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ASSESSMENT OF HUMORAL IMMUNE RESPONSE IN VACCINATED DOMESTIC DOGS AND CATS INTENDED FOR PET-TRAVEL FROM INDIA BY RAPID FLORESCENT FOCUS INHIBITION TEST (RFFIT)

Santosh AK[1], Isloor S[1],* Rathnamma D[1], Sharada R[3], Sunilkumar KM[6], Balamurugan V [4], Yathiraj S [5] , Satyanarayana ML[2]

[1]KVAFSU-CVA-Crucell Rabies Diagnostic Laboratory, Dept. of Microbiology, Veterinary College, KVAFSU, Hebbal, Bengaluru, Karnataka
[2]Department. of Pathology, Veterinary College, KVAFSU, Hebbal, Bengaluru, Karnataka
[3]Department of Microbiology, Veterinary College, KVAFSU, Hassan, Karnataka
[4]National Institute of Veterinary Epidemiology and Disease Informatics, Ramagondanahalli, Bengaluru
[5]Veterinary College, KVAFSU, Hebbal, Bengaluru, Karnataka
[6]Department of Animal Husbandry and Veterinary Services, Government of Karnataka, Shikaripura, Karnataka

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ABSTRACT

The present study evaluates humoral response in vaccinated domestic dogs and cats intended for pet-travel from India by Rapid Fluorescent Focus Inhibition Test (RFFIT). In present study, 184 serum samples from dogs (n=149) and cats (n=35), vaccinated within the period of one year against rabies were tested by RFFIT using PV-3462 strain of rabies virus and BHK 21 cells. Out of total studied 149 dogs samples (male-96, female-53), 122 showed titre ≥0.5 IU/ml and 27 below <0.5 IU/ml. Interestingly, all the 35 samples from cats showed titre ≥0.5 IU/ml. The protection observed in vaccinated dogs was 81.87 per cent and in vaccinated cats it was 100 per cent. The analysis showed serum sampling between 20-50 days will have higher percentage of vaccinates with neutralizing antibody titre ≥ 0.5 IU / ml than those collected < 20 days and > 50 days. Association of the age factor of the vaccinated dogs and getting varied neutralizing antibody titre was evident. Whereas gender and breed based on size did not reveal any statistically significant effect on antibody titre. Furthermore, Out of eight vaccine brands, only one (V8) yielded 100 percent (9/9) protection, whereas the remaining

* Corresponding author
E-mail: kisloor@gmail.com (Isloor S)

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seven resulted in 61.53 (8/13 in V1) to 90.90 (10/11 in V6) percent protection. The failure to protect 18.13 (27 / 149) per cent of dogs even after vaccination is alarming. The exact cause for failure to protect these vaccinated dogs is unknown; the possible reasons could be attributed to age, genetic profile, and brand of vaccine used and non-maintenance of cold chain of vaccine / sample of animal.

1 Introduction

Rabies is one of the terrifying infectious disease that has affected mankind since antiquity (Jackson, 2016). Yearly 60,000 human deaths due to rabies are reported worldwide (Hampson et al., 2015), of which 20,000 deaths are from India alone which accounts for one third of total deaths by rabies (Sudarshan et al., 2007; Sudarshan, 2017). Rabies is caused by virus belonging to family *Rhabdoviridae* and genus *Lyssavirus* causes acute progressive viral encephalitis (Rupprecht et al., 2002). The virus is made of five structural proteins among which glycoprotein is important major structural protein involved in induction of production and protection against rabies virus by anti-rabies neutralizing antibodies after vaccination (Perrin et al., 1985; Etessami et al., 2000). International travel and trade have increased dramatically during the past two decades. Global travel and trade present risks for rapid, long distance movement of a variety of infectious diseases including rabies virus (IMNA, 2010). Due to this, in the past few years, serological testing of dogs and cats has increased because many rabies free countries have amended their quarantine measures and adopted a scheme requiring rabies vaccination followed by a serological test (Mansfield et al., 2004). This scheme has been promoted by the WHO, OIE and the European Commission and allows the free movement of pets from countries without rabies, or where rabies is under control, to rabies-free countries. Pre-exposure vaccination is considered successful by WHO and OIE when the neutralizing antibody titre is at least 0.5 IU/ml in serum from vaccinated humans and animal (WHO, 2005; OIE, 2008). Rapid fluorescent focus inhibition test (RFFIT) is a gold standard test which is recommended for serological testing of pets intended for international trade (European Commission, 2003).

