Study on Differences Between Rabies Serology Methods Affect Cut-Off Values for Determination of Adequate Vaccine Response

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ABSTRACT

Vaccine equivalency, booster administration, and animal import decisions are based in part on the level of rabies virus neutralizing antibody (RVNA) in serum. The RVNA level 0.5 IU/mL is recognized by the World Health Organization to represent an adequate response to vaccination. This cut-off value was selected after expert review of serum neutralization testing (SN) of clinical trial samples. Other methods are currently or can be employed to measure rabies vaccine response. Commonly the same cut-off value, 0.5 IU/mL, is applied to provide information on which medical decisions are made. Studies have shown that although enzyme-linked immunosorbent assay (ELISA) and SN results are correlated, exact comparison cannot be ensured.

This study investigated whether use of the 0.5 IU/mL cut-off value can be interchangeably used between different methods to provide the same vaccine response interpretation. Serum from rabies vaccinated subjects grouped by vaccine and vaccination regimen type were collected on days 0, 14, 30, and 90. The serum samples were tested by both SN and ELISA methods. At each time-point, the percentage of subjects producing rabies virus neutralizing antibody (RVNA) or anti-rabies antibodies above an assigned cut-off as well as the individual result values were compared between groups. Using either the SN or the ELISA results to compare vaccine-type groups for all time-points produced similar vaccine equivalency conclusions, but the comparison of the vaccination-regimen groups produced different conclusions dependent on test method used. Overall, the greatest difference between test method results was from samples collected at day 14 and day 30.

Directly comparing SN to ELISA results of clinical trial samples and covering a time-point range encompassing the development and maturation of the humoral immune response, provides information on the kinetics of the rabies antibody response as defined by test method. Because SN methods measure neutralizing function and ELISA methods the binding function of rabies antibodies, the results are not expected to be equal; thus the cut-off values should be independently determined, not extrapolated between different methods.

Keywords: RVNA, SN, ELISA, Serum, World Health Organization.

1.0 Introduction

There are several different laboratory methods for detection and measurement of the rabies antibodies, yet the 0.5 IU/mL level is the one globally recognized marker of adequate response to vaccination in humans (World Health Organization, 2012). Often forgotten are the circumstances surrounding the origin of this determination. The World Health Organization (WHO) Expert Committee on Rabies (from the 3rd/1957 through the 6th/1973 reports) states that the vaccination response should be verified in the serum one month after vaccination by detection of RVNA, but no specific antibody level is specified. A Working Group convened during the 1978 Joint WHO/International Association of Biological Standardization

(IABS) symposium defined a cut-off value after review of RVNA levels obtained from several clinical trial studies for the newly developed cell culture rabies vaccines. The conclusion was presented "that the serum be tested four weeks after the last inoculation and at that time a minimum value of 0.5 IU per ml be attained to demonstrate seroconversion. In the WHO Expert Committee on Rabies Report from 1984, the 0.5 IU/mL cut-off value is described as the level expected one month after vaccination; and that booster vaccinations are required until that level is reached (World Health Organization, 1984). The key points are that the 0.5 IU/mL antibody level was determined based on results from serum neutralization methods (Mouse Neutralization Test or MNT and Rapid Fluorescent Focus Inhibition Test or RFFIT), was for specific time point (one month after vaccination), and was for a specified purpose (demonstrate seroconversion). To extrapolate this value to other methods, time points, and purposes is not supported without further investigation. In addition, referring to the level as protective is a misinterpretation of the original intent.

SN and ELISA rabies serology methods have been compared many times and the findings are similar: correlation is fair to good. This is not surprising as both methods are quantitating, in different ways, the specific rabies antibody response to rabies vaccination. The reasons SN and ELISA results cannot be considered statistically comparable for all samples are: the methods measure different characteristics of rabies antibodies (neutralizing function for SN and binding function for ELISA); and the normal antibody response to rabies vaccination is polyclonal, producing immunoglobulins with various epitope specificities, affinities, Ig subclasses, and neutralizing abilities--a unique polyclonal response per individual. This means the relationship of binding antibody measurement to neutralizing function will be variable between individuals. Similar issues have been described for discordant estimates of seroprevalence to mumps and measles using SN and ELISA methods.

This study investigated whether 0.5 Equivalent Units per milliliter (EU/mL) or another logical cut-off level could be determined by evaluation of the response as measured by ELISA using clinical trials samples (over set time points), analogous to how the 0.5 IU/mL adequate response level was established for SN methods.

