EVALUATION OF ANTI RABIES VACCINAL EFFICACY IN FREE RANGING DOG POPULATION IN BENGALURU

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By

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CERTIFICATE

This is to certify that the thesis entitled ***"EVALUATION OF ANTI RABIES VACCINAL EFFICACY IN FREE RANGING DOG POPULATION IN BENGALURU"*** submitted by **Ms. LEKSHMI J. DAS, I.D. No. MVHK-1633** in partial fulfilment of the requirements for the award of **MASTER OF VETERINARY SCIENCE in VETERINARY MICROBIOLOGY** of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, is a record of bonafide research work carried out by her, during the period of her study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

Place: Bengaluru

Date: 27.07.2018

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DEDICATED TO

MY BELOVED

PARENTS

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Bengaluru,

July, 2018 ***LEKSHMI J. DAS***

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LIST OF ABBREVATIONS/ACRONYMS

%	:	Per cent
/	:	Per
°C	:	Centigrade
ARF	:	Animal Rights Fund
BBMP	:	Bruhat Bengaluru MahanagaraPalike
C-ELISA	:	Competitive ELISA
CUPA	:	Compassion Unlimited Plus Action
CVA	:	Common wealth Veterinary Association
DLA	:	Dog leukocyte antigen
DNA	:	Deoxyribose Nucleic Acid
EDTA	:	Ethylene Diamine Tetra Acetic Acid
EIA	:	Enzyme immunoassay
ELISA	:	Enzyme Linked Immunosorbent
et al.	:	*et alia*
EU	:	Equivalent units
FAVN	:	Fluorescent Antibody Virus Neutralisation
FBS	:	Foetal bovine serum
Fig	:	Figure
Gm	:	Gram
GM	:	Growth Medium
H_2O_2	:	Hydrogen peroxide
H_2SO_4	:	Sulfuric acid
HRPO	:	Horse radish peroxidise
Hrs	:	Hours
I/M	:	Intramuscular
iELISA	:	Indirect ELISA
IU	:	International Unit

kDa	:	Kilo Dalton
KVAFSU	:	Karnataka Veterinary Animal and Fisheries Sciences University
M	:	Molar
MEM	:	Minimum Essential Medium
Mg	:	Milli gram
MHC	:	Major Histocompatibility Complex
Min	:	Minutes
mM	:	milli Molar
MNT	:	Mouse Neutralization Test
NC	:	Nucleocapsid
Ng	:	Nanogram
NIMHANS	:	National Institute of Mental Health and Neurosciences
OD	:	Optical Density
OIE	:	Office International *des* Epizooties
OPD	:	1, 2-phenylenediamine Dichloride
PBS	:	Phosphate Buffered Saline
PCECV	:	Purified chick embryo cell vaccine
PEP	:	post exposure prophylaxis
RABV	:	Rabies Virus
RFFIT	:	Rapid Fluorescent Focus Inhibition test
RNA	:	Ribo Nucleic Acid
RNP	:	Ribonucleo protein
Rpm	:	Revolutions per minute
Sf	:	*Spodopterafrugiperda*
SRV	:	SarvodayaSevabhaviSamstha
TCID50	:	50% Tissue Culture Infective Dose
VNT	:	Virus Neutralisation Test
WHO	:	World Health Organization

Introduction

I. INTRODUCTION

Rabies, a disease of great fear and intrigue, has been known to mankind since millennia. It is a fatal zoonotic viral disease present in all continents and endemic in most African and Asian countries. Rabies virus is the prototype virus of the genus *Lyssavirus* (from the Greek *lyssa*, meaning "rage") in the family *Rhabdoviridae* of the Order *Mononegavirales* causing fatal encephalomyelitis in animals and humans.

Prevalence of rabies is particularly high in India where, as many as 20,000 people die annually (Sudarshan, 2004) and main source of human infection (in more than 96% of the cases) is the unvaccinated free ranging dog population. Rabies circulates in two epidemiological cycles: an urban cycle involving maintenance of infection in dog population and a sylvatic cycle involving wildlife. Hence, dogs are the most important rabies reservoirs along with cats and wildlife.

Prevention of rabies in humans depends on a combination of interventions. These include provision of post-exposure prophylaxis (PEP) to exposed patients, Pre-exposure immunization of people at high risk of exposure, control of infection in animal reservoirs, and control of dog population (WHO, 2007). KVAFSU-CVA-Crucell Rabies Diagnostic Laboratory (OIE twinned) under Department of Microbiology has been testing animal brain samples from different parts of India for confirmatory diagnosis of rabies. In the past three years, surprisingly, 82% (58/70) of brain samples from free ranging dogs were found positive for rabies viral inclusions and hence such unprotected dogs play an important role in spreading rabies in both man and animals. Therefore, effective control of rabies in free ranging dogs is necessary. Their mass vaccination and sterilisation

through Animal Birth Control (ABC) programme are the current rabies preventive measure in India. In this programme, only a single dose of anti rabies vaccine was given to free ranging dogs whereas owned dogs receive initial two doses (including a booster) of vaccination followed by annual boosters. There is a need to find out efficacy of ongoing anti rabies vaccination in free ranging dogs through seromonitoring, in the backdrop of increasing reports of dog bites.

The effectiveness of rabies vaccination can be identified through detection and quantification of virus neutralizing antibodies in the serum based on inhibition of rabies infection *in vivo* in animals or *in vitro* in cell cultures (Atanasiu, 1973; Bourhy and Sureau, 1990). World Health Organization (WHO) recommends *in vivo* virus neutralization test on mice (VNT) and *in vitro* rapid fluorescence focus inhibition test (RFFIT) (Smith *et al.,* 1973) and fluorescent antibody virus neutralization (FAVN) (Cliquet *et al.,* 1998). The VNT on mice is time demanding and too expensive for routine use in virological laboratories. Recently, it has been replaced by sensitive, less expensive and more rapid *in vitro* tests.

Rapid fluorescent focus inhibition test (RFFIT) is a virus neutralization test. When a fixed amount of rabies virus and serial dilutions of serum is neutralized, the remaining virus in each dilution is inoculated into cultured cells, and assessed using a specific anti-rabies nucleoprotein antibody labeled with fluorescein isothiocyanate. The WHO and World Organization for Animal Health (OIE) recommends a neutralizing antibody titer of $\geq$0.5 IU/ml is adequate to prevent rabies (WHO, 2005; OIE, 2012). With regard to the preparation and performance of the test, RFFIT takes 24-48 hours and

includes the respective costs. There is no significant difference between RFFIT and the newer FAVN method recommended by OIE for examination of animal sera (Ondrejkova *et al.,* 2002). However, these VNTs are tedious and complicated to perform, making them unsuitable for large-scale sero-epidemiologic surveillance studies. To overcome this limitation, several enzyme-linked immunosorbent assay (ELISA) have been developed for detecting antibodies to rabies virus, using monoclonal based techniques (Cliquet *et al.,* 2000). The ELISA has several advantages as it takes less time and is easy to use; it does not require highly trained persons. Poor quality sera can cause cytotoxicity in VNT, which could lead to false positive results. For such samples, the use of an indirect ELISA has been shown to be as sensitive and specific as VNT and do not require high containment facilities and yield rapid results (Servat *et al.,* 2007). Commercially, G protein based ELISA kit is available to assess the levels of vaccinal antibodies. This will help in rapid screening of serum samples from vaccinated animals, but it is expensive. Considering this limitation, recently, a baculovirus expressed rabies virus G protein based in-house indirect ELISA (iELISA) has been standardized (Santosh, 2017). Application of both RFFIT and in-house iELISA for determination of anti rabies antibody levels in representative vaccinated dogs may provide indication about efficacy of current vaccination programme.

Considering these issues, it is proposed to undertake the screening of serum samples from free ranging vaccinated dogs in North and South regions in Bengaluru city with the following objectives;

i) To evaluate the anti-rabies vaccinal efficacy in selected free ranging dog population in Bengaluru.

ii) To evaluate Indirect Enzyme Linked Immunosorbant Assay with Rapid Fluorescent Focus Inhibition Test and for estimating anti rabies vaccinal antibodies in free ranging dog population.

Review of Literature

II. REVIEW OF LITERATURE

2.1 History

Rabies is one of the oldest recognized diseases and is a zoonotic disease which has been known for more than 4300 years (Takayama, 2008). It has been recognized in India since the Vedic period (1500-500BC) and was described in the ancient Indian scripture Atharvaveda, where Yama, the mythical God of Death, has been depicted as attended by two dogs as his constant companions, the emissaries of death. Also in historical references from ancient Mesopotamia, Greece and Rome provide glimpses of the recognition associating animal bite and the malady known as rabies (Rupprecht *et al*., 2006).

The first recorded description of canine rabies was done by Democritus (Baer, 2006). Aristotle wrote "the disease is fatal to the dog itself and to any animal that it may bite, man excepted" but it was unclear whether or not he thought humans were susceptible to rabies. Celsus (25 AD) was a Roman physicist who coined the term 'hydrophobia' and emphasized on the etiologic role of infectious saliva (Wilkinson, 1977).

Research on treatment of rabies started in 1885, when Louis Pasteur grew "street virus" in laboratory animals and found that it can reduce the virulence, or its ability to cause the disease in these animals. Using desiccated spinal cords from rabies infected rabbits, Pasteur developed the first vaccine against rabies and he used this vaccine for a nine year old boy, who had been bitten by a rabid dog, multiple times. The child, Joseph Meister, who had received that rabies vaccine first time on 6th July 1885 survived (Knipe

et al., 2007). Demand of Pasteur's vaccine increased during the first half of the twentieth century, but its efficacy remained questionable leading to improvements by Semple and Fermi. Wiktor and his colleagues in 1930s developed tissue culture vaccines against rabies to counter the neuroparalytic complication of vaccine developed by Semple and Fermi (Zuckerman *et al*., 2004).

2.2 Rabies in India

Rabies is primarily a disease of terrestrial and airborne mammals, including dogs, wolves, foxes, coyotes, jackals, cats, bobcats, lions, mongooses, skunks, badgers, bats, monkeys and humans, whereas dogs are the main reservoir of Rabies in India (Vaughn *et al.,* 1965). According to National Multicentric Rabies Survey, conducted by the Association for Prevention and Control of Rabies in India in collaboration with the World Health Organization, number of human death was recorded as 20,565 per year because of rabies and 91% of these were reported to be due to dog bites. India has approximately 25 million dogs, with an estimated dog:man ratio of 1:36 (Sudarshan, 2004). Domesticated dogs fall into 4 broad categories: pets (restricted and supervised); family dogs (partially restricted, wholly dependent); community dogs (unrestricted, partially dependent); and feral dogs (unrestricted, independent). Most dogs in India, perhaps 80 per cent, would fall into the last 3 categories (Chaudhuri, 2005) and that is major constrain of control of rabies. The dogs that are domesticated but not confined to an owner's home or property are considered free-roaming dogs. These free roaming dogs could be both owned and allowed to roam freely or stray (recently owned but lost from home or abandoned). Strays may also include quasi-owned animals that are cared for or considered to belong to the

neighbourhood. The term free-roaming simply describes a lack of confinement (Childs, 1990; Patronek, 1998).

2.3 Rabies virus

Rabies virus (RABV) is the prototype virus of the genus *Lyssavirus* (from the Greek *lyssa*, meaning "rage") in the family *Rhabdoviridae* (from the Greek *rhabdos*, meaning "rod") of the order Mononegavirales. Lyssa viruses consist mainly of RNA (2-3%), protein (67-74%), lipid (20-26%), and carbohydrate (3%) as integral components (percent of total mass) of its structure (Wunner, 2003). Globally the Lyssavirus have been subdivided into two phylogroups based on genetic relationship, Phylogroup I include Rabies lyssavirus, Aravan lyssavirus, Australian bat lyssavirus, Bokeloh lyssavirus, Duvenhage lyssavirus, European bat lyssaviruses type 1 and type 2, Irkut lyssavirus, Khujand lyssavirus, and the new Gannoruwa bat lyssavirus. Phylogroup II includes Lagos bat lyssavirus, Mokola lyssavirus and Shimoni bat lyssavirus. The remaining West Caucasian bat lyssavirus, Ikoma lyssavirus, and Lleida bat lyssavirus are not included in either of the phylogroups because the amount of genetic divergence and absence of cross-neutralization do not allow placement in a single phylogroup based on existing demarcation criteria (Gunawardena *et al*., 2016).

Rabies virus are bullet-shaped with a size of about 75 nm X 200 nm and can be roughly divided into a structural and a functional unit: the viral envelope and the ribonucleocapside or ribonucleoprotein (RNP) core. It has five monocistronic genes relate to five viral proteins: the nucleoprotein (N), Phosphoprotein (P), Virion-Associated RNA polymerase or Large Protein (L), Matrix protein (M) and Glycoprotein (G).

2.3.1 Nucleoprotein (N)

The Ngene codes for a nucleoprotein that encapsulates the viral unsegmented negative-stranded RNA. Flamand *et al.* (1993) through their rabies virus neutralization studies found that most abundant protein in RNP core is the Nucleoprotein (1325 or 1800 copies). The amino acid sequence of N is the most conserved of the viral proteins among the lyssa viruses (Marston *et al*., 2007). Even though it is highly conserved there is a relatively high degree of genetic diversity within short segments of the N gene between the genotypes (Bourhy *et al.,* 1993 and Kissi *et al.,* 1995). This variation in N protein was used for the analysis of phylogenetic relationship of lyssa viruses and nucleotide sequencing studies.

2.3.2 Phosphoprotein (P)

The P gene codes for a phosphoprotein, which is important not only for transcription and replication but also for interactions with cellular protein components during axoplasmic transport. Phosphoprotein contains 297 amino acids (38kDa) and is the least conserved of the five rabies virus proteins. P is a multifunctional, multifaceted protein and it interacts with N protein to form N-P complexes and acts as chaperone for newly synthesized N, preventing its polymerization and non-specific binding to cellular RNA (Mavrakis *et al.,* 2003). It was reported to specifically direct N encapsulation of the viral RNA (Chenik *et al*., 1994; Fu *et al.,*1994; Gigant *et al*., 2000). As a subunit of the RNA polymerase (P-L) complex, the P plays a pivotal role as a non-catalytic cofactor in transcription and replication of the viral genome. The P stabilizes the RNA polymerase

(L) and place the P-L complex on the RNA template, which otherwise not able to be carried out by L alone (Chenik *et al*., 1998; Fu *et al.,* 1994).