Any pet animal moving from India to most other countries is vaccinated and neutralizing antibodies checked for protective neutralizing antibody titre. In India, KVAFSU- CVA- Crucell Rabies diagnostic Laboratory, (a laboratory twinned under OIE programme with APHA, UK, and CDC, Atlanta) Veterinary college, Bangalore is regularly processing serum samples for RFFIT from different states. The present study was conducted to estimate the neutralizing antibodies to anti rabies vaccination in domestic dogs and cats from different states of India which were primarily intended to travel abroad.

2 Materials and Methods

2.1 Collection of Serum samples

Serum samples (n=184) of vaccinated dogs and cats from 11 different states of India were submitted to KVAFSU-CVA-Crucell Rabies Diagnostic Laboratory, Veterinary college, Bengaluru along with details of age, gender, breed, history of vaccination, date of serum collection and Microchip number. Of these 184 serum samples, 149 were from dogs (Males-96, Females - 53) and 35 from cats (Males – 18, Female - 17). These serum samples were stored at -20 °C until the test was performed

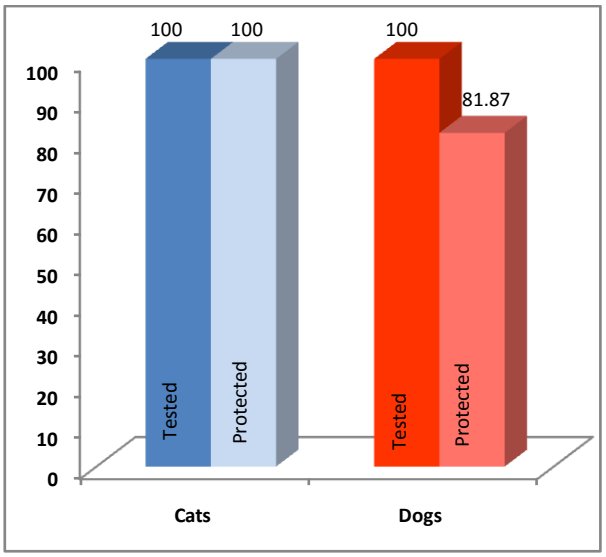

Figure 1 Results of anti- rabies neutralizing antibodies in dogs and cats

2.2 Rapid Fluorescent Focus Inhibition Test (RFFIT)

The RFFIT which is gold standard test was employed to assess the neutralizing antibodies against rabies according to Smith et al., 1996 and Neelufer et al., 2015. Initially, the test serum samples were diluted two fold and heat inactivated at 56°C for 30 min followed by further two fold serial dilution *viz*, 1:2, 1:4, 1:8, 1;16 in the 96 micro titre plate was carried out in duplicate and mixed

with 100 $TCID_{50}$ constant amount of PV-3462 (Dr. Larghi's strain) of rabies virus obtained from Pasteur Institute, Coonoor, Tamil Nadu. This was incubated for 90 minutes at 37°C for virus neutralization. Then 50 µl of BHK-21cells (25,000-30,000) suspended in 10% growth medium was added and incubated for 48hrs at 37^0C in 5% CO_2. The contents were then removed and 70% chilled acetone added to the wells and fixed for 30 minutes in -20 ℃. Then 50µl of Rabies anti-nucleocapsid based conjugate (Light diagnostic rabies DFA III cat # 6500) was diluted 1:100 with phosphate buffer saline (PBS) along with Evans blue as a counter stain at concentration of 0.001% and incubated at 37 ℃ for one hour. The microtitre plate was then washed with 1x PBS two times, observed under fluorescent microscope at 20X objective.

The neutralising antibody titre of test serum sample was determined by dividing the reciprocal of highest dilution of serum sample by reciprocal of highest dilution of WHO reference serum at which complete neutralization observed, then multiplied by unitage of WHO reference serum. The neutralising antibody titre was expressed in International Unit (IU) / millilitre of test serum. A antibody titre of equal or above 0.5 IU/ml was considered as protective.

2.3 Statistical analysis

A linear model was fitted with various factors like age, gender, breed and brand of vaccine with neutralizing antibody titre. GraphPad Prism-5 software was utilized for calculating mean, standard deviation, standard error. Chi-square (χ^2) test was employed to detect effect of breed, gender, vaccine brand and association between time intervals for blood sampling and antibody titre. A χ^2 test was used to analyze difference among the variables (Version 5; Graphpad Software Inc., La Jolla, CA, USA). The p value less than 0.05 concluded as statistically significant where as vice versa as statistically non-significant.