The objectives of this study were to characterize the kinetics of the rabies antibody response to vaccination as defined by an ELISA method that detects anti-rabies glycoprotein IgG; to evaluate the ELISA method "adequate response" level by comparing antibody measurement units (EU/mL for ELISA versus IU/mL for SN); and to determine the degree of agreement between ELISA results and SN results for evaluation of vaccines or vaccine regimens for individual and group response.

2.0 LITERATURE SURVEY

Catherine M. Sturgeon (2011) said because of this vulnerability, immunoassays are still used in clinical settings because of the potential for human error. Infrequent errors that occur because of the specimen's characteristics are particularly difficult to find. Cross-reacting substances, anti-analyte antibodies, and anti-reagent antibodies all have the potential to produce falsely high or low results, depending on the situation. High-dose hooking in the presence of extremely high analyte concentrations can lead to poor tumor marker results. Any specimen can give false results without warning, and there is no practical way to identify specimens that are more likely to cause issues during immunoassays. When results don't match the clinical picture, look for signs of interference. If an error occurs, it should be investigated as soon as possible because even the best-run laboratories can make mistakes. Staff at the clinic should be encouraged to contact laboratories with any questions about the results they have. Most laboratories have the ability to conduct investigations into possible interference, including tests for linearity on dilution,

recovery experiments, treatment with heterophilic blocking tubes, and confirmation using a different method. A specialist laboratory may be consulted if more in-depth studies are required. The key to minimizing the risk of clinical mismanagement due to undetected interference is to inform clinical and laboratory staff of the ever-present possibility of interference and to ensure that laboratory staff has access to brief clinical details.

Moore, Susan M. (2005) examined the relationship between the rabies vaccine viral seed strain and the challenge virus strain used to measure anti-rabies antibody levels after vaccination. A homologous and a heterologous testing system was used by RFFIT to compare serum samples from 173 subjects who had received either a purified Vero cell rabies vaccine (PVRV) made from the Pittman Moore (PM) seed strain of the rabies virus or a purified chick embryo cell rabies vaccine (PCECV) made from the Flury low egg passage (Flury-LEP) seed strain of the rabies virus. It was found that in the homologous system, subjects vaccinated with PVRV had a higher humoral immune response when challenged with CVS-11, compared to those vaccinated with PCECV, who responded better to Flury-LEP. The challenge virus in the assay for subjects vaccinated with PCECV was CVS-11, and Flury-LEP was used for PVRV-vaccinated subjects in the heterologous system. Despite the fact that the difference between the CVS-11 and Flury-LEP rabies virus strains in terms of G protein homology is only 5.8%, the use of a homologous testing system led to approximately 30% higher titers for nearly two-thirds of the samples from both vaccine groups when compared to a heterologous system. Using homologous or heterologous testing systems to assess the level of rabies virus-neutralizing antibodies allowed us to compare the immune responses after vaccination with the two different vaccines. Using a homologous testing system led to vaccine equivalence, but using a heterologous testing system failed to produce the same results. According to the findings of this study, the virus strain used in biological assays to measure the level of anti-rabies virus antibodies after vaccination could have a significant impact on the evaluation of rabies vaccines.

Kader Muhamuda (2007) estimated the level of neutralizing antibodies after rabies vaccination, three tests have been approved: the mouse neutralization test (MNT), the rapid fluorescent focus inhibition test (RFFIT), and the fluorescent antibody virus neutralization test (FAVN). These tests necessitate a high level of expertise and are typically only performed in specialized reference laboratories, making them inaccessible to the general public. Developing and evaluating a competitive ELISA test (C-ELISA) for neutralizing antibody estimation in order to make this test more widely available was the goal of this study. Rabies vaccines that are both effective and safe, as well as affordable, are still being sought. Vaccines against the avian paramyxovirus that causes Newcastle disease (NDV) have shown promise. This strain of NDV La Sota was engineered to express the rabies virus glycoprotein (RVG), and its efficacy as a rabies vaccine was assessed. Titers of the recombinant virus rL-RVG in allantoic fluid reached 1098 50 percent egg infective doses (EID50)/ml. A virus-like ability to spread was achieved by RVG expression, and RVG was found on the viral particle's surface. Adding RVG didn't affect the NDV vector's ability to infect mammalian cells with trypsin. A neutralization antibody against NDV was sensitive to rL-RVG and La Sota NDV, while a neutralization antibody against the rabies virus was resistant to it. Numerous species of animals, including cats and dogs, were given high doses of the recombinant vaccine rL-RVG without any adverse effects. After receiving an intramuscular dose of rL-RVG, the mice developed a strong anti-rabies virus antibody response and were completely protected against infection with rabies virus strains currently in circulation. Of primary importance, rL-RVG produced protective neutralization antibody responses against the rabies virus in dogs and cats that lasted for an extended period of time. For over a year, a low vaccine dose of 1083 EID50 provided complete protection against challenges with a

circulating rabies virus. Using an NDV-vectored vaccine, this study is the first to show that recipients develop long-lasting, systemic immunity against rabies.

