ELISA were 100% and 63.3%, respectively. A comparative evaluation of the estimation of rabies virus antibodies among free-roaming, vaccinated dogs in Bengaluru, India was done by Lekshmi *et al*., 2022 [7] in which they found that the sensitivity and specificity of the iELISA was 94.4% and 95.2%, respectively. Based on these studies we can infer that a quantitative ELISA may be a complementary tool for sero-monitoring immune responses of dogs and cats after rabies vaccination.

More studies with larger sample sizes are recommended to assess the statistical significance of each associated factor *viz*. age, sex, breed, vaccine brand, months since vaccination, number of vaccinations received etc on the immune response following antirabies vaccination. The study findings indicate the recommendation of booster dose after primary dose, annual boosters and vaccination campaigns which are necessary to maintain adequate protection levels and herd immunity.

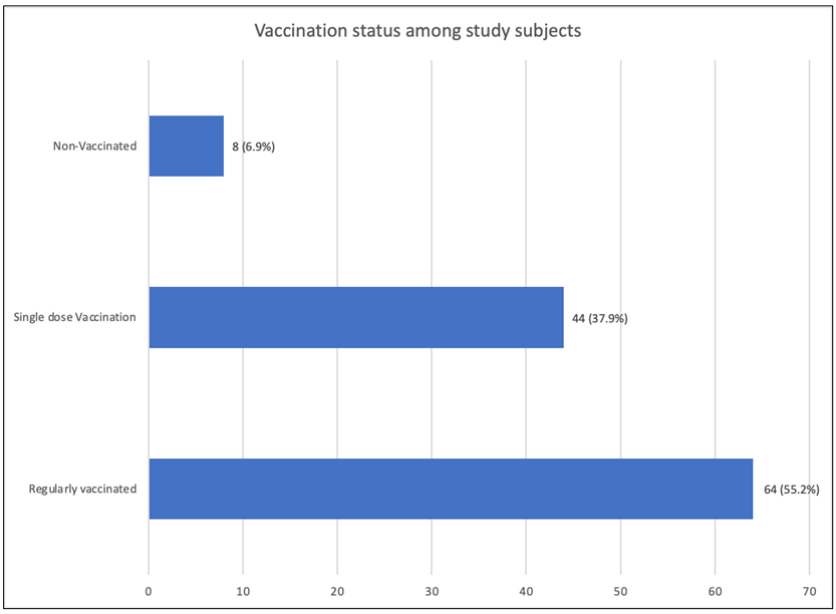



**Fig 1:** Vaccination status among study subjects

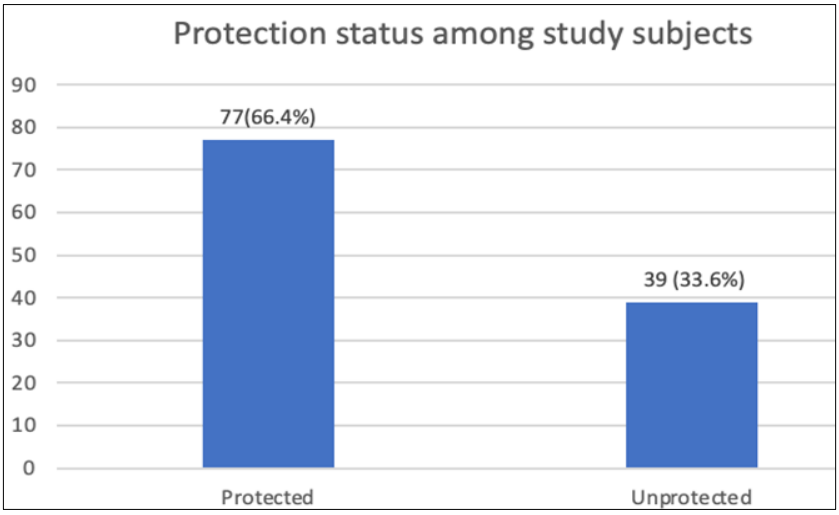
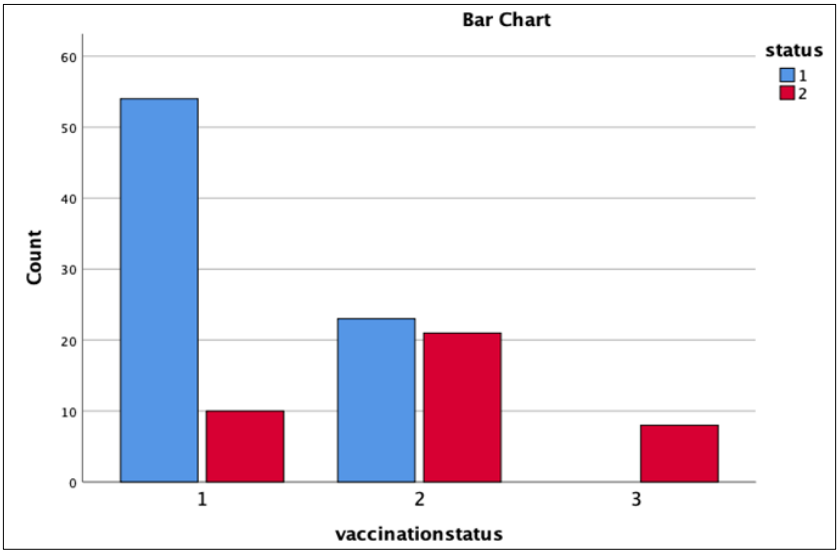
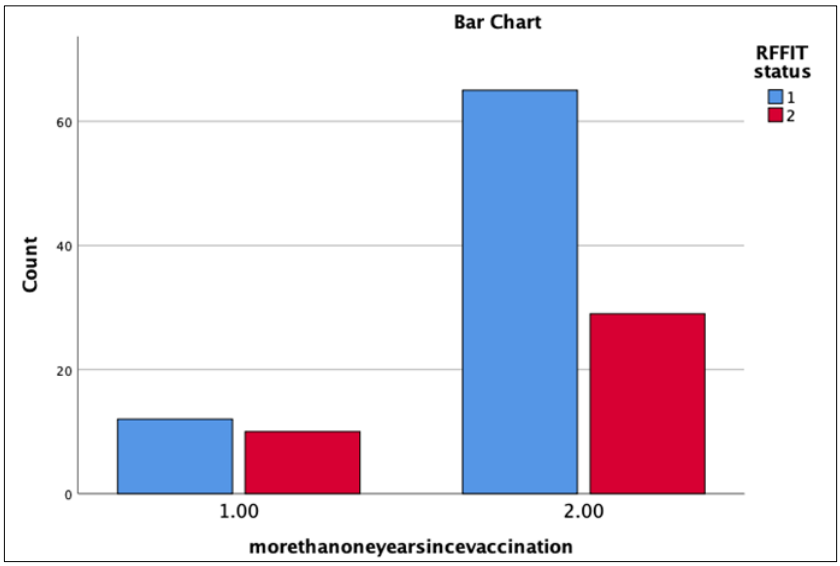



**Fig 2:** Protection status among study subjects




Status 1: Protected, Vaccination status 1: Regularly vaccinated, Status 2: Unprotected 2: One-Time vaccinated, 3: Unvaccinated

**Fig 3:** Protection status among different vaccination status groups




Status 1: Protected, Vaccination status 1: More than one year since vaccination, Status 2: Unprotected, 2: Less than one year since vaccination

**Fig 4:** Protection status among more than and less than one year since vaccination groups

**Conclusion**

In the multivariable analyses of the present study, the history of 2 or more vaccination was the only factor significantly associated with the proportion of binding antibody titres ≥0.5 EU/ml More studies with larger sample sizes are recommended to assess the statistical significance of each associated factors *viz*. age, sex, breed, vaccine brand, months since vaccination, number of vaccinations received etc on the immune response following antirabies vaccination. The study findings indicate a recommendation for booster dose after primary dose, annual boosters and vaccination campaigns which are necessary to maintain adequate protection levels and herd immunity. Moreover, we can infer that a quantitative ELISA may be a complementary tool for sero-monitoring immune responses of dogs and cats after rabies vaccination.

**Acknowledgement;** The authors are thankful to the Director, Animal husbandry department, Kerala for giving the opportunity to do this research work.

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