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Serosurveillance of rabies neutralizing antibodies in companion animals in Mumbai region by using indirect ELISA

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Abstract
Introduction: Rabies is a deadly viral zoonotic disease caused by the lyssavirus of the Rhabdoviridae family but preventable by vaccination, having significant public health apprehension. Preventive measures like mass anti-rabies vaccination are the best solution to eradicate the disease and control by adopting the "One Health Approach" through proper coordination and measures taken by the veterinary and human health sectors.
Method: A total of 216 sera samples were evaluated from companion animals by using Commercial ELISA kits (Plateliatm rabies II assay ad Vetarinarium Bio-rad Ref: 3550180 USA), out of which 141 were from pet animals and 75 were from community animals.
Result: In pet animals, the highest mean antibody titre was observed in group II (<2 - 5 years) in pet dogs at 0.714 EU/ml and a group I (6 months - 2 years) at 0.763 EU/ml in pet cats. A significant difference were observed between age groups of pet dogs. In community animals, variables such as interval after pre exposure ARV and sex of the animals were considered. A total of 75 serum samples were collected from the community animals, and highest mean antibody titre of 0.662 EU/ml and 0.865 EU/ml were observed in community dogs and cats of group I (<1 month after ARV). No significant differences were observed between gender and anti-rabies antibody titre.
Conclusion: Compared to pet animals, low seroprevalences of rabies antibodies in community animals indicated that community animals are still susceptible to rabies virus infection in and around Mumbai.

Keywords: Rabies, anti-rabies antibody, Anti-rabies vaccine, Dogs, Cats, seroprevalance

Introduction
Rabies is considered one of mammals' leading viral zoonotic diseases caused by the Lyssavirus under the Rhabdoviridae family and is are responsible for causing non-suppurative encephalomyelitis. (Cherian *et al.*, 2015) [5]. Rabies has been considered a severe problem since the beginning of the disease. The disease is most dangerous and characterized by intense thirst and, fear of water, hydrophobia (Sagar 2013) [17]. India reports 59.9% of human deaths, and globally about 35% of human rabies deaths have been recorded (WHO Expert Consultation on Rabies Report 2018). Several underlying causes are responsible for higher susceptibility in animals, including the species, age, genetic makeup, strain, biotype or dose of the virus, and exposure route. Between 20% and 50% of domestic animal species have rabies, including 48% of dogs, 21.9% of cats, 61.4% of cattle, 48.7% of caprines, and 45% of horses (WHO, 1998) [22].
The rabies virus is an enveloped, bullet-shaped, and negative-sense single-stranded RNA virus (Madhusudana *et al.*, 2008) [12]. The virus is characterized by five structural proteins, namely nucleoprotein (N), RNA-dependent RNA polymerase (L), a phosphorylated protein (P), a matrix protein (M), and an external surface glycoprotein (G). The ribonucleoprotein complex (RNP) is formed by N, P, and L proteins, an essential rabies virus antigen in the virus's life cycle. It is known to induce CD4+ T cells, promoting the production of rabies virus (RV) neutralizing antibodies (Dietzschold *et al.*, 1987) [7].
National Rabies Control Programme is the apex program in India for the control and surveillance of rabies in both humans and animals. A pilot project was launched on the 11th Five-year plan by the Indian, which was aimed to prevent and control rabies from five cities. This pilot study became the basis of establishing the National Rabies Control Programme in the 12th five-year plan to reduce human deaths due to rabies and implement a plan to control the disease in dogs. It mainly focused on two components, which are the human and animal components.

Under the animal component, strategies such as population survey of dogs, bulk vaccination of dogs, dog population management, and intense surveillance and response were adopted (NRCP, 2018) [16].

Successful vaccination programs can decrease the incidence of the disease. Endemic regions should implement strict vaccination campaigns for the stray population (Singh *et al*., 2011; WHO, 2013). Blue Cross of India, formed in 1959, came up with an idea of a humane method to manage the dog population in the 1960s. The Catch-Neuter-Vaccinate-Release (CNVR) program, later known as Animal Birth Control programs (ABC), successfully helped decline human rabies cases in areas around cities like Chennai Jaipur Kalimpong, and Bangalore. Since the ABC program started, the Blue Cross has successfully carried out the sterilization program and mass street dog vaccination in states like Tamil Nadu and Sikkim (Krishna 2010; BCI, 2019) [11, 4].