2.3.3 Virion-Associated RNA polymerase or Large Protein (L)

The L gene encodes a polymerase for RNA synthesis (Bradame and Tordo, 2001 and Rupprecht *et al*., 2002). This is the catalytic component of the polymerase complex, which along with the non-catalytic cofactor P was reported to be responsible for the enzymatic activities involved in viral RNA transcription and replication (Banerjee and Chattopadhyay, 1990).

2.3.4 Matrix Protein (M)

The M gene codes for a matrix protein. It is the smallest of rabies virion proteins and it confers the bullet shaped structure of the virion. M protein binds to condense the nascent nucleocapsid (NC) core into tightly coiled, helical RNP-M protein complex which forms a sheet around the NC core giving the structure of the virus (Mebatsion *et al.,* 1999)**.**

2.3.5 Glycoprotein (G)

The G gene produces a single trans-membrane glycoprotein which is assembled as a trimeric spike. This glycoprotein is responsible for the initial binding during infection of susceptible cells and is the only target for virus-neutralizing antibodies. It is a type I membrane glycoprotein with an N-terminal ectodomain, which extends outward to the plasma membrane and consist of a trans-membrane domain and a long C-terminal domain. This trans-membrane domain is organized into trimers in Golgi apparatus which

later form the G spikes embedded in the plasma membrane and on the virion surface (Gaudin *et al*., 1992 and Whitt *et al*., 1991). The G spikes in the viral envelope extend 8.3nm from the virus surface and represent the major surface protein of the virion (Sissoeff *et al.,* 2005) and it is reported to be critical for the induction of a host humoral immune response to rabies virus infection by acting as a target for cytotoxic T cells (Celis *et al.,* 1988 and Macfarlan *et al*., 1986).

2.4 Epidemiology

Dogs are the most susceptible and important reservoir species of rabies in Asia and Africa. In dogs, stray dogs are the major source of human bites and deaths, with more than 90 per cent of human rabies being transmitted from dog bites (Susilawathi and Darwinata, 2012). Skunk, raccoon, and fox rabies are found to be most prevalent in many parts of the US and Canada. Jackals, bat-eared foxes, and mongooses are involved in the transmission of this deadly disease in Africa. Many bat species harbour and transmit rabies and rabies-related viruses in Australia, Africa, Central and South East Asia, Europe, and many parts of the America. Mongooses (*Herpestes*spp.), foxes (*Vulpesbengalensis*), jackals (*Canis aureus*), and wolves (*Canis lupus*) have been considered as wildlife reservoirs of rabies in Bangladesh, India, and Nepal (Gongal and Wright, 2011). The geographical distribution of other lyssavirus are more localized: Lagos bat, Shimoni bat, Mokola, and Duvenhage viruses are detected only in Africa; European bat lyssaviruses, only in Europe; Australian bat virus, exclusively in Australia; Aravan, Khujand, Irkut, and West Caucasian bat viruses restricted to Asia (Kuzmin *et al.,* 2010).

2.5 Immune response in rabies vaccination

Vaccination is the only method to prevent rabies and Louis Pasteur pioneered this approach in 1885 using desiccated spinal cords derived from rabies-infected rabbits. Vaccines were then derived from neural tissue of variety of animal sources and were effective and affordable throughout the world.

Later, anti-rabies vaccine was developed by David Semple in India in 1911. From 1930s, the standardized Semple vaccine (5% sheep brain in carbolic acid) was used (Chakrabarti, 2010). However, the high content of myelin basic protein in this type of vaccine leads to fatal encephalitis though in small members (Hemachudha *et al*., 1987). Considering this, WHO recommended discontinuation of this vaccine from 1993. Alternative to this approach, inactivation of infected chick embryos (Koprowski and Cox, 1948) or inactivation of infected suckling mouse brain that has a lower level of myelin compared to the adult brain was recommended (Fuenzalida *et al*., 1964).

A new paradigm for rabies vaccines followed the development of cell culture for virus propagation. The first tissue culture vaccine was derived from virus grown in primary hamster kidney cells (Kissling, 1958), followed by growth affixed RABV in a human diploid cell line (Wiktor *et al*., 1964). An alternative to human diploid cell vaccine (HDCV) was the use of purified chick embryo cells (PCEC) (Kondo, 1965). These vaccines are now used successfully worldwide. Vos *et al*. (1999) conducted safety studies on SAD B19, an attenuated oral rabies vaccine and concluded that the vaccine can be used for oral vaccination campaigns. Preparation of rabies vaccine using vero cells

was standardized for the purpose of large scale production and became comparatively less expensive.

Antibodies have been shown to be critical for protection against the spread of RABV and the key target for antibodies is virus glycoprotein. Glycoprotein is the only surface-exposed protein on the virion particle and a number of antigenic sites to which neutralizing monoclonal antibodies bind have been identified on this protein (Seif *et al*., 1985; Prehaud *et al*., 1988). RABV glycoprotein has been expressed on the surface of the vaccinia virus (Wiktor *et al*., 1984), canary pox virus (Cadoz *et al*., 1992) and canine adenovirus (Yarosh *et al*., 1996) because of its application in eliciting immune response against the disease. Furthermore, a chimeric lyssavirus glycoprotein with segments derived from RABV and Mokola virus that provided immunity against more than one lyssavirus (Jallet *et al*., 1999) and Deoxyribonucleic Acid (DNA) vaccine with rabies virus glycoprotein cloned into a plasmid vector were developed (Lodmell and Ewalt, 2001)

Biswas *et al.* (1999) evaluated the protective efficacy of a DNA vaccine consisting of eukaryotic expression plasmid encoding the rabies G protein designated as pCMVRab. Level of anti-glycoprotein antibodies in the sera of mice which were inoculated with pCMVRab through I/M route, was higher than the minimum level of 0.5 EU/ml needed to resist experimental infection induced by the injection of wild rabies virus.

After rabies vaccination the standard methods for determining adequate immune response is by measuring virus neutralising antibodies using the fluorescent antibody virus neutralisation test (FAVN) / Rapid fluorescent focus inhibition test (RFFIT)

(Cliquet *et al*.,1998) in officially recognised laboratories. A serum titre of 0.5 IU/ml and above of rabies virus-specific antibodies is considered adequate protection against rabies. A titre below this level is considered as vaccination failure, leaving the dog less likely to be protected from the rabies virus (Fooks *et al*., 2002). Various factors influencing vaccination failures are:

2.5.1 Age

It has been reported that young dogs and old dogs do not show adequate immune response to vaccination unlike adult dogs. Dogs less than six months old and cats more than 14 years of age are reported to have lower antibody titres (Mansfield *et al.,* 2004). Younger dogs are at greater risk of not reaching protective antibody titres after their first anti-rabies vaccination. That risk can be minimized by the application of a second vaccination and blood sampling for antibody estimation (Jakel *et al*., 2008).

2.5.2 Duration after vaccination

Babboni *et al*. (2014) had reported that after one year of vaccination with booster only 54.7 per cent of the dogs maintained protective antibody levels against rabies.

2.5.3 Breed

Kennedy *et al*. (2007) had reported that small sized dogs elicited higher antibody levels than large breeds of dogs. Another observation was that the magnitude of response immediately following vaccination and duration of immunity varied between breeds of dog. This could be because of large genetic variations across breeds, whereas within each breed this variation is much more limited.

2.5.4 Management

Management of dogs plays an important role in successful vaccination. Puppies from non-vaccinated bitches responded well to vaccine after 4th week of age, showing progressive increase in virus neutralizing antibodies as measured by RFFIT. But puppies from vaccinated bitches responded only at 10th week of age although maternal antibody levels had decreased by 6th week of age (Aghomo *et al.*, 1990).

The importance of the interval between vaccination and antibody testing was demonstrated by Cliquet *et al*. (2003) and Mansfield *et al*. (2004) who recorded that the risk of test failure significantly increased when dogs were tested beyond six weeks after vaccination. The choice of the vaccine and the timing of blood tests are critical factors in achieving successful serological test results after rabies vaccination (Minke *et al*., 2009). Yale *et al.*(2014) showed that regular vaccination, regular exercise, companionship, non-descript breed, neutering, age above one year with annual booster dose of vaccination favoured long duration of immunity in the dog.

2.5.5 Gender

It was found that castrated mice responded to antigenic stimulation than un-castrated mice and had twice the number of T lymphocytes (Rife *et al.*, 1990). It was suggested that testosterone may affect the immune system through enhancement of suppressive activity in testosterone injected animals. It was also recorded that due to late thymus involution, immune system in females works longer and effectively against parasitic and infectious diseases (Aspinall, 2000)**.** Neutered animals responded better by maintaining a protective antibody titre to anti-rabies vaccination than unneutered animals

(Mansfield *et al*., 2004). However, Kennedy *et al*. (2007) demonstrated that gender of the dog does not have any significant effect on immune response to anti-rabies vaccination.

2.5.6 Genetic factors

One strong genetic factor known to influence immune response to vaccination is the Major Histocompatibility Complex (MHC). It has been previously identified that dog leukocyte antigen (DLA) polymorphism is related to both autoimmune susceptibility and infectious disease susceptibility (Kennedy *et al*., 2007).

2.5.7 Nutritional status

Nutritional status of dogs constitute an important factor in the outcome of vaccination as Cell-mediated and non-specific immunity are more sensitive to nutritional deficiency than humoral immunity (Scrimshaw and Sangiovanni, 1997). Vanloveren *et al*. (2001) indicated that nutritional status as well as individual nutrients in food can affect vaccination titre.

2.5.8 Stress

Stress in case of vaccinated animal has been associated to a certain extent of failure of vaccination. Stress is known to reduce immunity as reported by Vanloveren *et al*. (2001).

2.5.9 Endoparasitism

The infestation with endoparasites has been attributed to a factor responsible for vaccination failure. Puppies suffering from immunosuppression and not treated with anti-

helminthic, significantly lower specific antibody levels after anti rabies vaccination was demonstrated on day 28 by Mojzisova *et al*. (2007).

2.5.10 Multiple vaccinations

Multiple vaccinations could result in better immune response. Single vaccinated pets had significantly lower rabies antibodies than dogs vaccinated twice or more, and a rapid decrease of rabies antibodies was seen in primo vaccinated dogs (Cliquet *et al*., 2003). Hirayama *et al*. (1990) found that the titres declined in 120 days when rabies vaccines were administration without booster and 40 per cent of the animals did not have protective titres. However, with booster the drop of serum antibody titres (<0.5 IU/ml) in dogs occurred only after 180 days (Babboni *et al.*, 2014).

2.5.11 Storage condition of vaccine

Proper storage of anti-rabies vaccine generally used with reference to cold chain maintenance is important. Dogs receiving properly stored and improperly stored vaccine produce significant difference in antibody response (Smith *et al*., 2017).

2.6 Anti rabies antibodies in free ranging dog population

Globally, rabies kills around 60,000 people annually of which most (99%) cases are transmitted by domestic dogs (Hampson *et al.*, 2015; Knobel *et al*., 2005; WHO, 2016). So, elimination of dog mediated rabies in most important criteria in control of this disease. Countries like India, free ranging dog population is the major group of domesticated dog population. The Western Hemisphere and countries in Asia has demonstrated the effectiveness and sustainability of vaccinating dogs by combining

massive dog rabies vaccination with coordinated efforts of the medical and veterinary sectors including education about responsible pet ownership, rabies awareness campaigns, and access to post exposure prophylaxis (Rupprecht *et al*., 2002; Vigilato *et al*., 2013). The World Health Organization recommends that at least 70 per cent of the dog population be vaccinated to control and potentially eliminate dog rabies (WHO, 2016).

Presently control of dog population is done by four methods, (1) trap, remove and euthanize, (2) trap, remove and either relocate or place in kennels, (3) trap, neuter and return or the more-complete program of trap, test for infectious diseases, vaccinate, alter, return and monitor (TTVAR-M) and (4) do nothing. The American Veterinary Medical Association, the American Humane Association and The Humane Society of the United States have supported the idea of TTVAR-M as a rational and effective control method (Kristensen, 1980; Remfry, 1980; Neville and Remfry, 1984; Neville, 1989; Tabor, 1989; Zaunbrecher and Smith, 1993; Remfry, 1996).

Kasempimolporn *et al*. (2007) investigated the rabies antigen and antibody prevalence among stray dogs in Bangkok, Thailand. A total of 3314 stray dogs were captured at the Veterinary Public Health Division, Health Department, Bangkok Metropolitan Administration during December 2003-June 2004 from streets and public places of 50 districts in Bangkok as part of a campaign for sterilization and vaccination against rabies. General data about the dogs, such as estimated age, sex, ownership status, and location of capture were recorded. Determination of rabies antibody in serum was done by an enzyme-linked immunosorbent assay (ELISA).

Ogawa *et al.* (2009) estimated Rabies Immune Status of Dogs brought into the Hyogo Prefecture Animal Well-Being Center, Japan. In Japan a rabies control programs have been enforced by the Rabies Prevention Law since 1950 which included quarantine of imported animals, compulsory registration and vaccination of domestic dogs and capture of stray dogs. The neutralizing antibody titre for rabies was measured using the indirect immunoperoxidase virus neutralizing test (Ogawa *et al*., 2008).

Singh *et al*. (2011) studied prevalence of rabies antibodies in street and household dogs in Chandigarh, India and serum samples were collected from 100 street and 50 household dogs and tested for the presence of anti rabiesantibodies by ELISA. Olugasa *et al.* (2011) also conducted a study on prevalence of antibody against rabies among confined, free- roaming and stray dogs in Ilorin, the capital city of Kwara State, Nigeria. The sera were collected between June and December 2008 from apparently healthy dogs which includes 116 confined 61 free- roaming and 13 stray dogs. A quantitative, indirect enzyme linked immunosorbent assay (iELISA), the Platelia^{TM} Rabies II kit (Bio-rad, Marnes-la-Coquette) was used for detecting anti rabiesantibodies and results were expressed in equivalent units per ml (EU/ml) (Feyssaguet *et al*., 2007).

Savaliya *et al*. (2015) conducted a study to detect the vaccinal anti-rabies antibodies in pet dog serum samples and to relate the antibody status with various factors and assessment of anti-rabies antibody status in stray dog population. For that 53 serum samples were collected from unvaccinated stray dogs from Animal Birth Control Programme (ABC) unit, Ahmedabad and 107 pet dog serum samples were collected from TVCC, College of Veterinary science and Animal Husbandry, Anand and from

Veterinary Poly Clinic, Vadodara. Anti rabiesantibody levels in these serum samples were detected by PLATELIA™ RABIES II ASSAY *Ad Veterinarium*(Ref: 355-0180) BIORAD ELISA kit.