Jinying Ge (2013) said that in order to make an accurate rabies diagnosis before a person dies, pathologists must use techniques that are fast, accurate, and sensitive. Molecular amplification procedures, in vitro virus isolation, immunological methods, and histochemical analysis are all used in conjunction to try to figure out what's wrong with the patient. Once thought to be fatal as soon as symptoms appeared, modern medicine now offers potentially life-saving treatment for this disease. Medical intervention efforts, on the other hand, necessitate an early and accurate diagnosis of the clinical disease. It is possible to perform IFA testing on cerebrospinal fluid and serum samples in a short period of time, but this has not yet been thoroughly investigated. The specificity of this assay must be understood because false-positive IFA results can have a significant impact on patient treatment and outcomes. 135 samples of cerebrospinal fluid and serum from patients with viral encephalitis or a suspected viral infection caused by an agent other than the rabies virus were tested for immunofluorescence activity (IFA). According to the findings, the rabies IFA test can produce false-positive results. Seven of the 135 cerebrospinal fluid samples tested showed staining patterns that were morphologically similar to antirabies staining. There were also immunoglobulins bound to cell culture substrate antigens in the majority of cerebrospinal fluid specimens from encephalitis patients. Cross-reactive antibodies were frequently found in encephalitis cases linked to the West Nile and Powassan flaviviruses, which raised serious safety concerns. To avoid false positives in rabies tests performed on human samples, IFA should not be the sole basis for initiating antirabies treatment.

Matthew Reed (2004) said that viral encephalitis that can be fatal, rabies is caused by viruses of the Lyssavirus genus. A bite from a canine carrying the classical rabies virus usually results in infection. Contact with bats, on the other hand, causes a small number of cases. The majority of lyssavirus genotype variants, also known as strains, are found in bats. Geographical distribution and species exclusivity are common features of lyssaviruses, which are only found in bats. Most of them have been linked to human rabies and, in some cases, to domestic animals that have been exposed to them. Many diagnostic techniques fail to distinguish the rabies virus from other genotypes. This means that some human and animal cases may go unreported. On top of all of that, current vaccines are only partially effective against specific genotypes.

Johnson, N. (2004) said that Viral encephalitis of the Family Rhabdoviridae, Genus Lyssavirus, causes rabies, an acute, progressive, and fatal disease. The rabies virus stands for the entire family. Warm-blooded vertebrates can be experimentally infected, but bats and mammalian carnivores are the main primary hosts for disease perpetuation. Among the important wild carnivores are foxes, raccoons, skunks, and mongooses, among others. The dog is the global reservoir. Due to a variety of factors, including economics, ecology, and ethical considerations, traditional rabies control methods such as population reduction programmes supported by the government have failed. Immunizing domestic dogs with traditional veterinary vaccines administered intravenously, on the other hand, was responsible for the worldwide abolition of dog-transmitted rabies. The idea of wildlife vaccination was conceived in the 1960s based on the basic concept of applied herd immunity, and modified-live rabies viruses were used in the 1970s for the experimental oral vaccination of carnivores. The first field tests for rabies virus vaccines using attractive baits were conducted in Switzerland in 1978 as a result of the development of safe and effective vaccines. As a result, vaccine quality and production have improved thanks to advances in

recombinant virus design and production, as well as the ease with which millions of edible baits can be distributed across vast geographic areas. Rabies has all but vanished from Western Europe as a result of widespread oral vaccination programmes targeting the red fox that used hand and aerial distribution of vaccine-laden baits. In southern Ontario, the same startling discovery was made. Oral vaccination programmes targeting raccoons, grey foxes, and coyotes were also successful in the United States in the 1990s.