"One Health" strategy should be adopted to create a herd immunity of safe and appreciable levels by vaccinating the local populations of dogs, educating the community, and managing the human population. These strategies are well proven to work in developed and developing countries (Nale *et al*., 2021) [15]. OIE and WHO recommend that all canine anti-rabies vaccines have protective titre values equal to or above 0.5 EU/ml for at least one year and two years, respectively, as the vaccine is one of the most pivotal factors to control rabies (Yale *et al* 2021) [25]. Therefore it is suggested that continuous sero-monitoring should be followed after vaccination to check the efficacy of the vaccination programs and herd immunity (Debnath *et al*., 2019; Aiyedun 2013) [6, 1]. As per the WHO, the epizootiological baseline for maintaining herd immunity in a community against rabies should have 70 to 80% vaccinated population. (Nale *et al*. 2021) [15]. Nowadays, enzyme-linked immunosorbent assay (ELISA) test kits have become available to detect the level of anti-nucleoprotein antibodies, which helps check the degree of humoral immunity after vaccination. The importance of verifying the effectiveness of rabies vaccination has shown signs in recent years to evaluate the effectiveness of campaigns that principally work to eradicate the disease and international travel of domestic dogs and cats to rabies-free countries. In order to assess the status of the animals as protected species in the Mumbai region, the current study intends to quantify the amount of neutralizing antibodies against the rabies virus in sera of dogs and cats from both domestic and community animals.

Material and Method

Study area and sample size

A serosurveillance study was carried out to determine the immunisation status of both community and domestic animals (dogs and cats) in the Mumbai and Navi Mumbai region from August 2019 to January 2021. A total of 216 serum samples were obtained from community and pet cats and dogs who had received anti-rabies vaccinations in the past, with the only information available being age and sex. The necessary permission for the study was obtained from the Institutional Biosafety Committee (IBSC) of Mumbai Veterinary College, Mumbai vide Resolution No 2.1.4. (Lr.No. BVC/Dean/VPH/IBSC/234 of 2018 dated 29/08/2018).

Sample collection

For the sero surveillance study, blood samples were collected aseptically from community dogs and cats using sterile disposable 22 G scalp vein needles and syringes. Blood was collected in vacutainer vials and allowed to clot by keeping the serum vial undisturbed for 1 hour at room temperature and was then transported to the laboratory on ice. Serum was collected after centrifuging, transferred to sterile microtubes, and stored at -20^{0}C until further use. All the processing of blood samples work was performed by taking appropriate biosafety precautions, including Personal Protective Equipment (PPE) such as an apron, protective gloves, N95 masks, head caps, and goggles. A total of 141 serum samples were received from rabies vaccinated pet dogs and cats in the Mumbai region. The 141 serum samples were further categorized depending on the age of the animals into three groups. Group I consisted of animals belonging to the age group of 6 months to 2 years, group II consisted of animals between the age group of 2 – 5 years, and group III animals were between the ages 5 – 10 years old.

Seventy-five serum samples were collected from rabies vaccinated community animals from various NGOs around Mumbai and Navi Mumbai region. The 75 serum samples collected from community dogs and cats were categorized into three groups based on the time interval after the last anti-rabies vaccination. Group I consisted of animals whose blood samples were collected within one month of ARV, group II included animals between one to three months, and group III included animals between three to six months after the last ARV

Serosurveillance of rabies antibodies in dogs by using ELISA

Commercial ELISA kits (Plateliatm rabies II assay ad Vetarinarium Bio-rad Ref: 3550180 USA) were used for the detection of antirabies antibodies. The ELISA Kit is based on the principle of indirect ELISA. Rabies glycoproteins, extracted from the inactivated and purified virus membrane, were used to coat the microplate wells. The enzymatic conjugate consisted of Proteins of protein A obtained from Staphylococcus aureus coupled with peroxidase. The kit also consisted of a positive control which was calibrated according to OIE standards. This allowed for the determination of the quantitative and qualitative analysis of antirabies antibody titre. The test was performed as per the protocol provided by the manufacturer. For obtaining the optical density, the ELISA microplate reader was adjusted at 450-620 nm. These OD values were plotted using the standard curve method to procure the titre values and were expressed in equivalent units (EU/ml).

Statistical Analysis

The statistical analysis was done by using Web Access Server Protection version (WASP) 2.0 software (Snedecor and Cochran, 1994) [20]. Mean, Standard deviation, minimum values, maximum value, variance, and standard error were calculated for community dog and cat sera samples. T-test was performed to detect the effects of sex on mean EU/ml values in vaccinated dogs and cats. Chi-square test was performed to see the impact of persistence of antirabies

antibodies after pre-exposure vaccination in community dogs and cats and the difference between the mean EU/ml values in dogs and cats.