Pimburage *et al*. (2017) conducted a comparative study to investigate virus neutralizing antibody titre development in stray and domestic dogs. Previously vaccinated stray dogs (n=47) and previously unvaccinated stray dogs (n= 47) from Kalutara district of Sri Lanka were selected for the study and serum was collected day 0 before vaccination and on day 30, 180, 360 post vaccination. Rapid Fluorescent Focus Inhibition Test (RFFIT) was performed to determine the titres of rabies virus neutralizing antibodies.

2.7 Rabies serology

Mouse Neutralization test, Enzyme Linked Immunosorbent Assay (ELISA), Fluorescent Antibody Virus Neutralization (FAVN) and Rapid fluorescent Focus Inhibition Test are the major tests used for detection of anti rabies antibodies.

2.7.1 Mouse Neutralization test

Webster and Dawson (1935) used an *in vitro* test for determining rabies virus neutralization antibodies. Dilution of sera with a constant virus dose, incubated for 1.5 hours, and inoculated intacerebrally into weaning mice was done. Animal in which neutralization of virus did not occur showed clinical symptoms and was studied.

Louie *et al*. (1975) compared MNT with the RFFIT for measuring anti-rabies antibody. It was concluded that RFFIT can be substituted for MNT for estimating anti

rabies antibodies with reference to reproducibility accuracy and convenience of the test. Fitzgerald *et al*. (1979) had also carried out comparative study of Mouse Neutralization Test (MNT) and Rapid Fluorescent Focus Inhibition Test for detecting anti rabies antibodies and found that MNT and RFFIT can be used interchangeably.

Fitzgerald *et al*. (1979) suggested that mouse neutralization test can be considered as a standard test for anti rabies antibody titre estimation in which a constant dose of previously titrated challenge virus with a series of different dilution of serum was tested using mice as an indicator system.

Webster and Casey, (1996) suggested that RFFIT is a very good alternative for MNT since it is more sensitive, easy to perform, less time consuming and more humane in nature. Devi *et al*. (2018) conducted a study on the effect of a specially formulated mineral supplement in shaping the humoral immune response of dog pups in response to anti-rabies vaccination. 226 dog serum samples were tested for antibody titre against rabies vaccine using Rapid Fluorescent Focus Inhibition Test (RFFIT) and *in vivo* Mouse Neutralization Test (MNT) after post supplementation of formulated mineral supplement up to 28 days and both test showed high correlation. Study showed that mineral supplementation prior to the anti-rabies vaccination lead to elicit quick and high level of protective antibodies.

2.7.2 Rapid Fluorescent Focus Inhibition Test (RFFIT)

Smith *et al*. (1973) developed RFFIT for determining rabies neutralizing antibody titre in human and animal sera. During this test different dilutions of test serum and a fixed amount of challenge virus standard were added together for neutralization of virus

and extent of neutralization was detected by inoculating cell culture. Later, the presence or absence of virus was examined using Fluorescent Antibody Technique. Result obtained was correlated with that of MNT. Later this test was recommended by WHO as a standard test to determine rabies virus neutralizing antibody titre along with MNT.

Kurz *et al.* (1986) conducted a study in which the RFFIT results were statistically analyzed and compared with those of MNT. An anti-rabies immunoglobulin of human origin and the WHO standard serum were analyzed on 20 consecutive days to determine the reproducibility of RFFIT and found that it is excellently correlated with MNT and a better reproducibility was found.

The WHO Geneva convention (1992) recommended that MNT can be replaced by the RFFIT, since it is rapid and sensitive. VNT on mice was time consuming and too expensive for routine use in virological laboratories. Meisner *et al.* (1997) conducted a comparative analysis of RFFIT and MNT and reported equivalent results but RFFIT was reported to be a more rapid technique and was less expensive and less tedious than the MNT.

Cliquet *et al*. (1998) evaluated 414 serum samples from unvaccinated and vaccinated dogs using FAVN test, the RFFIT and the MNT and found that they are well correlated. Cleaveland *et al.* (1999) carried out a sero survey of rabies in Tanzania using RFFIT and Liquid phase blocking ELISA in vaccinated and unvaccinated dogs. Serum samples were collected from 567 unvaccinated dogs and 240 vaccinated dogs. Madhusudana *et al*. (2001) compared the immune response to rabies vaccine inoculated

by intramuscular route and intracerebral route using RFFIT in mice. There was an early and profound increase in the antibody response to intracerebral route.

In India, the RABV neutralising antibodies estimation is done at different laboratories such as National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore (Mani and Madhusudana, 2013) and Dept. of Animal Biotechnology, Madras Veterinary College, Chennai (Jemima *et al.*, 2014), Pasteur Institute, Coonoor and Indian Immunological, Hyderabad. Kramer *et al.* (2009) determined the potency of two inactivated rabies vaccines for veterinary use by mouse challenge test and RFFIT. Both tests were correlated well and RFFIT can be used for testing potency of vaccines.

Moore *et al*. (2015) compared anamnestic responses to rabies vaccination in dogs and cats with current and out-of-date vaccination status. Serum samples from 74 dogs and 33 cats analyzed for anti rabies antibodies by means of a rapid fluorescent focus inhibition test. Neelufer (2016) conducted a study to develop a cell culture based rabies neutralization assay for assessing the efficacy of prophylactic rabies vaccination in dogs and to investigate the factors influencing the antibody response against rabies in vaccinated dogs. 150 serum samples from in and around Bengaluru were subjected to RFFIT and found that only 58 per cent of dogs showed a protective antibody titre. Factors like vaccine brand and breeds of dogs have significant influence in immune response.

Immanuel and Shanmugavel, (2017) conducted a study to find out the most effective vaccine against rabies infection in Tuticorin and Bangalore. The blood samples (n=100) collected on days (14, 28) post intramuscular vaccinations in people were tested by RFFIT (Rapid Fluorescent Focus Inhibition Test). Four different types of rabies

vaccines (PCEV, Rabipur, India), PVRV (Verorab, France), HDCV (Mireux, France), PDEC (Vaxirab, India) were tested. Although all available types of vaccines had elicited the required minimum rabies virus neutralizing antibody (titre above 0.5 IU), the Purified Vero Cell Vaccine PVRV elicited the maximum neutralizing antiviral antibodies in the vaccine recipients.

2.7.3 Fluorescent antibody virus neutralisation (FAVN)

Cliquet *et al*. (1998) developed and evaluated a test called FAVN, which was adaptation of the original RFFIT. The principle of the FAVN test is same as that of RFFIT but difference is in interpretation. In RFFIT, wells showing 50 per cent fluorescence were considered for estimation of titre. Whereas, in FAVN, 'all or nothing' principle was employed *i.e*., dilution of serum sample at which 100 per cent virus is neutralized in 50 per cent of wells was taken as its rabies virus neutralising antibody titre. They compared FAVN, RFFIT and MNT using serum samples from vaccinated and unvaccinated dogs and found three tests have good agreement.

Ondrejkova *et al.* (2002) conducted study on canine sera by VNT on mice, RFFIT and FAVN. The comparison of rabies antibody titres determined in vaccinated dogs using VNT and FAVN methods showed 86.6 per cent correlation, while those obtained by RFFIT and FAVN correlated in almost with 95 per cent of cases. Zhang *et al.* (2009) performed FAVN to detect rabies virus neutralising antibodies after oral vaccination of dogs with baits containing recombinant rabies canine adeno virus type 2 vaccine. The anti-rabies antibody titre after two years was above 0.5 IU/ml in 90.8 per cent animals.

2.7.4 Enzyme Linked Immunosorbent Assay (ELISA)

The enzyme immuno assays were developed by Perlman and Engvall (1971) and it uses a solid phase enzyme immunoassay (EIA) to detect the presence or absence of antigen / antibody. Gangadhar(1993) conducted a study on 1311 pet dog serum sample by indirect ELISA for detecting antibody response to different vaccines at different period. A micro plate coated with rabies glycoprotein extracted from the inactivated and purified virus membrane was utilized. Test serum samples were added followed by addition of enzymatic conjugate. On addition of substrate chromogen, presence of antibodies was indicated by development of colour. More titre was found up to eight months after booster vaccination and gradually declined in ten months.

Cliquet *et al.* (2004) developed a qualitative indirect ELISA for the measurement of rabies virus specific antibodies in vaccinated dogs and cats. They found that a strong correlation between ELISA and gold standard fluorescent antibody virus neutralisation (FAVN) test and although the ELISA has a lower sensitivity than the FAVN test, it is a useful tool for rapidly screening serum samples from companion animals. Yang *et al.* (2007) developed a double antigen sandwich ELISA to measure antibodies against rabies virus in dogs. 500 serum samples were screened and results showed that the newly developed ELISA had a sensitivity of 92.67 per cent and specificity of 95.15 per cent when compared to standard neutralisation assays.

Servat *et al.* (2007) evaluated a quantitative indirect ELISA to detect rabies antibodies in vaccinated domestic and wild carnivores. The Platelia^{TM} Rabies II commercially available test kit was found to be highly specific (more than 98%) using a cut off value of 0.5 IU/ml. This ELISA is the only one certified and prescribed by the

OIE. Mugale *et al.* (2013) conducted a study on 183 rabies suspected cases of small and large animals presented from July 2010 to June 2011 at Veterinary Clinical Complex, GADAVASU, Ludhiana in Punjab. The immune status was determined in 180 dogs and three large animals using ELISA. In vaccinated dogs, rabies virus specific antibodies were detected in 70.55 per cent. None of the unvaccinated dogs and large animals showed protective antibody titre and was found that antibody level was directly proportional to the number of vaccine shots.

Starodubova *et al.* (2018) compared anti rabies DNA vaccines for now, its potential to induce virus-specific antibody production. Sera samples from mice were tested by ELISA 21 days after immunization. The recombinant consensus glycoprotein G-cons was used for this purpose.

2.8 Comparison of ELISA and RFFIT

Bahloul *et al.* (2005) studied antibody response of dogs reared either in experimental kennel or living in field conditions after vaccination with a cell culture derived vaccine and they found that in experimental condition both ELISA and RFFIT techniques were well correlated but in field condition ELISA yielded upper estimates. Although RFFIT is a cumbersome test should be considered as a reference rabies antibody assay technique. A seroconversion threshold of 0.5 IU/ml should be cautiously considered and a higher threshold (1 IU/ml) could be more appropriate in the evaluation of rabies immunity in the field in order to marginalize the interfering factors.

Feyssaguet *et al.* (2007) compared an ELISA (PLATELIA^{TM} RABIES II) developed for rabies envelope glycoprotein antibody detection or titration and its

comparison to reference method (RFFIT) in a multicenter study and found that sensitivity reached 98.6 per cent and the specificity 99.4 per cent. They tested 1348 human serum sample from vaccinated and non-vaccinated people with both the tests and concluded that PLATELIA™ RABIES II ELISA kit is safe, rapid and can be considered as a useful alternative to the neutralisation test.

Muhamuda *et al.* (2007) developed and evaluated a competitive ELISA for estimation of rabies neutralizing antibodies after post-exposure rabies vaccination in humans. The C-ELISA was designed based on competition between a murine neutralizing monoclonal antibody (Mab) and the antibodies in serum of vaccinated people. Serum samples (n=990) were tested from 250 people who had been administered purified chick embryo cell vaccine (PCECV) and serum samples were collected on days 0, 14, 30 and 90 post-vaccination, and were tested by C-ELISA. They found that the C-ELISA was 100% specific and sensitive in comparison to RFFIT.

Manickam *et al*. (2008) examined the efficacy of two commercially available rabies vaccines and the efficacy of a five doses vaccination regimen in 40 healthy street dogs which were selected and challenged intramuscularly and all serum samples were screened by RFFIT and ELISA. Both vaccines were found safe and effective in preventing rabies when inoculated intramuscularly applying the five doses regime (0, 3, 7, 14 and 28 days).

Shyamsundar *et al.* (2014) subjected 91 serum samples from different breeds of domestic dogs and 40 serum samples from street dogs using PLATELIA™ RABIES II ASSAY Ad Veterinarium and standard RFFIT test. When the results obtained in both

these tests were compared, of the 50 domestic dog serum samples for which both ELISA and RFFIT were employed, 42 were positive by RFFIT, but only 40 were positive by ELISA. Furthermore, the anti-rabies antibody titre of serum samples of 38 domestic dogs evaluated by ELISA were tabulated based on (i) age (0-3yrs, > 3-6yrs and > 6yrs), (ii) vaccine brand (V1 and V3) (iii) breeds of dog (Pomeranian, Labrador Retriever, 47German shepherd, and others) and (iv) sex of animal (Male and Female). Upon statistical analysis, it was found that there was no significant (P>0.05) difference in anti-rabies antibody titre due to age, vaccine brands, breed or sex.

Santhosh (2017) developed a rabies virus glycoprotein basedELISA and compared it with standard test RFFIT. A recombinant pETRVL-G plasmid having G gene of Dr. Larghi's strain of rabies virus was cloned into pFastBacTM1 vector in DH5α *E.coli* and transposed into DH10 Bac and the resultant recombinant baculovirus were used to express rabies virus G protein. The crude lysate of baculovirus infected Sf-21cells was used in ELISA. Serum samples from 247 vaccinated dogs were tested by RFFIT and ELISA and found that the newly developed ELISA has 90 per cent sensitivity and 80 per cent specificity compared to the RFFIT.

Materials and Methods

III. MATERIALS AND METHODS

The present study was carried out to monitor seroconversion in free ranging dogs in and around Bengaluru, Karnataka and the study was conducted at the KVAFSU-CVA-Crucell Rabies Diagnostic Laboratory (OIE twinned) Department of Veterinary Microbiology, Veterinary College, Hebbal. The materials utilized and methods followed were as follows.

3.1 Collection of serum samples from Free ranging dog population in and around Bengaluru

3.1.1 Serum sample collection

Blood sample from free ranging dog population were collected during a period of January to June 2018 from different wards of Bengaluru with the help of Non Governmental Organizations from Northern and Southern parts of Bengaluru who were undertaking rabies vaccination in their respective areas. The list of NGOs who helped in sample collection was depicted in Table 1.