3 Results

Out of 149 vaccinated dogs, 122(81.87%) dogs possessed anti-rabies neutralising antibody titre of ≥0.5IU/ml. Interestingly, all 35(100%) vaccinated cats had neutralising antibody titre ≥0.5IU/ml

3.1 Persistence of anti-rabies neutralizing antibodies and best window period for serum collection

The animals were divided into 3 sampling time groups *viz*., short, normal and long. This analysis showed that the 20-50 days sampling period had a higher percentage of dogs with neutralizing antibody titre ≥0.5 IU/ml_than the other two groups in dogs (Table 1). This is the best window period for sampling which is having least failure rates (14.55 per cent) with in dogs and higher mean antibody titre (8.654 IU/ml) in cats.

Data were assessed in terms of protective or non-protective percentage of neutralizing antibody and mean neutralising antibody titre (Table 2). In the present study, the data was tabulated and analysed for association of various factors with anti-neutralising antibody titre. The statistical analysis with chi square test suggests that there is association of age of vaccinated dog and protective neutralizing antibody titre is not significant because p values for this is less than 0.05. The adult (1 year - 5 years) and older (>5 years) dogs revealed a higher percentage of dogs with neutralizing antibody titre ≥ 0.5 IU / ml at 76.38 and 94.47 respectively compared to young dogs with 65

Table 1 Best window period for serum sampling to find out neutralizing antibody titre.

		No. of animals with ≥0.5 IU/ml	No. of animals with <0.5 IU/ml	Total	Mean Titre	Std error	Per cent	Chi square Test
Below 20 days (Short)	Dogs	05	02	07	1.500	0.6892	71.42	Dogs 0.5742^{ns}
	Cats	02	00	02	2.250	1.750	100	
20-50 days (Normal)	Dogs	47	08	55	2.330	0.4739	85.45	
	Cats	13	00	13	8.654	3.075	100	
>50 days (Long)	Dogs	70	17	87	2.477	0.2940	80.45	
	Cats	20	00	20	2.625	0.8069	100	
Total		**157**	**27**	**184**				

Table 2 Effect of age, gender, brand of vaccine, and breed of animals on neutralizing antibody titre of vaccinated dogs

Factors	Levels	No. of dogs with ≥0.5 IU/ml	No. of dogs with <0.5 IU/ml	Total	Mean Titre	Std error	Percent Protection	Chi square test
Age	Young (≤ 1 year)	13	07	20	2.654	0.863	65.00	0.0222**
	Adult (1 year-5years)	57	17	74	1.824	0.246	76.38	
	Older (>5 years)	54	03	57	2.426	0.491	94.47	
Gender	Male	77	19	96	1.821	0.260	80.20	0.5148ns
	Female	45	08	53	1.841	0.228	84.90	
Vaccine brand	V1	08	05	13	1.688	0.3889	61.53	0.2074ns
	V2	35	04	39	1.714	0.2063	89.74	
	V3	21	04	25	1.571	0.3507	84.00	
	V4	13	03	16	1.769	0.5846	81.25	
	V5	12	04	16	1.682	0.3889	75.00	
	V6	10	01	11	2.167	0.8079	90.90	
	V7	07	03	10	1.286	0.4983	70.00	
	V8	09	00	09	1.333	0.3909	100.00	
Breed	Large	07	02	09	1.667	0.4859	77.77	0.2117ns
	Medium	56	13	69	1.604	0.2057	81.15	
	Small	50	10	60	1.810	0.2432	83.33	

per cent. Whereas, gender, brand of vaccine, breed, had no statistical effect (p> 0.05).

3.2. Effect of age, gender, brand of vaccine, and breed of animals on neutralizing antibody titre in vaccinated cats

Interestingly, all the 35 cats were having the protective titre of neutralising antibody against rabies (Table 3). The mean titre of adult cats was high compared to young and old. Both the genders showed similar protective neutralizing antibody titre and vaccine brands *viz*., V2, V3, V1, and V4 resulted in higher mean antibody titre in decreasing order. In breed wise analysis, non-descript cats revealed good mean antibody titre than the Persian and domestic short hair.

3.3 State wise - neutralizing antibody titres in dogs

The maximum number of serum samples were received from Maharashtra (59) and Karnataka (54) with protective neutralizing antibody titre showing (51/59) 86.44, (44/54) 81.48 percent respectively. From other 8 states the serum samples received varied from 01 to 10. It indicates more number of pets from Maharashtra and Karnataka are being transported out of India compared to other states (Table 4).