Results

Serosurveillance of rabies antibodies in dogs and cats by ELISA

Status of antirabies antibodies in vaccinated pet dogs and cats

Out of the 141 sera, samples, 107 were from dogs, and 34 were from cats. From the 107 dog sera samples, 104 (97.19%) and all 34 (100%) cats showed rabies antibody titre equal to or above the cut-off value of 0.5EU/ml. In dogs, the sex-wise distribution of serum revealed that 65/66 (98.48%) male dogs and 39/41 (95.12%) bitches showed rabies antibody titre equal to or above the cut-off value of 0.5 EU/m.

Sex-wise distribution in the cats revealed all 17 male cats and 17 female cats had rabies antibody titre equal to or above the cut-off value of 0.5 EU/m.

a) Persistence of anti-rabies antibodies after pre exposure antirabies vaccination (ARV) in pet dogs and cats based on their age groups

The persistence of antirabies antibodies after pre-exposure antirabies vaccination (ARV) in pet dogs and cats were determine by divided serum samples (n=141) into 3 groups based on their age groups. Group-wise, the serum samples were 60 in Group I, 64 in Group II, and 17 in Group III. Group I, II, and III revealed, 60 (100%), 63 /64 (98.43%), and 15 /17 (88.23%), respectively, showed a cut-off value of 0.5 EU/ml.

A higher mean value was observed in group II dogs (0.714) (fig 1 In vaccinated pet dogs, the mean antibody titer was 0.674 EU/ml and 0.658 EU/ml in groups I and III, respectively. The relationship between the antirabies antibody titre and age of vaccination in pet dogs and cats was evaluated by statistical analysis using the chi-square test which showed a significant difference ($p \leq 0.05$).

Higher mean antibody titre in vaccinated cats was reported in group I 0.763EU/ml (fig 1). The mean antibody titre was 0.699 EU/ml and 0.622 EU/ml in groups II and III. The Chi-square test of pet cats was null as all the samples were positive

Status of antiraabies antibody in vaccinated community dogs and cats

Out of the 75 sera samples collected 47 (62.66%) showed a positive antirabies antibody titre of above 0.5 EU/ml which included 59 samples from dogs and 16 samples from cats. 36/59 (61.01%) dogs and 11/16 (68.75%) samples showed rabies antibody titre equal to or above the cut-off value of 0.5 EU/ml.

b) Persistence of anti-rabies antibodies after pre exposure antirabies vaccination (ARV) in community dogs and cats.

To obtain antirabies antibodies' persistence after pre-exposure ARV in community dogs and cats, the serum samples were divided based on the base of time interval after pre-exposure ARV in 3 groups.

In group, I 34/41 (82.92%) animals had a protective titre, while in groups II and III, 8/17 (47.05%) and 5/17 (29.41%) were observed, respectively.

A higher mean antibody titre, was observed in group I in vaccinated community dogs of 0.662 EU/ml, and mean antibody titre, in vaccinated community dogs of groups II and III were 0.454 EU/ml and 0.459 EU/ml, respectively (Figure 2). Statistical analysis revealed a significant difference ($p \leq 0.05$) between antirabies antibody titre of vaccinated community dogs and duration from the last ARV.

The higher mean antibody titre in vaccinated community cats was reported in group I of 0.865 EU/ml. The mean antibody titre in vaccinated community cats (Figure 2) in group II and III was 0.516 EU/ml and 0.125 EU/ml, respectively. Statistical analysis revealed a significant difference ($p \leq 0.05$) antirabies antibody titre of vaccinated community cats when categorized in different age groups.