Free ranging dogs captured for routine vaccination programme undertaken by Non Governmental Organization (NGO) in Bengaluru (Sarvodaya Sevabhavi Samstha, Animal Rights Found, Compassion Unlimited Plus Action) were selected for sampling. These NGOs were claimed that they were vaccinating these animals yearly. While collection of blood samples, details of each dog were recorded using a free ranging dog data collection web application developed by Ashoka Trust for Research in Ecology and the Environment (ATREE) for understanding the rabies mechanism at population level

under One-health Rabies Project headed by ATREE under Department of Biotechnology. With help of this web application, name of the NGO, geo-tagged photos of dogs, colour, body condition score and unique code for each dog were recorded using smart phone. The Unique code includes NGO's name, Ward No. from where the sample collected and sample number like SRV-90-01 was a unique code given to sample No.01 collected from ward 90 with the help of NGO Sarvodaya Sevabhavi Samstha (SRV).

Whole blood (2-3ml) was collected into a serum vacutainer from cephalic vein/saphenous vein after sterilizing that area using 75% isopropyl alcohol. The samples were allowed to clot at room temperature and centrifuged at 4000 RCF for 3 min within 2-3 hrs of collection to separate the serum samples. The serum samples were stored at -20ºC till further analysis. A total of 250 samples were collected from 18 different wards of Bengaluru (Fig. 1) during this study.

Fig.1: Map of Bengaluru indicating wards from which serum samples were collected from free ranging dogs

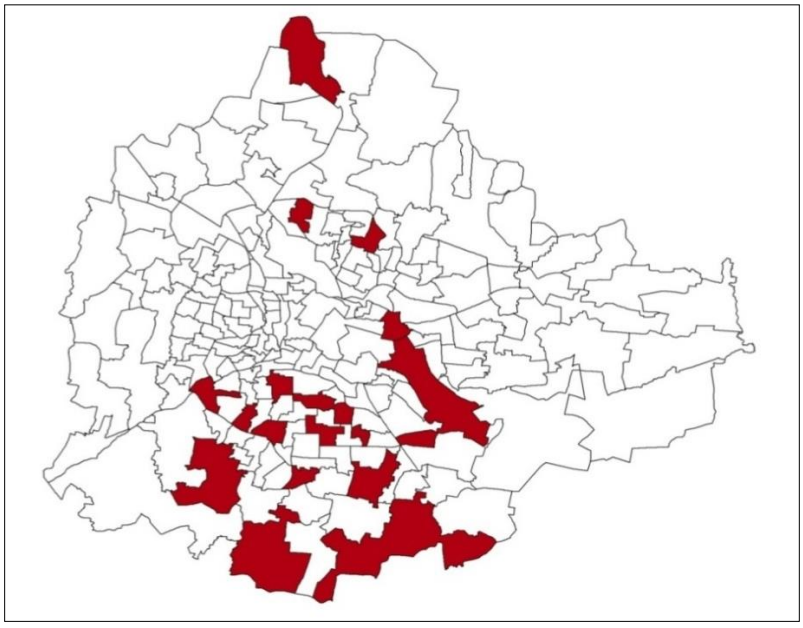

Table 1: NGOs and respective wards in Bengaluru from where samples collected

Sl. No.	BBMP Wards	NGOs
1	90	SRV(Sarvodaya Sevabhavi Samstha)
2	32	
3	114	
4	19	
5	2	
6	153	ARF(Animal Rights Fund)
7	173	
8	142	
9	158	
10	165	
11	168	
12	171	
13	163	
14	188	CUPA(Campassion Unlimited Plus Action)
15	184	
16	196	
17	186	
18	192	

3.2 Sero-monitoring of rabies in dogs using Rapid Fluorescent Focus Inhibition Test

3.2.1 Preparation of media

The Baby Hamster Kidney 21 (BHK-21) cell line was maintained using following media and reagents.

1. Phosphate Buffered Saline (PBS)
2. Growth Medium (GM)
3. Maintenance Medium (MM)
4. Trypsin EDTA
5. Tryptose Phosphate Broth

3.2.1.1 Phosphate Buffered Saline (pH 7.2)

1. NaCl : 8.00 g
2. Na_2HPO_4 : 1.15 g
3. KH_2PO_4 : 0.20 g
4. KCl : 0.20 g
5. H_2O : 1000 ml

Reagents 1, 2, 3, 4 and 5 were added to distilled water and the volume was made up to 1000ml. Then it was sterilised by autoclaving at 121 ºC at 15 psi for 15 min.

3.2.1.2 Growth Medium (GM)

Growth medium was prepared by adding 15.6 g powdered Sigma-Aldrich Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM, D8900-10X1L) to 970 ml of Milli-Q double distilled water and supplemented with 1.2gm/L of sodium bicarbonate. To this media 1% penicillin (10,000 units), streptomycin (10mg) and Amphotericin B (25µg/ml) (Himedia, A002-20ml) were added. The medium was then mixed and filtered through positive pressure with membrane filter of size 0.22 µm, supplemented with 10 per cent each of foetal bovine serum (FBS) (Himedia, Ref.RM1112) and tryptose phosphate broth (Himedia, Ref. M1710) to get a complete growth medium for cell propagation**.**

3.2.1.3 Maintenance Medium (MM)

Growth medium was prepared by adding 15.6 g powdered Sigma-Aldrich Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM, D8900-10X1L) to 970 ml of Milli-Q double distilled water and supplemented with 1.2gm/L of sodium bicarbonate. To this media one per cent penicillin (10,000 units), streptomycin (10mg) and amphotericin B (25µg/ml) (Himedia, A002-20ml) were added. The medium was then mixed and filtered through positive pressure with membrane filter of size 0.22 µm, supplemented with 2% foetal bovine serum (FBS) (Himedia, Ref.RM1112) and 10% tryptose phosphate broth (Himedia, Ref. M1710) to get a complete growth medium for cell propagation**.**

3.2.1.4 Preparation of trypsin EDTA

3.2.1.4.1 Preparation of trypsin EDTA stock solution (10X)

Trypsin	:	2.0825 gm
Sodium chloride	:	0.85 gm
Distilled water	:	100 ml

Trypsin and Sodium chloride was added in to distilled water and volume was made up to 100ml. Then it was filtered using 0.22μm filter and stored at -80 °C until further use.

3.2.1.4.2 Preparation of 53 mM of EDTA stock solution

EDTA	:	1.9729 gm
Distilled water	:	100 ml

EDTA was mixed to distilled water and volume was made up to 100ml. Then the solution was sterilized by autoclaving at 121 ºC at 15 psi for 15 min and stored at 4°C until use.

3.2.1.4.3 Working stock of 1X trypsin

10X trypsin	:	10 ml
53 mM EDTA	:	01 ml
Sterile PBS	:	89 ml

10x trypsin and 53 mM EDTA were added to sterile PBS and volume was made up to 100ml.

3.2.2 Rapid Fluorescent Focus Inhibition Test (RFFIT)

Following equipment and chemicals were used for conducting RFFIT

- Centrifuge
- Water bath
- Deep freezer (-20 ºC)
- Pipettes (5ml, 1ml)
- Multi-channel pipette (20-300 µl)
- Assay medium (MEM with 10% FBS)
- Purified rabies immunoglobulin Reference standard (obtained from Pasteur Institute of India, Coonoor, Tamil Nadu)
- Rabies virus (PV 3462 strain-Dr. Larghi's strain)
- BHK 21 cells with concentration of 3.0×10^5 cells/ml
- Cell culture flask (T25-25cm^2, T75-75cm^2)
- Sterile 96 well tissue culture plate
- CO_2 Incubator
- 70 per cent chilled acetone

- Anti-Rabies Nucleocapsid Conjugate (Fluorescein Isothiocyanate, FITC) (Fujirebio Diagnostics, Inc.Cat# 525202)
- Sterile Phosphate buffered saline
- Evan's blue counter stain (Final concentration 0.001%)
- Inverted fluorescent microscope (Carl Zeiss, Axiovert)

3.2.2.1 Revival of BHK-21 Cells

1. The stored BHK-21cells at -80 °C were revived by thawing cryovial containing frozen cells by gentle agitation in a water bath at 37 °C for two min.
2. The cryovial was removed from water bath and decontaminated by spraying with 70% isopropyl alcohol.
3. The contents were transferred to sterile 15 ml centrifuge tube containing nine milliliter of growth medium. The dimethyl sulfoxide (DMSO) was removed by gentle centrifugation for 10 min (125 x *g*) at room temperature. The supernatant was discarded and the cells were suspended in 2 ml of growth medium. This cell suspension was transferred into T25 containing 6 ml of 10 per cent growth medium and incubated at 37 °C at 5 per cent CO_2 concentration
4. Cell culture was examined after 24 hr for checking cell growth.

3.2.2.2 Maintenance of BHK-21 cells

Once the confluent monolayer was formed, the cells were sub-cultured and maintained by following procedure (Table 2).

1. The spent medium was removed and flask was washed with PBS to remove the dead cells, serum components and accumulated metabolic wastes.
2. Trypsinisation of the monolayer was done to get the associated cells in dissociated and discrete form. About 2 ml of 0.25% trypsin (pH 7.6 to 7.8) was added to T25 and kept at 37°C for 1-2 minutes.
3. After the cell detachment from T25 wall, fresh growth media about 6-8ml was added and mixed by pipetting.
4. After monolayer formation growth media was replaced by maintenance media.

Table 2: Maintenance of cells

Sl. No.	Size of Tissue culture bottle (cm^2)	Volume of Cell growth medium (ml)	Approximate volume of Trypsin EDTA (ml)
1	25 cm^2(T25)	8 ml – 10 ml	2 ml (2 times)
2	75 cm^2(T75)	25 ml- 30 ml	5 ml (2 times)

3.2.2.3 Total Cell count

The protocol for total cell count was set according to Phelan (2007). Haemocytometer was used to determine the concentration of cells that is required in each stage to add the number of cells in microtitre plates in appropriate volume to each well. The cell suspension to be counted was collected in a sterile container. A drop of the cell suspension after thorough mixing was taken using capillary tube and placed on the edge of the 'V' shape of the Neubauer counting chamber without any air bubbles or gaps in between the squares. The cells were loaded under the cover slip without overflow. The

chamber was then viewed under 10X objective lens of microscope and the cells present in four corner squares were counted by the following formula.

$$C = n/v$$

Where, C = Cell concentration

n = average number of cells/mm^2 areas

v = volume counted = 10^{-4}

thus C = n X 10^4

3.2.2.4 Preparation of anti-rabies nucleocapsid (FITC) conjugate

Commercially available anti-rabies FITC monoclonal antibody conjugate (Fujirebio Diagnostics, Inc. Cat # 525202) was used in the study. A working solution of conjugate was made to optimize the exact dilution of the conjugate needed. For making working solution serial two fold dilutions of the conjugate like1:10, 1:20, 1:40 etc. were prepared in PBS. Dilution that provided good resolution and high fluorescent staining with minimal background fluorescence was considered as the end-point dilution of the conjugate. In the present study conjugate was used at 1:20 dilution and the working solution prepared was filtered using 0.45µm filters and stored for use at 4 ºC till further use.

3.2.2.5 Counter staining

Evans Blue (0.5 per cent in PBS, Sigma, Product # E0133) was used as a counter stain which was added to the working dilution of the conjugate. The stock solution (0.4 per cent Evans Blue in PBS) was aliquoted and stored at 4°C for up to six months. The cells when stained with counter stain notably appear red, but care was taken that staining

was not strongly red as to diminish the specific green fluorescence produced by the conjugate. In the present study, Evans Blue was used at a final concentration of 0.00125%. Diluted conjugate after addition of the counter stain were filtered using 0.45µm syringe filters.

3.2.2.6 Titration of stock virus

The rabies virus strain PV3462 (Dr. Larghi's strain) and BHK-21cells maintained in the Rabies diagnostic Laboratory, Department of Microbiology, Veterinary College, Hebbal, Bengaluru were used. Titration of the virus in the stock was carried out to estimate the infectious unit of virus and stored at -80 °C. The virus stock was serially diluted using the cell culture growth medium as diluent and log dilutions from 10^{-1} to 10^{-6} were prepared. Five wells were maintained for each dilution.

3.2.2.6.1 Virus dilution

Five sterile centrifuge tubes were labelled starting from 10^{-1} to 10^{-5} and 900µl of cell culture growth medium was taken into all tubes and 100 µl of virus stock was added to first test tube (Table 3). After mixing, 100 µl from tube no.1 was transferred to tube no.2 and similar serial dilutions were was carried out till tube no.5 and finally 100 µl was discarded from that tube. All these diluted virus solutions were added into 5 different wells in a microtitre plate. The cells in tissue culture flask which had formed a monolayer of BHK-21 cells was trypsinised and a homogenised cell suspension was made using the cell growth medium in such a way to get approximately 25,000 to 30,000 cells per 50µl of cell suspension as per the Section 3.2.2.2 and 3.2.2.3. Cell control and virus control was maintained by adding 50µl of cell suspension with cell culture medium and100µl

neat virus with media respectively. The plate was incubated in CO_2 incubator at 37 ºC temperature and 5 per cent CO_2 atmosphere for 48 hrs.

After 48hrs the cell culture fluid from all the wells was removed and 50 µl of 70 per cent chilled acetone in PBS was added and plate was kept at - 20 ºC for 30 min. After 30 min, acetone buffer was removed using multi-channel pipette and the plate was air dried. In the next step 50µl of working dilution (1:50) of anti rabies N-protein monoclonal antibody based-FITC (Fujirebio Diagnostics, Inc.Cat # 525202) conjugate was added and the plate was incubated at 37 ºC for an hour for identifying rabies virus infected cells. Later, the content in all the wells were discarded and washed twice using PBS. All the wells were then examined for the presence or absence of viral inclusions as apple green coloured fluorescent particles in the cytoplasm of cells using Fluorescent microscope.

Table 3: Dilution of virus

TubeNo.	**1**	**2**	**3**	**4**	**5**
Virus	**100**µl	**100**µl	**100**µl	**100**µl	**100**µl
Diluent	**900**µl	**900**µl	**900**µl	**900**µl	**900**µl
Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}

3.2.2.6.2 Interpretation

All the fields in each of the wells were examined and all or none principle was adapted to evaluate the presence or absence of viral inclusions. The observations at various dilutions of virus were recorded (Table 4). The virus titre was estimated by using Reed-Muench method (1938) in terms of $TCID_{50}$.

3.2.2.7 Heat inactivation of serum samples

The serum sample were initially diluted by adding 220µl of growth medium to 220µl of serum and then kept it in water bath set at 56ºC for 30 min for inactivating the complements.