4 Discussion

The study was conducted to estimate the neutralizing antibodies to anti rabies vaccination in dogs and cats intended to travel abroad from India. Interestingly, all the cats (n=35) were showing titre above or equal to 0.5 IU/ml which is required and recommended by OIE and WHO for protection (WHO, 2005; OIE, 2008). In dogs (n=149), 81.87 per cent revealed protective titre of neutralizing antibodies. This confirms that cats respond better than dogs as observed by Cliquet et al. (2003). Similar study was conducted in United Kingdom by Mansfield et al. (2004) and obtained 95.88, 94.83 percent of vaccinated dogs and 97.17, 97.33 percent of vaccinated cats showing protective antibody titre tested

Table 3 Effect of age, gender, brand of vaccine, and breed of animals on neutralizing antibody titre in vaccinated Cats

Factors	Levels	No. of cats with ≥0.5 IU/ml	No. of cats with < 0.5 IU/ml	Total	Mean Titre	Std error
Age	Young (≤ 1 year)	2	0	2	0.50	0
	Adult (1 year- 5years)	25	0	25	5.75	1.788
	Older (>5 years)	8	0	8	2.50	0.763
Gender	Male	18	0	18	4.306	1.712
	Female	17	0	17	5.882	2.035
Vaccine brand	V1	7	0	7	4.35	1.996
	V2	6	0	6	11	4.669
	V3	4	0	4	9.2	7.587
	V4	4	0	4	3.125	1.663
Breed	Persian	10	0	10	5.25	1.828
	Non Descript	5	0	5	8.2	5.97
	DSH	14	0	14	4.75	2.204

Table 4 State wise protective neutralizing antibody titre of dogs

S. No	State	No. of animals with ≥ 0.5 IU/ml	No. of animals with < 0.5 IU/ml	Total	Per cent protected
1.	Maharashtra	51	08	59	86.44
2.	Karnataka	44	10	54	81.48
3.	Haryana	07	00	07	100
4.	Andra Pradesh	03	01	04	75.00
5.	Punjab	07	03	10	70.00
6.	Kerala	00	01	01	0
7.	Tamilnadu	01	01	02	50.00
8.	West Bengal	00	01	01	0
9.	Gujarat	02	01	03	66.66
10.	New Delhi	07	01	08	87.50
		122	**27**	**149**	**81.87**

by two different laboratories by Fluorescent Antibody Virus Neutralization (FAVN) test. Shyamsundar et al. (2014) observed 84 percent vaccinated dogs showing protective neutralizing antibody titre by RFFIT. In contrast to this study, Neelufer et al. (2015) and Savaliya et al. (2015) reported low, *viz*. 58 and 62.36 percent of vaccinated dogs with protective neutralizing antibody titre by RFFIT, respectively.

The interval between vaccination and blood sampling is one of the main significant factors in determining host response. Rabies vaccination produces a typical antibody curve with response going down over the period. In this study, the best window period for sample collection was between 20 to 50 days of post vaccination which showed better protective neutralizing antibody titre percent *i.e.*, 85.45 and 100 per cent in dogs and cats respectively

compared to short (< 20 days) and long sampling days (> 50 days) of collection. This fact was earlier confirmed by researchers like Kennedy et al. (2007) and Mansfield et al. (2004).

The variation in production of neutralizing antibody response to length of interval of sampling following vaccination relates to response kinetics from primary vaccination. After primary vaccination, isotype shift from an Ig-M response to an Ig-G as immune response develops and optimum measurement in an appropriate window of time should measure this effect. Measurement at too early a stage captures only an Ig-M response, but will not confirm whether class switching to IgG response progresses. Measurement at later stage point of time may show lower antibody levels, but this may not relate to lack of immune protection. As the immunoglobulin measure may be proportionally more accounted for by Ig G (Kennedy et al., 2007).

The Chi square test in the present study, indicated the association between age and production of neutralizing antibodies against rabies where significant difference was observed between different age groups ($p<0.05$) in dogs. Ability of younger (< 1year) dogs and cats to develop protective neutralizing antibody titre is less compared to adult and older dogs. This could be attributed to immune system being less efficient (Kennedy et al., 2007). Mansfield et al. (2004) showed that animals less than one year old have an increased risk of having poor FAVN titres. Previous researchers like Aghomo et al. (1990), Kennedy et al. (2007) confirmed that young dogs can produce rabies antibodies from four weeks of age, when the titre of maternal antibody waned. This interference by maternal antibodies and less matured immune system explains the poorer immune response in young animals**.** Furthermore, in dogs it was observed that older dogs (>5 years) revealed higher protective neutralizing antibody titre than adults (1-5years) and young (less than 1 year) dogs. This observation in contrast to Mansfield et al. (2004) and Kennedy et al. (2007), where they observed poor protective neutralizing antibody titre in older dogs than adults and opined that this may be due to reduction in immune regulation thought to occur.