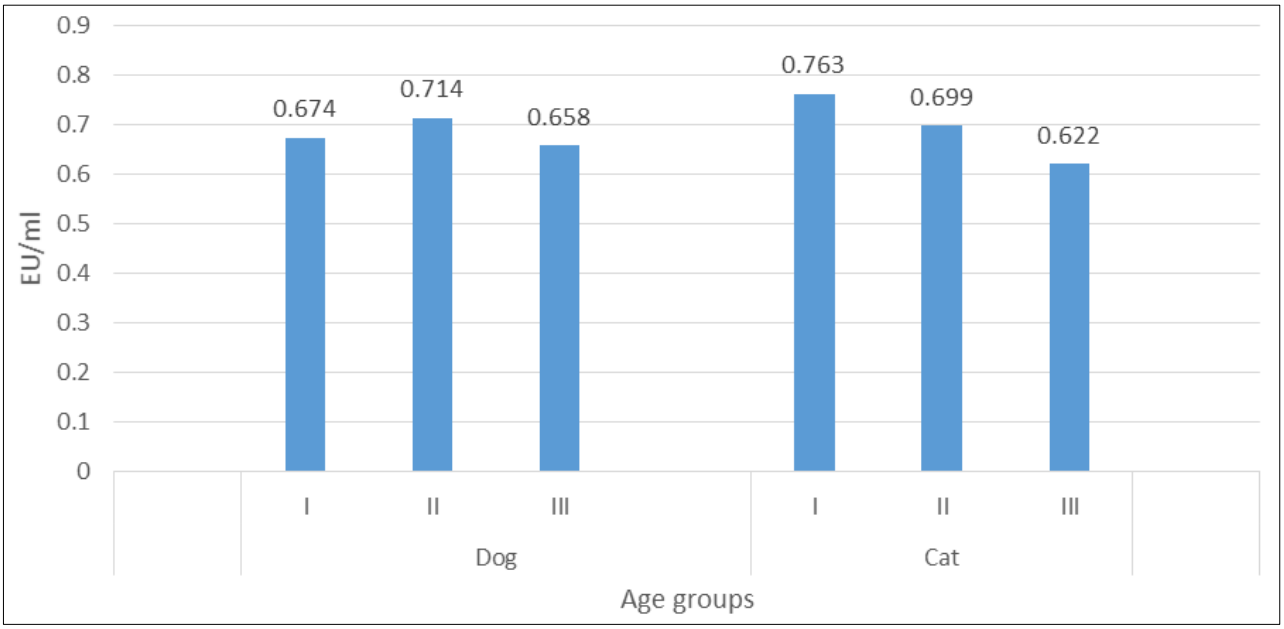

Fig 1: Statistical analysis of anti-rabies antibody titres at different age groups after vaccination in pet dogs and cats

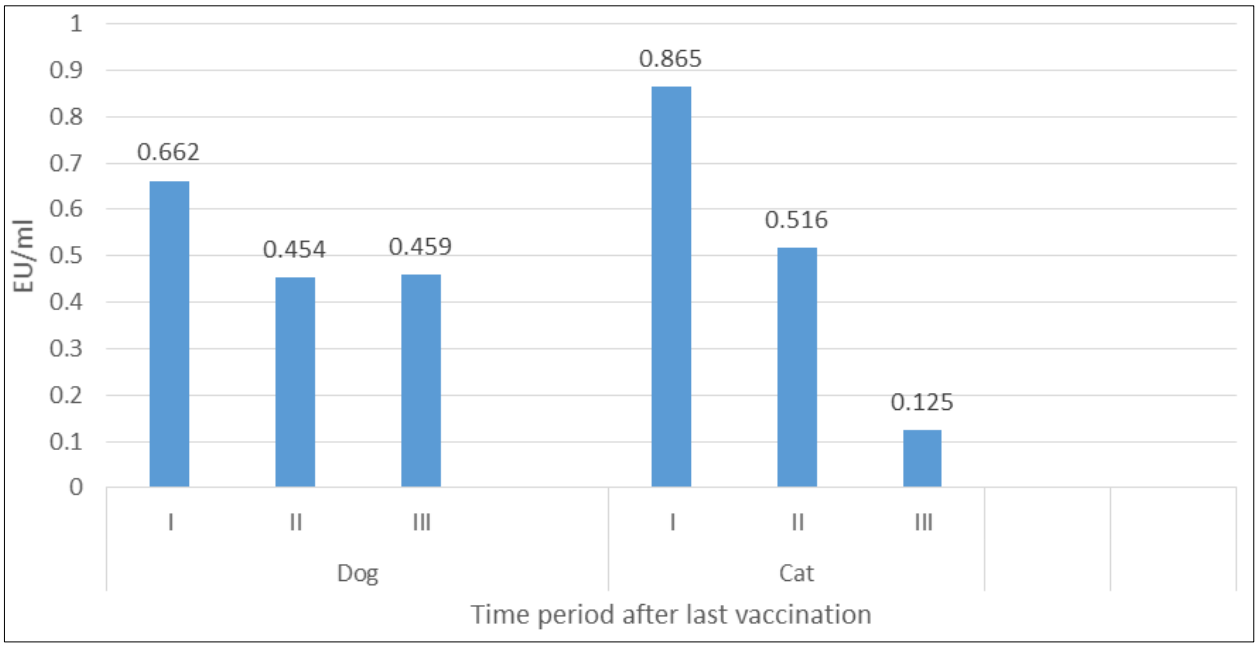

Fig 2: Statistical analysis of anti-rabies antibody titres at different time intervals after vaccination in community dogs and cats