Table 4: Layout of microtitre plate for virus titration

	1 10^{-1}	**2** 10^{-2}	**3** 10^{-3}	**4** 10^{-4}	**5** 10^{-5}							
A						**CC**						
B						**CC**						
C						**CC**						
D						**CC**						
E						**CC**						

3.2.2.8 Assay protocol

The test protocol standardised by Neelufer *(*2016) was employed. Flat bottomed 96 well micro titre plates were used or the assay and 100µl of cell growth medium was added to rows from B to D and F to H and 100µl of test sera to row A, B and E, F except in cell control and virus control. A serial doubling dilution of inactivated serum sample was prepared by transferring 100µl from second row to third row then to fourth row using a micropipette (Table 5) to arrive up to 1:16 dilution from A to D. This step was repeated for E to H. A 100 $TCID_{50}$ virus suspension was prepared from titrated PV 3462 (Dr.

Larghi's strain) working bank and 100µl of diluted virus that contains 100 $TCID_{50}$ was added to all wells except cell control wells and the plates incubated at 37 °C for 90 min with 5 per cent CO_2 for neutralisation. 200µl of growth medium was added to the cell control wells.

After the neutralization step 60µl of two to three days old BHK-21cells (25,000 to 30,000 cells/well) were added to all the wells and incubated at 37 °C at 5 per cent CO_2 for 48 hr. The WHO reference serum having 2 IU titre showed complete neutralisation at 1:8 dilution. This criterion was used in the formula given below to calculate the anti-rabies antibodies titre.

3.2.2.9 Fixation and staining

- The assay plates were observed under the microscope at 20X for cell growth after 48hrs.
- The medium was decanted from the plate without disturbing the monolayer and 100µl 70 per cent ice cold acetone was added and the plates were incubated for 30 min at -20 °C. Acetone was discarded using multi-channel pipette and allowed the plate to air dry.
- The monolayer was covered by the addition of 50µl of rabies anti-nucleocapsid conjugate working solution and the plates were incubated in CO_2 incubator at 37 °C at 5 per cent CO_2 tension for 60 min.
- After incubation, conjugate was discarded and the plates were washed by filling and emptying the wells with sterile phosphate buffered saline.
- The plates were observed under fluorescent microscope by using 20 X objective.

Table 5: Layout for RFFIT

R	C											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	**1:2**										**Ref S**	**VC**
B	**1:4**											
C	**1:8**											
D	**1:16**											
E	**1:2**											**CC**
F	**1:4**											
G	**1:8**											
H	**1:16**											

C: Column R: Row VC: Virus control CC: Cell control REF.S: Reference serum

3.2.2.10 Interpretation

Highest dilution of serum at complete neutralization of virus (no fluorescence) was noted and comparing with the reference serum anti rabies antibody titre of samples were recorded using following formula,

$$\text{Reciprocal of highest dilution of test serum showing complete neutralization of virus infectivity} = \frac{\text{Neutralisation of virus infectivity x Unitage of reference serum test}}{\text{Reciprocal of highest dilution of reference serum showing complete neutralisation of virus infectivity}}$$

3.3 Indirect Enzyme Linked Immunosorbent Assay (ELISA)

In this studyindirect ELISA developed and standardized by Santosh (2017) was used. Recombinant rabies virus G protein expressed in baculovirus (BVES) was used as antigen.

3.3.1 Reagents

3.3.1.1 Antigen coating buffer (Carbonate-bicarbonate buffer, pH 9.6±0.05) Stock solution:

Solution A:

Sodium carbonate (anhydrous)	:	1.06 g
Distilled water	:	50.00 ml

Solution B:

Sodium bicarbonate	:	0.84 g
Distilled water	:	50.00 ml

Solution A and B were stored at 4^{o}C.

Working solution (0.05 M, 1X)

Solution A	:	3.50 ml
Solution B	:	8.50 ml
Distilled water	:	38.00 ml

The working solution was freshly prepared each time before use.

3.3.1.2 Calcium –magnesium free phosphate buffered saline (CMF-PBS)(0.1 M, pH 7.2)

Sodium chloride	:	8.00 g
Disodium hydrogen phosphate	:	1.21 g
Potassium chloride	:	0.20 g
Potassium dihydrogen phosphate	:	0.20 g
Distilled water	:	1000.00ml

The solution was sterilised by autoclaving at 121^{o}C for 15 min at 15 psi and stored at 4^{o}C in aliquots of 100 ml.

3.3.1.3 Wash buffer: Working solution

CMF-PBS (pH 7.2 ± 0.2)	:	100.00 ml
Tween-20	:	0.05 ml

This solution was freshly prepared before use.

3.3.1.4 Serum conjugate dilution buffer (1% BSA in PBST)

Bovine serum albumin (Sigma)	:	1.00 g
CMF-PBS (pH 7.2 ± 0.2)	:	100.00 ml
Tween-20 (Himedia Pvt. Ltd.)	:	0.05 ml

The solution was freshly prepared before use.

3.3.1.5 Rabbit anti-dog Ig G HRP conjugate (Sigma, USA)

Rabbit anti-dog Ig G Horse Raddish Peroxidase conjugate was used as secondary antibody.

3.3.1.6 Chromogen solution:

One tablet of O-phenylenediamine-dihydrochloride(OPD) weighing five milligram, obtained from Sigma chemicals (USA), was dissolved in 12.5 ml of distilled water and stored at -20^{o}C in falcon tubes wrapped with aluminium foil till further use.

3.3.1.7 Substrate solution:

3 per cent H_2O_2 : 1 ml

The solution was stored at 4 °C until further use.

3.3.1.8 Stopping solution (Hydrochloric acid, 2.5 N)

Hydrochloric acid (35%) : 22.7 ml

Distilled water : 77.3 ml

This solution was stored in amber coloured bottle at room temperature until further use.

3.3.1.9 Carrier surface:

Nunc Maxisorp plates with 96 flat bottom wells were used as the carrier surface.

3.3.2.1 Source of serum samples

Serum samples collected from free ranging dogs in and around Bengaluru for which the RFFIT titre had been determined were tested usingELISA. Strong positive

serum control (Post anti-rabies vaccinal serum from dog having 8 IU/ml RFFIT titre) and Negative serum control from unvaccinated healthy dog having RFFIT titre less than 0.5IU were selected. The control serum and test sera samples were maintained in the KVAFSU-CVA-Crucell Rabies diagnostic Laboratory, Veterinary College, Bengaluru.

3.3.2.2 Anti dog Ig G HRP conjugate preparation

Anti-dog Ig G HRP conjugate at 1:15000 dilutions in conjugate dilution buffer **(**1% BSA-PBST) was used.

3.3.2.3 Recombinant RABV-G protein antigen dilution:

Recombinant RABV-G protein antigen was prepared in carbonate-bicarbonate buffer (pH 9.6 ±0.05) to provide a concentration of 500 ng/100 µl/well.

3.3.2.4 Serum dilution

Strong positive serum control (8 IU/ml) and negative serum control were diluted with serum dilution buffer **(**1% BSA-PBST) to provide a dilution of 1:100 was used. Samples were also diluted to 1:100.

3.3.3 Protocol of ELISA for measuring serum IgG response using rabies virus recombinant glycoprotein (RABV-G) antigen

1. Initially, 500 ng/100µl of RABV-G BVES expressed antigen were prepared in coating buffer (pH 9.6 ± 0.05) and 100µl of these antigens were added to each well. The plates were then incubated at 4^{o}C overnight.

2. After incubation, the contents of the wells were discarded and the plates were washed two times with PBS and gently tapped over a fresh tissue paper.

3. Diluted serum sample were added from column 3 to 12 in duplicate wells. The appropriate controls *viz*., Positive control serum (C++: post anti rabies vaccinal sera of dog having RFFIT titre of 8.0 IU), (C++: 1:2 to 4.0 IU/mL, 1:4 to 2.0 IU/mL, 1:8 IU/ml to 1 IU/ml, 1:16 equal to 0.5 IU/ml,1:32 to 0.250 IU/ml, 1:64 to 0.125IU/ml) negative control serum(C-: sera from unvaccinated healthy dog), conjugate controls (CC: no sera added) and blank wells were maintained in column 1 and 2 in quadruplicates.

4. The plate was then incubated at 37 ^{o}C for 120 min in the orbital shaker at 40 rpm and the plates were washed trice with wash buffer.

5. 100µl of 1: 15,0000 dilutions of rabbit anti-canine IgG HRP conjugate in dilution buffer was added to each well and incubated at 37 ^{o}C for 60 min. in the orbital shaker at 40 rpm.

6. The content of the wells were discarded and the plates were washed three times with wash buffer and gently tapped over a fresh tissue paper.

7. One hundred microliter of freshly prepared chromogen-substrate solution containing OPD and three per cent H_2O_2 as substrate (4 µl/ml of OPD) was added to each well and the plate was kept at room temperature for 15 min.

8. The content of the wells were discarded and the plates were washed four times with wash buffer and gently tapped over a fresh tissue paper.

9. Finally, 50µl of 2.5 N HCl was added to each well to stop enzyme-substrate reaction.

10. Absorbance values were read and recorded at 492 nm using ELISA reader.

3.3.3.1 Calculation of Percent of Positivity (PP) values

Percentage of positivity for each reading was calculated using the following formula,

$$PP = \frac{\text{OD of the test serum X 100}}{\text{OD of the positive control}}$$

3.3.3.2 Sensitivity and Specificity

To compare the sensitivity and specificity of various tests, the statistical formula given by Thrusfield (2007) was used (Table 6).

The notations used were,

a = Number of sample positive by both standard test and test to be compared.

b = Number of sample positive by test to be compared but negative by the standard test.

c = Number of samples negative by test to be compared but positive by the standard test.

d = Number of samples negative by both the tests.

Sensitivity: it is the capacity of the test to detect the protective animals, compared to the standard tests.

$$\text{Sensitivity} = \frac{a}{a+c} \text{ X } 100$$

Specificity: it is the capacity of the test to detect the un-prote tive animals, compared to the standard test

$$\text{Specificity} = \frac{d}{b+d} X100$$

Table 6: Two sided Contingency table

	Standard test			**Total**
Test		**Positive**	**Negative**	
	Positive	A	b	a+b
	Negative	C	d	c+d
	Total	a+c	b+d	

3.3.3.3 Kappa Value

Kappa is the ratio of the observed agreement beyond chance to the maximum possible agreement beyond chance. The value observed were compared with those suggested by Altman (1991).

3.3.3.4 Statistical analysis

Spearman rank correlation was employed to compare the association between the RFFIT titre (IU/ml) and the ELISA test OD derived titre (IU/ml). Tukey box and whisker plot diagrams were plotted to show the mean, median and distribution of different inter quartile range of anti-rabies antibody titre with respect to the dogs in different zones and those vaccinated by NGOs.

Results

IV. RESULTS

In present study, anti rabiesvaccinal efficacy in free ranging dog population in Bengaluru was studied using Rapid Fluorescent Focus Inhibition Test and compared it with iELISA.

4.1.1 Estimation of $TCID_{50}$

The virus was stored at -80 ^{0}c and before using for RFFIT, titre was estimated using Reed and Muench method (1938) (Table 7).

Table 7: Titration of virus using Reed-Muench method

Virus dilution	Infection ratio	Infected	Uninfectd	Accumulated values		Infection ratio (I/I + U)	Percent (I/I+ U)*100
				Infected (I)	Uninfected (U)		
10^{-1}	5/5	5	0	18	0	18/18	100
10^{-2}	5/5	5	0	13	0	13/13	100
10^{-3}	5/5	5	0 ↓	8	0	8/8	100
10^{-4}	3/5	3	2	3	2	3/5	60
10^{-5}	0/5	0 ↑	5	0	7	0/7	0

Since, 50% end point is seen to lie between 10^{-4} and10^{-5}, It will be located at the proportionate distance from 10^{-4}

$$\text{Proportionate distance} = \frac{\text{Infectivity above 50\%-50}}{\text{Infectivity above 50\%-Infectivity below 50\%}}$$

$$= \frac{60 - 50}{60 - 0}$$

$$= 0.16$$

The log of the dilution above 50 per cent was 10^{-4}

The proportionate distance was 0.16

The log of dilution factor was -1

Hence the 50 per cent end point is calculated in the following (log ID50)

= (log dilution above 50 per cent) + (Proportionate distance × log dilution factor)

= (- 4) + (0.16× -1)

= 4.16

Therefore, **$TCID_{50} = 10^{-4.16}$/ 0.1 ml**

The end point dilution is the dilution that will infect 50 per cent of the test units inoculated referred as one TCID50.

So, **100 $TCID_{50} = 10^{-2.16}$/ 0.1 ml**

Antilog 2.16=144.54

Original virus stock was diluted by 144.5 times to get 100 $TCID_{50}$ virus.

4.2 Estimation of anti rabies antibody titre using Rapid Fluorescent Focus Inhibition Test

Serum samples from vaccinated dogs were subjected to RFFIT as mentioned in Section 2.3.5 and the plates were observed under an inverted fluorescent microscope (with excitation filters of 480 nm and emission at 530 nm. The virus control with 100 $TCID_{50}$ (Fig. 2), cell control (Fig. 3) and the serum samples with insufficient neutralising anti-rabies antibodies resulted in growth of virus which was visualised by Fluorescent

microscope (Fig. 5). Whereas, the serum samples containing neutralising anti-rabies antibodies did not show fluorescence, since the virus was neutralised by the antibodies in the test/reference serum and not available for DFA Test (Fig. 4).

250 serum samples from free ranging dogs were subjected to RFFIT and out of this, 125 (50%) had protective anti rabies antibody titre of equal to or > 0.5 IU/ml.

4.2.3.1 Level of protective anti rabies antibody titre in dogs of different regions of Bengaluru

In the present study 250 serum sample were collected from North (n=97) and South (n=153) parts of Bengaluru where mass vaccinations were undertaken by Sarvodaya Sevabhavi Samstha (North) Animal Rights Fund and Compassion Unlimited Plus Action (South). In Northern part 65.97 per cent of free ranging dogs were found protected against rabies. Whereas, it was 40.52 per cent in Southern part of Bengaluru (Fig.6). Also Tukey box and whisker diagram plotted to show mean, median and distribution of inter quartile range in Fig.7.