HogenEsch et al. (2004) studied the effect of age on the immune response. However, they did not find any difference in IgM and IgG levels among the adult and old dogs. They confirmed pre-vaccination titre was higher in older dogs than in adults. Furthermore, they did not find any difference in post vaccination titre against rabies among adult and old dogs, this supports the findings of present study. The other possible explanation for higher protective neutralizing antibody titre in older dogs (94 percent) may be due to more number of booster vaccinations than the adults (76 percent) and young dogs (65 percent).

Influence of gender on neutralizing antibody titre in case of both dogs and cats was analysed by using chi square test which indicates that there is no significant difference ($p>0.05$) between the titre of two different genders in both species of animals. This is in agreement with the study conducted by Mansfeild et al. (2004), Jakel et al. (2008), Shyamsundar et al. (2014), Savaliya et al. (2015) and Neelufer et al. (2015) in dogs. But contrasting results were observed by Mansfield et al. (2004) in case of vaccinated cats, where male cats showed lesser percentage of protective neutralizing antibody titre compared to females. This was attributed to suppression of cytokine production by gonadal steroid hormone (Schuurs & Verheul., 1990; Rife et al.,1990; Verthelyi & Klinman., 2000).

Various vaccine brands such as V1, V2, V3, V4, V5, V6, V7, V8 and V1, V2, V3, V4 were used in dogs and cats respectively. Of these, only brand V8 provided 100 per cent protection (9/9) and all the other vaccine brands except V1 (61.53 percent) and V7 (70 per cent) provided satisfactory seroconversion ranging from 75 percent (12/16 in V5) to 90.90 (10/11in V6). In cats, higher seroconversion was obtained by all vaccine brands. The performance of each vaccine brand vary as they are produced by different manufacturers having different formulation, concentration, integrity of antigen content, adjuvant and maintenance of cold chain until its use, as reported by Kennedy et al. (2007). Although there is apparent, relative variation in the performance of vaccine brands, the statistical analysis of vaccine brands and antibody titres by chi square test did not showed significant difference ($P>0.05$). Similar observations was recorded by Shyamsundar et al. (2014) and Savaliya et al. (2015). The contrasting reports on influence of vaccine brands on antibody titre were also reported by Mansfeild et al. (2004) and Neelufer et al. (2015).

In the present study, small breeds (50 / 60, 83.33 percent) compared to larger (7/9, 77.77 percent) and medium (56/69, 81.15 per cent) sized breeds showed higher mean protective neutralizing antibody titres (1.810 IU/ml). However, there was no significant difference ($P>0.05$) in the titre of neutralizing antibodies among different breeds (based on size) when analysed by chi square test. Similar observations were recorded by Shyamsundar et al. (2014) and Savaliya et al. (2015). In contrast, to these results, Kennedy et al. (2007) observed five percent difference in log titre between breeds based on body size. There is clear existence of general relationship between the animal size and level of antibodies responses (Mansfield et al., 2004).The larger dogs are more likely to have deeper subcutaneous fat for injection, deposition and sequestration of rabies antigen known to reduce the level of immune response as compared to smaller breeds (Ellis, 1993; Keating & Noble, 2003). In the present study, unequal sample size of breeds based on size may be the reason for not observing the difference among them.

Failure to protect 27 dogs even after vaccination is alarming and it may be attributed to single or multiple factors acting synergistically, like age, where 6/27 were found below one year of age, genetic profile of individual dog is different, as haplotype of specific breeds of dogs is a factor which leads to a difference in immune response to vaccination (Kennedy et al., 1999), reproductive status (M=17/19, 89.5 percent were intact, Female=7/8, 87.5 were intact), brand of vaccine used and the inappropriate window period of serum sample collection in 74 percent (20/27) of dogs that failed the test (<20 days=3/27, 11 percent ; >50days=17/27, 63 percent).

Conclusion

In the present study immune response of vaccinated domestic dogs and cats intended for pet-travel from India was studied by Rapid Fluorescent Focus Inhibition Test. The observed protective anti-rabies neutralizing antibody titre in dogs and cats were 81.87 and 100 per cent. Study showed that cats are better responders than dogs. The best window period for serum sampling is between 20 to 50 days post vaccination. Statistically, the age of the dog showed association for higher neutralizing antibody titre whereas gender, vaccine brands did not reveal any statistically significant association in conferring protective neutralizing antibody titre.

Conflict of interest

Authors would hereby like to declare that there is no conflict of interest that could possibly arise.

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