Effect of gender on anti-rabies antibody titre

Sex-wise distribution of serum samples in male community dogs revealed 19/30 (63.33%) and bitches 17/29 (58.62%). In cats, 2/6 (33.33%) serum samples of male cats and 9/10 (90%) from female cats showed a protective level of rabies antibody titre. Male and female samples were statistically analyzed by t-test to determine the gender-wise relationship between anti-rabies antibody titer in community dogs and cats. The gender-wise data revealed no significant association.

Discussion

All over the world, rabies is considered one of the most lethal diseases to humans and animals. Therefore it is of utmost importance to vaccinate against rabies as a preventive measure. WHO has advised a serological test, ELISA to measure and determine the antirabies antibodies for the movement of animals from one country to other. OIE and WHO recommend that all antirabies vaccines protective titre values equal to or above 0.5 EU/ml for at least 1 year and 2 years, respectively, as the vaccine is one of the most pivotal factors in controlling rabies (Yale *et al.* 2021) [25]. For pet dogs and cats, yearly vaccination is essential to maintain the serum titre of 0.5 IU/ml and above antirabies antibodies (WHO 1992) [21]. The present study observed that in pet animals, out of 141 samples, 104/107 (97.19%) from pet dogs and 34/34 (100%) from pet cats, showed rabies antibody titre equal to or above the cut-off value of 0.5 EU/ml. In dogs the sex-wise distribution of serum revealed that, 65/66 (98.48%) male dogs and 39/41(95.12%) bitches showed rabies antibody titre equal to or above the cut-off value of 0.5 EU/ml. Sex-wise distribution in the cats, revealed all 17 male cat and 17 female cats had rabies antibody titre equal to or above the cut-off value of 0.5 EU/ml. A similar observations were reported by Santosh *et al.* (2017) [18], who collected 184 serum samples from vaccinated dogs and cats over one year. Out of 184 serum samples studied 122/149 (81.87%) were from dogs (male - 96, female - 53), and all 35 (100%) serum samples were from cats and showed titre ≥0.5 IU/ml. Knoop *et al.* (2010) [10] screened 284 and 120 serum samples from vaccinated pet dogs and cats respectively, under the pet travel scheme of Germany. Of the 120 cats, 99 (82.5%) showed threshold levels of above 0.5 IU/ml and from the 222/284(78.1%), dog sera showed a protective titre equal or above 0.5 IU/ml. Jeoung *et al*., (2021) [8] collected 990 sera samples from companion animals from Seoul, South Korea, for evaluation of the seroprevalence of rabies virus in dogs and cats. From the 990 samples 547 (55.3%) samples showed protective antibody levels. Singh *et al.* (2011) [19] and Yale *et al.* (2021) [25] noted similar observations, who recorded 16% and 40%, respectively.

The persistence of antirabies antibodies after pre-exposure antirabies vaccination based on their age groups of pet dogs and cats was determined by dividing the samples into three groups. In dogs, higher mean value was observed in group II in dogs (0.716). Statistical analysis revealed a significant difference ($p \leq 0.05$) antirabies antibody titre of vaccinated pet dogs, while in cat's higher mean antibody titre was noticed in group I 0.763EU/ml and a significant difference was noticed,

In India, dogs are considered one of the primary sources of rabies and are also responsible for spreading it to various other domestic animals (Aravindh Babu *et al.*, 2011) [2]. According to WHO, 35172 human deaths (59.6% of global deaths) per year in Asia are likely due to dog-mediated rabies, the high presence of dog populations, underreporting, and lack of sufficient data on rabies cases. The ABC program initiated by Blue Cross has successfully carried out mass sterilization and vaccination to keep street animals at check-in states like Tamil Nadu and Sikkim (Krishna *et al*., 2010; BCI, 2019) [11, 4]. Various NGOs in Mumbai and Navi Mumbai region like SPCA, IDA (Mumbai and Navi Mumbai), Ahimsa, etc. carry out regular Vaccination and ABC programs. In this study, a representative 75 serum samples was collected from the organizations mentioned above and NGOs. 59 were collected from community dogs and 16 were collected from community cats. It was observed that out of the 75 serum samples, 47/75 (62.66%) showed rabies antibody titre equal to or above the cut-off value of 0.5 EU/m. The results showed that 47 (62.66%) animals showed a protective titre of above 0.5 EU/ml against rabies antibodies titer suggesting that there is and prevalence of 61.01% and