4.3 Indirect Enzyme Linked Immuno Sorbent Assay

Indirect ELISA standardised by Santosh (2017) was employed for all 250 samples after RFFIT. The average of all control ODs were plotted against corresponding neutralising antibody titres and made into graph which gave a hyperbola (Fig. 8). The test samples (n=250) were subjected to RFFIT and recombinant iELISA and results were compared and analysed by Spearman rank correlation. Results showed a correlation coefficient (r) of 0.22 ($P < 0.05$) (Fig.9)

4.3.1 Anti rabies neutralising antibody titre by iELISA in dogs

The serum samples after testing by RFFIT for estimation of anti-rabies neutralizing antibodies were again analysed by iELISA for estimation of anti-rabies neutralizing antibodies (Fig.10). Out of 250 serum samples 126 (50.4%) serum samples were having neutralising antibody titre more than or equal to 0.5 IU/ml (Cut off value 57.09 or above) whereas 124 (49.6%) serum samples were having neutralising antibody titre less than 0.5 IU/ml (Table. 8).

Table 8: Percentage of protection by iELISA by comparing OD values with RFFIT titre

N= 250	Protected	Unprotected
Number of sample	126	124
Percentage	50.4	49.6

4.4 Sensitivity and Specificity

The sensitivity and specificity of iELISA was found to be 94.4, 95.2 per cent respectively. Kappa value of 0.89 was recorded (Table 9).

Fig. 2: Virus control with 100TCID$_{50}$ (10X)

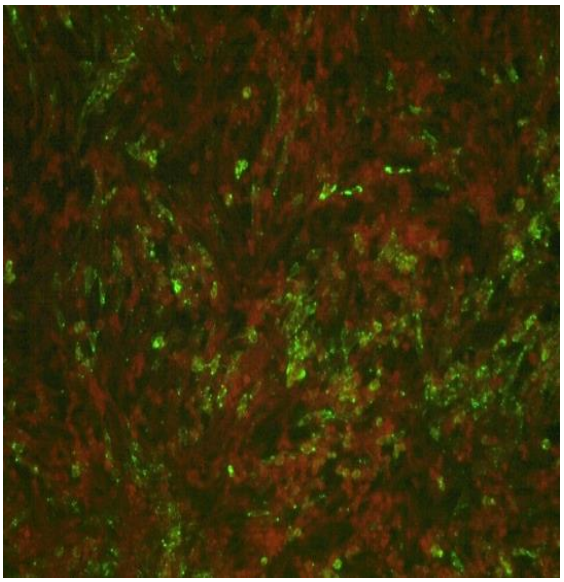

Fig. 3: Cell control (10X)

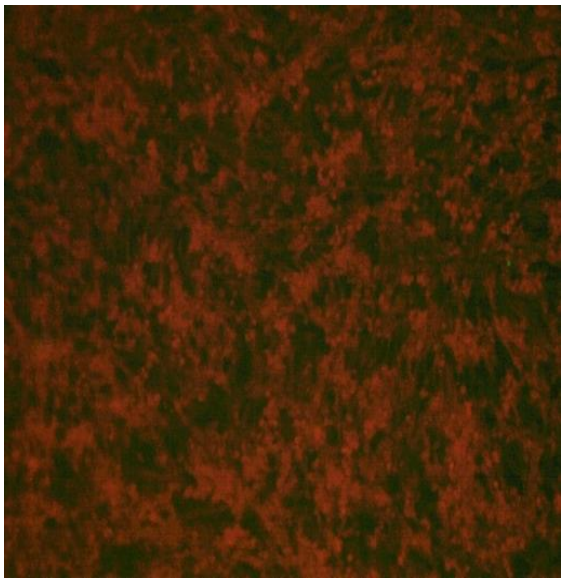

Fig. 4: Complete neutralization of virus at 1:8 dilution (10X)

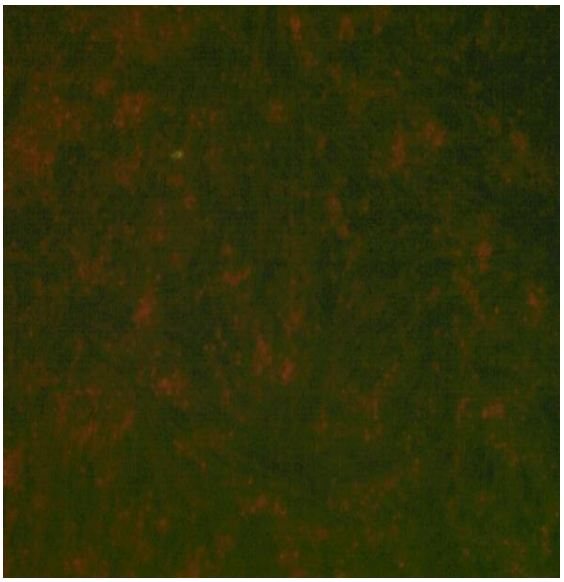

Fig. 5: Incomplete neutralization of virus at 1:16 dilution (10X)

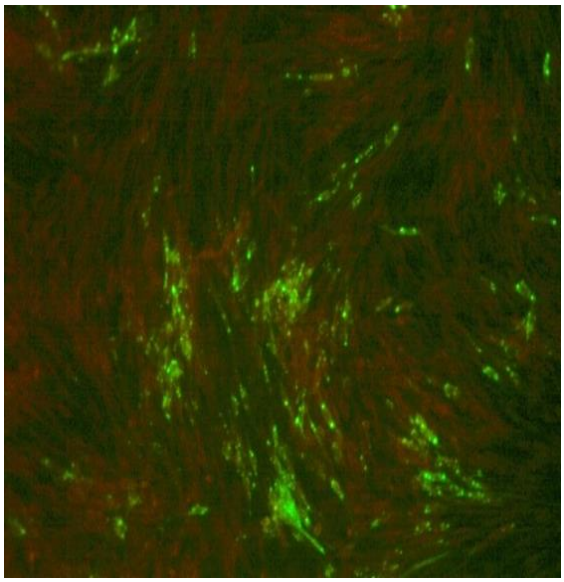

Fig. 6: Percentage of dogs with protective titre against rabies in South and North regions of Bengaluru

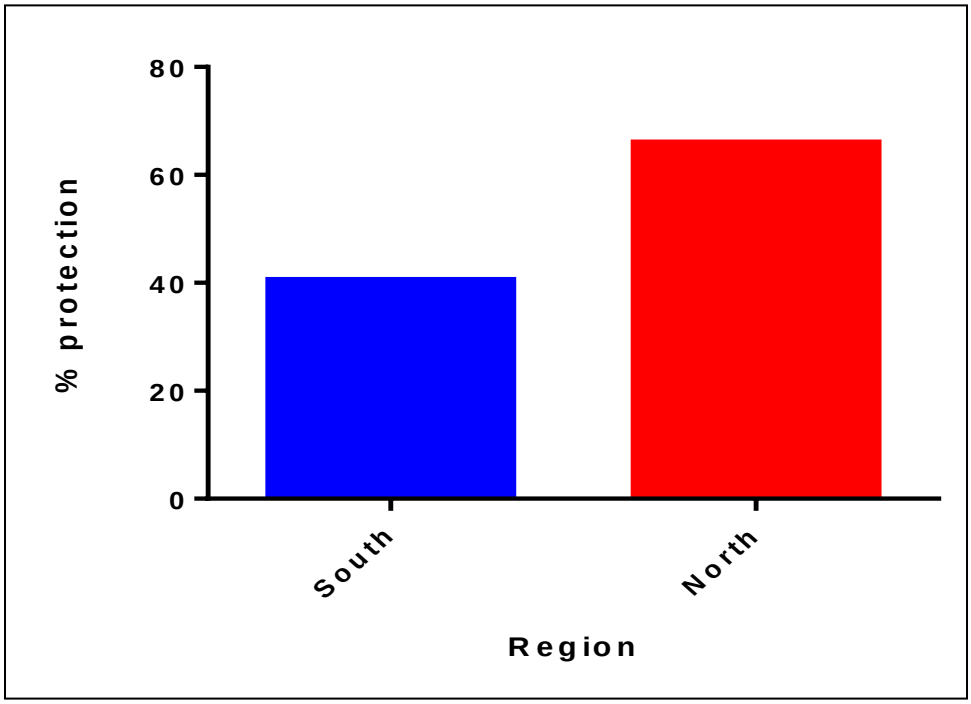

Fig. 7: Tukey Box and Whisker Diagram-Region wise level of protective titre against rabies in dogs

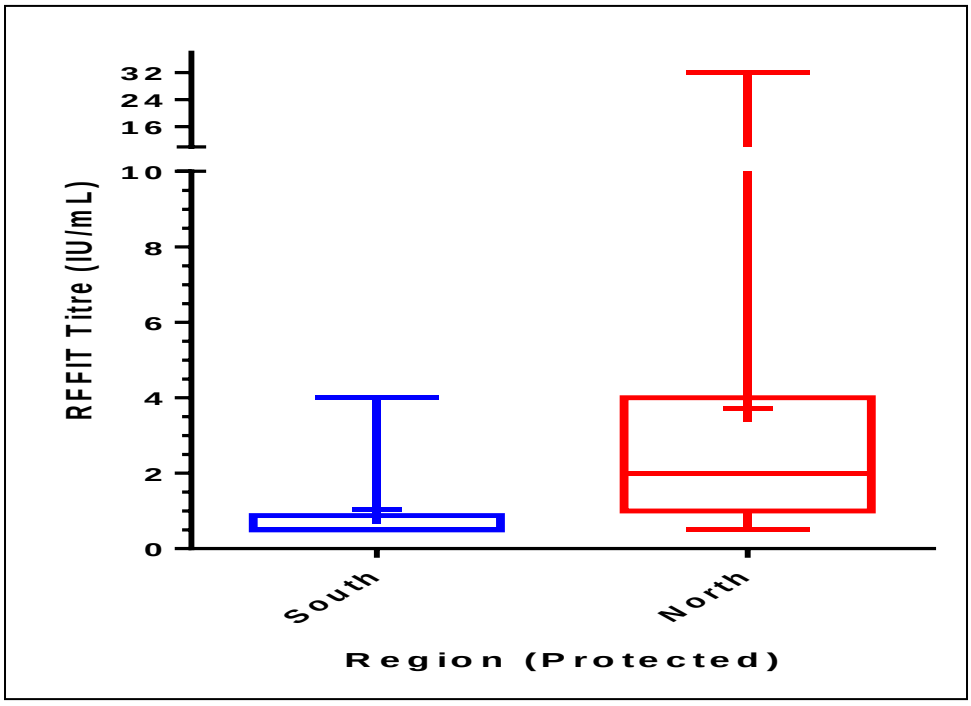

Fig. 8: Graph Showing Neutralising antibody titre versus PP values

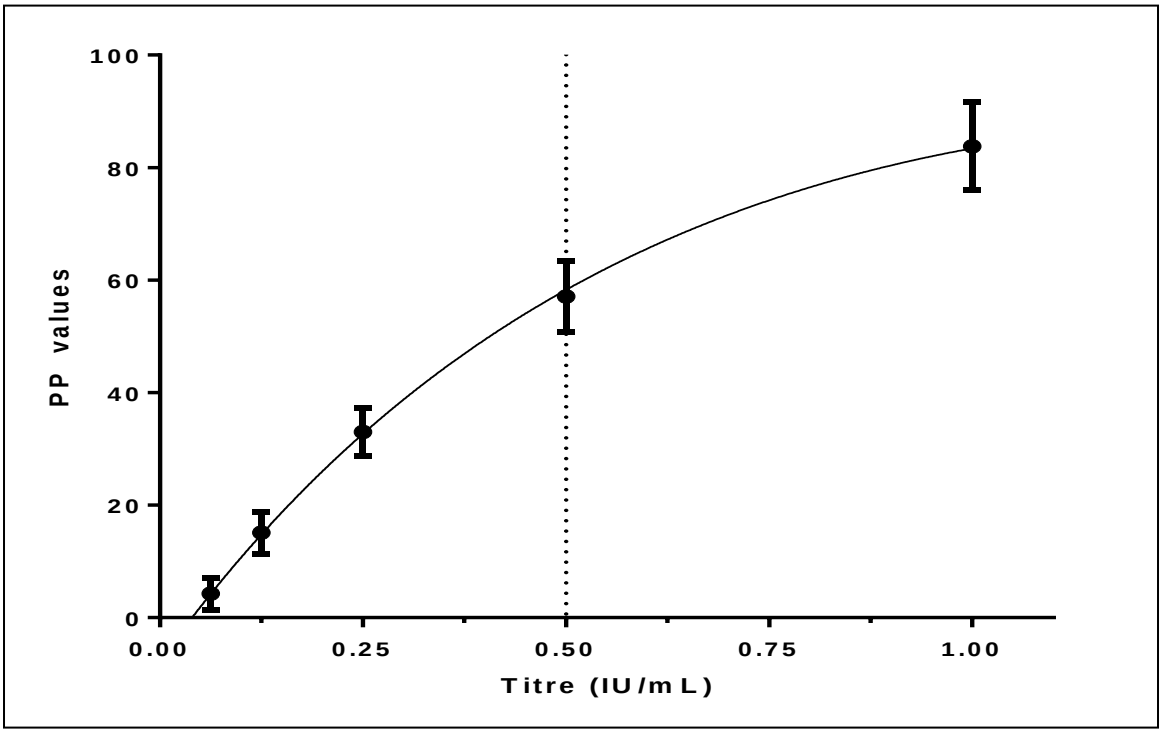

Fig. 9: Spearmen Rank Correlation for neutralising antibody titre(IU/ml) versus iELISA(IU/ml)

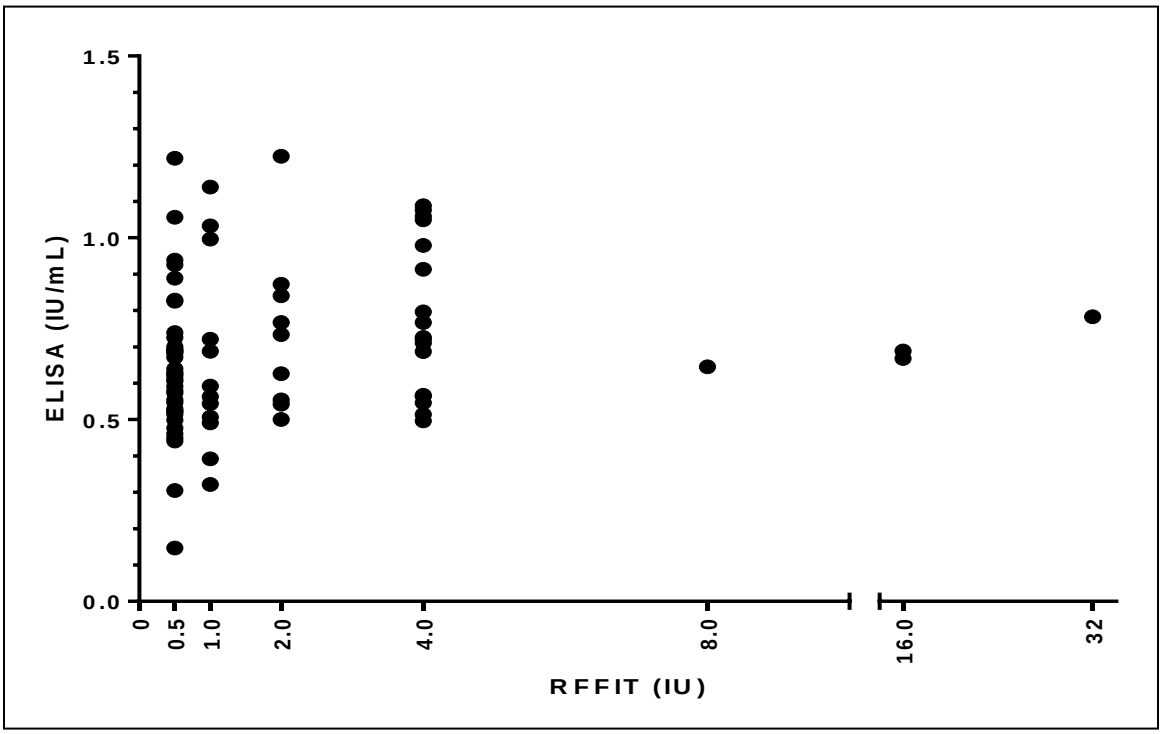

Fig.10 In- house iELISA plate for seromonitoring vaccinal antibodies against rabies

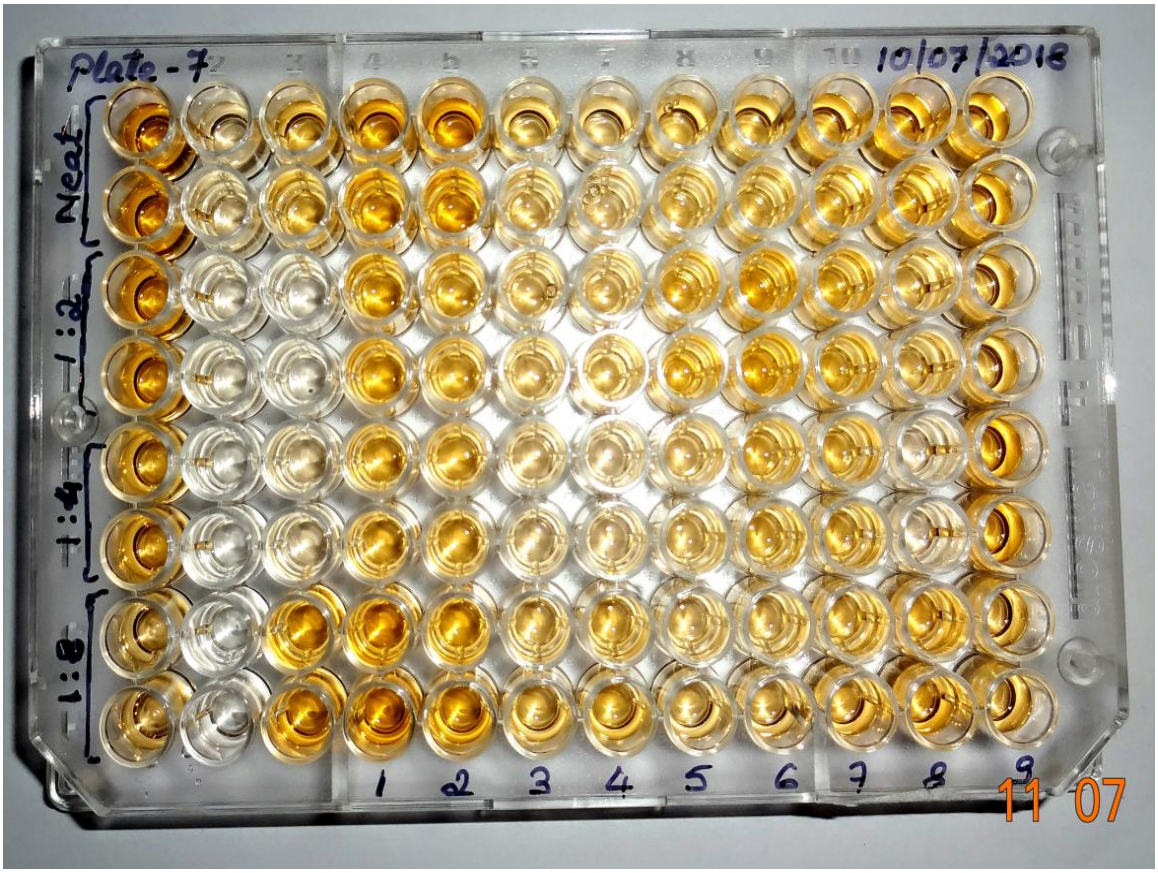

Table 9: Two sided Contingency table-Sensitivity and Specificity of iELISA

Test		RFFIT		Total	Sensitivity	Specificity	Kappa
		Positive	**Negative**				
G-protein iELISA	Positive	118	6	124	**(118/125)X100=94.4%**	**(119/125)X100 = 95.2%**	**0.89Perfectagreement**
	Negative	7	119	126			
Total		**125**	**125**	**245**			

Discussion

V. DISCUSSION

Rabies is an acute and fatal encephalitis caused by an RNA virus belonging to the genus *Lyssavirus* of the family *Rhabdoviridae*. As per World Health Organization (WHO), nearly 50,000 people worldwide die of the disease each year (WHO, 1992). Of all the rabies deaths reported worldwide, nearly 80 per cent is contributed by the South East Asian region and urban rabies continues to be a major threat in many countries (Acha and Arambulo, 1985). The incidence of rabies is particularly high in Bangladesh, Pakistan, and India (Sudarshan, 2004). Dog bite is the principal mode of infection in these countries. The dog population is nearly 37.5 million in South East Asia with an annual increase of 10%. In India, a national multicentre survey has revealed an annual incidence of 20,000 human deaths due to rabies and >95% fatal rabies cases are due to dog bite (Wilsmore *et al.,* 2006). A number of rabies cases are being reported in several cities including Bengaluru across the country.

In countries where the rabies is endemic, WHO recommends a preventive vaccination program for dogs (WHO, 2005). It is important to determine the level of anti rabies antibodies in animals to know the efficacy of control measures (Clark and Wilson, 1996). However, performance of these tests requires specialized reference laboratories with expertise. Recently, enzyme-linked immunosorbant assay (ELISA) kits have become available which detect the level of anti-nucleoprotein antibodies which are a good predictor of degree of humoral immunity after vaccination. These kits have been evaluated and found to have good sensitivity and specificity (Servat *et al.,* 2007; Shyamsunder *et al.,* 2014). Recently, G protein gene was cloned and expressed in *E.coli*

(Sharada, 2015) as well as baculovirus (Santosh, 2017) have been reported to have sensitivity and specificity of 90 per cent and 80 per cent respectively. Also, the test can be performed within a single day without the need of a sophisticated laboratory set up. The need to verify the effectiveness of rabies vaccination has become widespread in order to assess the efficacy of campaigns aimed at the eradication of the disease. Also, it is important particularly in the context of international trading of domestic carnivores from infected to rabies free territories. The WHO recommends that at least 70 per cent of the dog population should be immunized against rabies to minimize the risk of its re emergence (WHO, 2005**)**, and this recommendation is particularly important in urban areas of India with large free ranging population like Bengaluru. Furthermore, unvaccinated stray dogs may be a risk for the circulation of rabies and its transmission to humans. An understanding of rabies seroprevalence in free ranging/stray dogs is important for an accurate assessment of the risk of rabies in Bengaluru, but its seroprevalence was not surveyed earlier. Although many campaigns of mass rabies vaccination have been conducted in various parts of the world, there are only limited data regarding the serum antibody response in vaccinated populations of dogs (Blancou *et al.,* 1986; Haddad *et al.,* 1985; Koutchoukali *et al.,* 1985; Toma *et al.,* 1985) especially in countries where canine rabies is enzootic. The present study aimed to test for the presence of anti rabies vaccinal antibodies against the rabies virus in serum samples of free ranging in Bengaluru, India, in order to ascertain the immune status of the dog population.

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 and Kahn *et. al*., 2008). At present, only two neutralization tests FAVN or the RFFIT are gold standards and recommended for serological testing of pets intended for international trade or travel (European Commission, 2003). However, although they offer the most valuable and reliable way of assessing the efficacy of rabies vaccination by detecting neutralizing antibodies in serum samples, these methods are time-consuming, expensive, require highly trained technicians and special laboratory facilities including bio-containment while handling the live virus (Kahn *et. al*., 2008) and dependent on fluorescent microscope.

However, these virus neutralization tests are tedious and complicated to perform, making them unsuitable for large-scale sero-epidemiologic surveillance studies. To overcome this limitation, several ELISA have been developed for detecting antibodies to rabies virus, using monoclonal based techniques (Cliquet *et al.,* 2000). The ELISA has several advantages as it takes less time and is easy to use; it does not require highly trained persons. Poor quality sera can cause cytotoxicity in VNT, which could lead to false positive results. For such samples, the use of an indirect ELISA has been shown to be as sensitive and specific as VNT and do not require high containment facilities and produce rapid results (Servat *et al.,* 2007). Commercially, G protein based ELISA kit is available to assess the levels of vaccinal antibodies. This will help in rapid screening of serum samples from vaccinated animals but it is expensive. Considering this limitation, recently, a baculovirus expressed rabies virus G protein based indirect in-house ELISA has been standardized (Santosh, 2017). Application of both RFFIT and iELISA for determination of anti rabies antibody levels in representative vaccinated dogs may provide indication about efficacy of current vaccination programme.

5.1. Collection of serum samples

In all, 250 serum samples from free ranging population from 18 wards coming under north and south zones of Bengaluru were collected and tested by Rapid Fluorescent Focus Inhibition Test (RFFIT) and iELISA in OIE twinned KVAFSU-CVA-Crucell Rabies Diagnostic Laboratory, Department of Microbiology, Veterinary College, KVAFSU, Hebbal, Bengaluru. Previous researchers like Neelufer *et al.,* 2015 and Shyamsunder *et al.,* 2014 collected serum samples from vaccinated pet dogs in Bengaluru, Karnataka. In this unique study only free ranging dogs were covered which are responsible for dog bite menace and spreading of rabies.

5.2. Estimation of anti-rabies vaccinal antibodies by RFFIT

Rabies is hundred per cent fatal but can be prevented by vaccination. Neutralizing antibodies are known to be the most reliable indicator of successful vaccination (Clark and Wilson, 1996; Moore *et al*., 2015) in order to ensure satisfactory protection against rabies (Aubert, 1992; Brown *et al*., 2011). A vaccination to be valid when the neutralising antibody titre is equal or above 0.5 IU/ml in the serum of vaccinated animals and humans (OIE, 2012 and WHO, 2005).

The RFFIT procedure standardised by Neelufer, 2016 was used for assessing the neutralising antibodies in the serum samples. Of the 250 dogs serum samples collected from 18 different wards 125 serum samples were having antibody titre of equal to or above 0.5 IU/ml, indicating 50 per cent to have protective level of neutralising antibody. Similar results were reported by Watanabe *et al.,* 2013, who found that only 51.1 per cent of mass vaccinated dogs which received single shot of vaccine against rabies had

protective antibody titre against rabies. Babboni *et al*., 2014 reported that 54.7 per cent of the dogs maintained protective antibody levels against rabies. Pimburage *et al*., 2017 found that 59.58 per cent of free ranging dogs were having protective antibody titre by RFFIT. In the present study, we observed that only 50 per cent of the free ranging dogs had protective level of anti rabies antibodies. This is in spite of the fact that a number of dogs are being caught by the NGOs for sterilization and subsequent vaccination followed by their release into the community. Thus any bite by a free ranging dog in Bengaluru poses the risk of transmission of rabies virus if present in the saliva of dog. There are very few studies which highlight antibodies against rabies in house hold or free ranging dogs. Recently, a study from Japan showed that only 27.7 per cent of dogs had protective immune status (Ogawa *et al.*, 2009). A seroprevalence study from Bangkok, Thailand had reported the antibody prevalence of 49-86% in stray dogs (Kasempimolporn *et al.*, 2007). However, to the best of our knowledge, no study from India except that Singh *et al*. (2011) who reported a mere one per cent protective antibody levels in the street dogs vaccinated against rabies in Chandigarh.

5.2.1. Factors affecting RFFIT titre of vaccinated dogs

In present study, no effect was found on antibody response to rabies vaccination by gender. Study conducted by Mansfield *et al.* (2004) suggests that there is no effect of gender on immune response to rabies vaccination in dogs. Kennedy *et al*. (2007) demonstrated that gender of the dog does not have effect on immune response to anti rabies vaccination. Neelufer (2016) and Santosh (2017) found similar results.

5.3. Level of protection in different regions Bengaluru

In the present study, 250 serum samples were collected from North (n=97) and South (153) parts of Bengaluru where mass vaccination was undertaken by Sarvodaya Sevabhavi Samstha (North) and Animal Rights Fund and Compassion Unlimited Plus Action (South). In Northern part 65.97 percent of free ranging dogs were found protected against rabies whereas, it was 40.52 percent in Southern part of Bengaluru. Aiyedun and Olugasa, 2013 conducted similar survey of rabies antibody profile in Ilorin, Kwara state, Nigeria and found that a sero-prevalence rate of 53.70%, 43.60% and 25% for Ilorin East, West and South respectively. Possible reasons for difference in the protective percentage in different regions may be due to lack of consistent vaccination, vaccine keeping quality and vaccination failure and the brand of vaccine used. 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).