68.75% in community dogs and cats respectively of antibody against rabies in the representative samples obtained from regions of Mumbai and Navi Mumbai these are following the observations of Kasempimolporn *et al*., (2007) [9], who conducted a seroprevalence study of 3314 serum samples from stray dogs in Bangkok, Thailand and reported that the antibody prevalence was in the range 49–86% in stray dogs. Nale *et al.,* (2021) [15] screened 120 serum samples from the Mumbai and Navi Mumbai region of Maharashtra, India, out of which 47 (39.2%) showed a protective antibody titre of above 0.5 EU/ml.

In a previous study, Nale (2019) [14] observed only 39.2% antibody status in community animals in 2017-18. Whereas in the present study, there is an improvement in vaccinal antibody status in community animals with an upward trend of 62.66% in between years 2019-21. This indicates that the CNVR program has shown a positive direction and is closer to the expected 70% vaccinal status to attain herd immunity

The highest mean antibody titre was observed in group I of both community dogs (0.662 EU/ml) and cats (0.865 EU/ml), consisting of animals of age < 1 month after ARV. The overall seropositivity percentage in community animals observed 62.66%, species-wise the overall highest seropositive percentage observed in cats, 68.75% (11/16), followed by 61.01% (36/59) dogs protective anti-rabies antibody titre. Similar observations was observed by Nale *et al*., (2021) [15], who evaluated 120 dog serum samples in Mumbai and Navi Mumbai region. The least literature is available regarding the anti-rabies antibodies in community cats. Santosh *et al*. (2017) [18] concluded that a higher percentage of dogs with neutralizing antibody titre ≥ 0.5 EU/ml was seen when serum samples were collected between 20-50 days after vaccination.

It can be concluded that gender/sex of the animal has no effects on the anti-rabies antibody titre in both community dogs and cats. These were in concordance with the study done by Berndtsson *et al*., (2011) [3]. They evaluated 6789 samples from vaccinated dogs and studied various factors affecting the success of rabies vaccination in Sweden. Nale *et al.,* (2021) [15] also observed similar facts when they screened 120 serum samples from stray dogs. Mansfield *et al*., (2004) [13], observed that there were no significant effects of gender in dogs; however, a significant effect was observed in the sex of the animal in the case of cats.

Conclusion

The sero-surveillance studies revealed that the overall seroprevalence of anti-rabies antibodies in pet animals was 97.87%, and in community animals was 62.66%. The finding of sero-surveillance in pet animals is high compared to community animals due to regular vaccination carried out in pet animals and pet owners' awareness of the importance of rabies vaccination. No variations were observed in vaccinated pet animals, based on age groups. There was no significant effect of gender on anti-rabies antibody titre recorded in vaccinated community animals. The low level of seroprevalence in community animals, below the World Health Organization (WHO) prescribed, indicated that the community animal population is still susceptible to rabies virus infection. A sequel of these suggests that the community animals pose a risk to other animals and raise a public health concern in the human population.

Ethical statement

The study was conducted with the necessary approval from the Institutional Biosafety Committee (IBSC) of Mumbai Veterinary College, Mumbai vide Resolution 2.1.4. (Lr.No. BVC/Dean/VPH/IBSC/234 of 2018 dated 29/08/2018).

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Supplementary material

1. Anti-rabies antibodies titres of vaccinated pet dogs and cats from group I aged 6 months to 2 years.
2. Anti-rabies antibody titres of vaccinated pet dogs and cats from group II aged > 2 years to 5 years.
3. Anti-rabies antibody titres of vaccinated pet dogs and cats from group III aged >5 years to 10 years.
4. Anti-rabies antibodies titres of vaccinated community dogs and cats from group I
5. Anti-rabies antibodies titres of vaccinated community dogs and cats from group II
6. Anti-rabies antibodies titres of vaccinated community dogs and cats from group III

Conflicts of interest: All the authors have no conflicts of interest to disclose

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