5.4. In-house iELISA for estimation of anti rabies vaccinal antibodies in free ranging dogs

Indirect ELISA was employed by different researchers worldwide for determination of anti-rabies antibody titre (Gangadhar, 1993; Cliquet *et al.*, 2004; Servat *et al*., 2007; Singh *et al*., 2011; Santosh, 2017). In the present study, an indigenously developed ELISA (Santosh, 2017) has been used for the quantification of anti rabies antibody titres in dogs. The average of all control ODs were plotted against corresponding neutralising antibody titres and a graph was plotted. Out of 250 serum

samples tested, 126 serum samples showed protective antibody titre equal or above 0.5 IU/ml which accounts for 50.4 per cent (Cut off value 57.09 or above) whereas, 124 (49.6 per cent) serum samples were having neutralising antibody titre less than 0.5 IU/ml (Table 8). The ELISA was employed in view of the ease, rapidity, safety in its application and its comparable sensitivity (94.4 %) and specificity (95.2 %) to the gold standard RFFIT (Table 9).

The major advantages of the ELISA test is that it can be done in a day, does not require the use of live virus and can be performed without the need for specialized laboratory containment. This is in contrast to 3 days required for conventional rabies antibody virus neutralization assays. Hence, ELISA is a valuable screening tool for detection of rabies antibody to know the vaccination status of animals.

Chomel *et al*., 1988 performed serological survey of mass vaccinated dogs (After 12 months) by random selection of samples. They observed 97 per cent of dogs had protective antibody titre that is more than 0.5 IU/ml. Later, Singh *et al.,* 2011 studied the prevalence of rabies antibodies in street dogs in Chandigarh, India by iELISA. They found that only one per cent of street dogs were having protective antibodies. Recently, Olugasa *et al*., 2011 also studied prevalence of antibody in Kwara State of Nigeria and reported that 23 out of 61 free roaming and 1 out of 13 stray dogs, were having protective antibody titre against rabies virus.

5.5 Comparison and evaluation of iELISA and Rapid Fluorescent Focus Inhibition Test (RFFIT) for estimation of anti rabies vaccinal antibodies in dog

Rapid Fluorescent Focus Inhibition Test is a cell culture based technique. RFFIT detects neutralizing antibodies and is an OIE/WHO recommended technique but in this technique live virus needs to be handled, BSL laboratory facilities are required and it makes the laboratory workers at risk. Also, it requires highly trained personnel and a minimum of 48 hours to arrive at results. In view of these, research has been directed to use a simple, safe and rapid technique for detection of anti rabies antibodies by ELISA.

In all, 250 serum samples were tested by both RFFIT and ELISA of which 125 were found to possess protective level of neutralising antibodies by RFFIT and 126 by iELISA. Subsequently, thirteen out of 250 discrepant samples which included seven samples positive by RFFIT but negative by the iELISA and six serum samples were negative by RFFIT but positive by ELISA (Table 9).

The sensitivity was about 94.4 and specificity was 100 per cent and kappa value was about 0.89 which indicated perfect agreement between the RFFIT and ELISA (Table 9).

Cliquet *et al*., 2003 reported that the sensitivity of ELISA versus FAVN was about 93 per cent and 73 per cent at different labs *viz*., Veterinary Laboratories Agency, UK and AgenceFrançaise de Sécurité Sanitaire des Aliments (AFSSA), Nancy respectively. The specificity they observed was about 97.3 per cent. Wasniewski *et al*., 2014 evaluated I ELISA (Platelia™) with FAVN as internal study between the laboratories. They found sensitivity of 78.2 per cent and specificity of 100 per cent.

Earlier researchers like Welch *et al*., 2009 attempted to evaluate indirect ELISA based on purified G protein of rabies with RFFIT for determination of human anti rabies virus antibodies. They observed sensitivity and specificity of 91.7 per cent and 73.0 per cent respectively. Santosh, 2017 found that sensitivity was about 90.56, specificity was 80 per cent and kappa value was about 0.611 which indicated good agreement between RFFIT and ELISA.

Even though ELISA measures a different set of characteristics of the rabies-specific immunogloblin response compared with neutralization assays and it is not directly comparable to neutralization assay results (Irie and Kawai, 2002), still, ELISA based on detection and titration of anti-glycoprotein antibodies may be a method of choice on par with RFFIT owing to some advantages like, it's safe handling (handling of live virus not required), does not require cell culture facility, independent of expensive fluorescent microscope, user friendly and rapid. It can be used as tool for seromonitoring of anti rabies antibodies in India, as rabies is endemic throughout the main land. Further attempts can be done to increase the specificity and sensitivity of indirect ELISA by using purified recombinant RABV-G protein produced.

Rabies is preventable through vaccination, public awareness, responsible ownership, sustained collaboration among stakeholders and elimination of stray dog population (Knobel *et al*, 2005). Although numerous studies have been conducted on rabies in India, comprehensive information on rabies control is mostly limited in the literature. This study is therefore a community-based investigation of rabies antibody profile to study the efficacy of vaccination. We determined that 50 percent of vaccinated

dogs did not exhibit protective levels of neutralising antibody titre within 1 year of vaccination, which indicates that vaccination is yet to be effective for establishing herd immunity.

A previous study also demonstrated that the neutralising antibody titre dropped to ≤0.5 IU/ml in a 50 per cent of dogs vaccinated within 120 days after a single vaccination (Minke *et al*., 2009). In the current study, however, the neutralising antibody levels in dogs that had received at least a single vaccination were estimated. The low prevalence of antibody against rabies observed in this study indicates lack of consistent vaccination programme and/or vaccination failure in some cases. Further, as a part of ABC programme, free ranging dogs of > 6 months are subjected to Catch Neuter Vaccinate and Release (CNVR) unlike in ownership dogs which are vaccinated at the 2.5 to 3 months age itself followed by a booster after 21 days to 1 month. However, the free ranging dogs are deprived of such boosters. The data analysis was done statistically and based on the findings of this study, the control of rabies in dogs through vaccination remains the only cost effective way to control rabies in dogs and to sustainably protect human from contracting the disease in Bengaluru.

The low seroprevalence in free ranging dogs could also be attributed to poor nutritional status of free ranging dogs. Furthermore, there is need for animal health authorities to utilize sero-surveillance for pre- exposure anti-rabies vaccination of dogs in Bengaluru, if the city is to achieve rabies control. Low vaccination coverage and ineffective management of stray dogs are the most likely reasons for the dog rabies elimination program's lack of success. Public-health authorities consider mass

vaccination of dogs the primary tool for control of rabies. Generally, stray or semi-owned dogs are captured only once for vaccination annually. WHO-coordinated research might bring about oral dog rabies vaccination as an adjunct to parenteral mass vaccination. This might facilitate rabies control where dogs are difficult to vaccinate by injection.

Developing a mass vaccination and sterilization campaign in Bengaluru city alone without considering neighbouring areas is unlikely to be successful. Translocation of dogs from neighbouring regions is also believed to have occurred and could be the source of infection. Furthermore, dogs vaccinated in the field may have an antibody titre lower than those in dogs vaccinated experimentally and kept under ideal conditions (Precausta *et al*., 1985). The mass vaccination campaign on war footing if conducted in a short period (1 month) allows to cover a higher percentage of dogs. Such vaccination levels must be sufficient to break dog-to-dog transmission cycles. Furthermore, without measuring immunogenicity (both humoral and cell mediated) in an animal following vaccination, it is difficult to consider whether an animal is well protected or not. Irrespective of this hypothesis, based on the results of our study, it is necessary to vaccinate puppies at an early stage with a booster at a suitable interval with annual boosters thereafter, in order to improve herd immunity. This could interrupt rabies transmission by both urban and sylvatic cycles. By this way vaccination could take the lead role in eradicating rabies from India. Additionally, focal vaccination campaigns are to be conducted promptly whenever cases of rabies appear in any neighbourhood.

The control of rabies in dogs through vaccination remains the only cost effective way to sustainably protect human from contacting the disease. This view is supported by

the findings of Beran and Frith (1988), Olugasa *et al*, (2011) who argue that vaccination of dogs and cats remains the most important strategy in rabies control and campaigns in Africa, while effective publicity, legislative and judicial support enhance adequate coverage of dog and cat population to reach 70-80 per cent epizootiological baseline.

In conclusion, the present study was the first comparative study conducted to investigate neutralising antibody titre development (humoral immunity) in free ranging dogs in Bengaluru after an anti-rabies vaccination. The outcome of the study revealed that sero prevalence achieved from the present vaccination campaigns in the city is low to protect the dog and human populations.

The low sero prevalence of antibody against rabies observed in this study may indicate a combination of factors including lack of stable anti rabies vaccination programmes for the majority of dogs and vaccination failures in some of the cases. Such low prevalence of antibodies favours large scale epizootic or focal outbreaks with an increased risk for humans. The results of this study suggests that the current annual rabies dog vaccination program must be reviewed and strengthened given that at least 70 percent of dogs are vaccinated each year. Therefore, the present may be the appropriate time period to consider updating the rabies prevention system.

There is a need to strategize the control activities which requires coordination among the regional Government and Non Government agencies. Also there is urgent need to have a regular census of dogs so as to tackle the menace of free ranging dogs on a war footing. Also more effective municipal licensing of pet dogs is needed and awareness campaigns about the vaccination schedule need to be carried out. Periodic surveys need

to be conducted to test the status of anti rabies IgG among dogs so as to ascertain the attainment of WHO protective levels. Community education efforts should address the importance of dog ownership and movement restriction and the need to vaccinate young dogs.

The mass vaccination of dogs against rabies is the most rational strategy for interrupting the natural transmission of rabies. Currently, monitoring and evaluation of dog vaccination exercises were lacking yet essential to achieving effective and efficient population sero conversion. Therefore, there is an increasing need for the concerned to utilise a combination of serosurveillance and modern geographic information systems to focus pre exposure anti rabies vaccination of dogs.

In India, 'Mission Rabies' programme implemented in Goa and Chattisgarh states for mass vaccination of dogs is reported to be successful. Mass vaccination in these two states has now stopped the chain of transmission of the disease. This observation justifies the need for regular rabies vaccination campaigns in other states as well to prevent the spread of rabies. Knowledge of the neutralising antibody level against rabies virus is required to evaluate herd immunity of dogs in mass vaccination campaigns (Manalo *et al.*, 2017). The 50 per cent of vaccinated street dogs which are having protective titre of antibodies is not enough to protect against rabies. Additional seroprevalence surveys should be conducted on previously vaccinated free ranging dog population from other geographical areas to determine the actual state of rabies immunity in the dog population.

The present study will be useful in formulating the anti rabies vaccine strategies for dogs and also provide an impetus among people to take initiative to launch the

awareness against rabies vaccination and ultimately control and eliminate ‘Canine mediated rabies in India by 2030’.

Summary

VI. SUMMARY

The present study was carried out with an objective to evaluate the anti rabies vaccinal efficacy in free ranging dog population in two different zones of Bengaluru using Rapid Fluorescent Focus Inhibition Test (RFFIT) and to evaluate an indirect Enzyme Linked Immunosorbant Assay (ELISA) with RFFIT for estimating anti rabies vaccinal antibodies.

This study was aimed to check the efficiency of current anti rabies vaccination programmes conducted by Non-Governmental Organizations like Sarvodaya Sevabhavi Samstha, Animal Rights Fund and Compassion Unlimited Plus Action in different zones of Bengaluru.

Samples were collected from 18 wards of Bengaluru North and South with the help of NGOs who were vaccinating in respective area. A total of 250 serum samples were collected during the study period (January to June 2018) from free ranging vaccinated dogs. In this study, post vaccinal seroconversion in free ranging dogs were assessed by targeting neutralizing antibodies to find out the reach of ongoing rabies control programme in Bengaluru. Currently available tests for assessing anti rabies vaccinal antibodies are RFFIT and FAVN. Since both these tests are expensive, time consuming and require special laboratory facilities, ELISA which is devoid of all these disadvantages is recently replaced these tests. Thus, in the present study a recombinant rabies glycoprotein based ELISA was evaluated against the reference test RFFIT.

Serum samples collected were tested for anti rabies vaccinal antibodies using RFFIT and it was found that 50 per cent of the tested free ranging dogs were having protective titre against rabies. Further, the samples from North zone of Bengaluru showed a better seroconvesion (65.97%) compared to that of the South zone. However, both the zones had not attained the WHO prescribed 70 per cent epizootiological baseline of herd immunity in a population. Sarvodaya Sevabhavi Samstha was the NGO that had undertaken vaccination in North zone which showed the highest percentage of seroconversion in the present study. Whereas samples from Compassion Unlimited Plus Action (20.28%) and Animal Rights Fund (57.14%) showed a lesser seroconversion suggesting a lower level of protection against rabies in South zone of Bengaluru.

An ELISA developed and standardized by Santhosh (2017) was evaluated by comparing it with the standard RFFIT results. By iELISA, 50.4 per cent serum samples showed per cent positivity (PP) of more than 57.09 cut off value corresponding to 0.5IU/ml of RFFIT titre. Specificity and sensitivity of iELISA was found to be as high as 95.2 per cent and 94.4 per cent respectively. Results were analysed statistically and the kappa value (>0.80) suggested a perfect agreement between results of iELISA and RFFIT. This suggests the use of the iELISA developed, owing to its ease of handling and cost effectiveness than the currently available techniques. This test can be adopted for further seromonitoring studies on a larger scale. Similar survey need to be carried out in other cities of India for obtaining a true picture of the effectiveness of rabies control programmes in India. Regular vaccination followed by serosurveys is the need for effective control of rabies in free ranging dogs.

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Abstract

VIII. ABSTRACT

The present study was undertaken to evaluate the anti rabies vaccinal efficacy in free ranging dog population in Bengaluru and comparison of an iELISA with RFFIT. Serum samples from 250 free ranging dogs were collected for the study and tested by RFFIT as well as iELISA. In all, 18 wards from the North and South zones of Bengaluru were covered during the sample collection with the help of three NGOs, who claimed that those animals were annually vaccinated. So, the post vaccinal efficacy was studied and it was found that, out of 250 dogs 125 were having a protective anti rabies antibody titre by RFFIT accounting for 50 per cent of seroconversion. Samples from North zone were having a better seroconversion level (65.97%) than south zone of Bengaluru. By iELISA, 126 dogs showed a per cent positivity (PP) more than 57.09 (cut off) accounting for 50.4 per cent of post vaccinal seroconversion. A kappa value of >0.80 suggested a perfect agreement between the results of RFFIT and iELISA. The sensitivity and specificity of iELISA was found to be 94.4 per cent and 95.2 per cent respectively. This ELISA can be used in large population surveys instead of RFFIT to overcome the disadvantages associated with it. The present study emphases regular rabies vaccination followed by seromonitoring of free ranging dogs in Bengaluru.