Blanton JD (2007) said that in 2006, the CDC received 6,940 reports of rabies in animals and three reports of rabies in humans, an 8.2 percent increase from the 5,417 animal cases and one human case reported in 2005 from 49 states and Puerto Rico. Approximately 92% of the cases involved wildlife, while only 8% involved pets. The following are the relative contributions made by the various animal species: a total of 2,615 raccoons (37%), 1,692 bats (24%), 1,494 skunks (21.5%), 427 foxes (6.2%), 318 cats (4.6%), 82 cattle (1.2%), and 79 dogs were rounded up (1.1 percent). Cases in 2006 increased across all groups except cattle when compared to 2005's reported numbers. Eleven of the twenty eastern states where raccoon rabies was enzootic reported an increase in the number of rabid raccoons in 2006 and reported cases increased by 3.2% overall compared to 2005. The number of rabies cases in skunks across the country increased by 6.1% from 2005 to 2006. Texas once again had the most rabid skunks (n = 351) and the most rabies cases per capita in the entire state (889). Rabies cases linked to the dog/coyote rabies virus variant have not been documented. An outbreak of this canine rabies virus variant was discovered near the US/Mexico border in March 2004. The fact that the dog/coyote rabies virus variant has not been detected in the United States for the second year in a row suggests that the virus is no longer in circulation, according to surveillance data. Rabies cases in foxes have increased by 13.6% since 2005, according to the CDC. This is because there have been more foxes diagnosed with the rabies virus variants specific to Arctic foxes (Arctic Fox Rabies) in Alaska, Texas Gray Fox Rabies (Texas Gray Fox Rabies), and Virginia Raccoon Rabies (Virginia Raccoon Rabies). Bat rabies cases increased by 14.5% from 2005 to 1,692, making them the second most commonly reported rabid animal after raccoons, with a total of 1,692 bat rabies cases reported. While cattle rabies cases decreased by 11.8 percent, cat and dog rabies cases increased by 18.2 percent. Rabies cases in horses and mules also rose. Rabies cases in sheep and goats also rose. Rabies cases in mongooses have increased by 9.2% in Puerto Rico, while rabies in domestic animals has increased by 20% as a result of spillover infection from mongooses. In 2006, rabies in humans was found in three different states: Texas, Indiana, and California. Bat rabies virus variants were found in Indiana and Texas, while a dog in the Philippines was to blame for the case in California.

Jesse D Blanton (2014) said that as a result of their wide geographic distribution, high rabies virus susceptibility, and propensity to live in areas near human habitations and domestic animals, skunks are an important rabies vector species in North America. Oral vaccination is a cost-effective and socially acceptable method of controlling rabies in terrestrial wildlife. However, controlling rabies in skunks has proven particularly difficult due to the lack of an oral vaccine for this species. In this study, we examined the striped skunk's (Mephitis mephitis) antibody response to ONRAB (®) and tested the vaccine's protection against the rabies virus. Three-hundred-and-twenty-one onrus were given ONRAB (®) vaccine bait, twenty-five onrus were given ONRAB (®) by direct instillation into the oral cavity (DIOC), and ten onrus were not given any kind of ONRAB vaccine. Six weeks before the treatment, a blood sample was taken from controls and vaccines, and then again five and seven weeks after the vaccination (PV). The rabies antibody was found using a competitive ELISA (RAb). All skunks' pre-vaccination sera were negative for RAb, as were the sera of all other controls in the serology study. Detectable RAb was found

in 58% (18/31) of the baited skunks and 100% (25/25) of the ONRAB (®) DIOC-treated skunks at 7 weeks post-vaccination (PV). The rabies infection killed all ten of the controls. The 247-day PV challenge was successfully completed by 100% (23/23) of the skunks given ONRAB (®) DIOC. Skunks exposed to ONRAB (®) baits survived at a rate of 81% (25/31). All 18 skunks in the bait group had detectable RAb at 7 weeks post-partum and survived the challenge. Seven more skunks that had no detectable RAb by PV week 7 survived as well. ONRAB® is a safe and effective oral rabies vaccine for skunks based on the results of serology and challenge tests, which show no notable pathology in study animals.

Susan M. Moore (2017) said that Even though humoral immune effectors receive more attention when it comes to rabies prevention, both cell-mediated and humoral immune effectors are important when it comes to fighting rabies infection. Oral rabies vaccination (ORV) of wildlife reservoir populations has been used for decades to control the spread of the rabies virus among wild hosts. Rabies antibody levels (and assays to measure them) that confer resistance to the rabies virus are still hotly debated. Our unpublished animal studies on the induction of rabies binding and neutralizing antibodies following oral immunization of animals with live attenuated or recombinant rabies vaccines are examined in this paper as correlates of protection against lethal rabies infection in captive challenge settings, using data from published literature and our own. Studies conducted by us show that even though serum neutralization test results are expected to reflect in vivo protection, day 28 ELISA results were a better predictor of survival than serum neutralization test results on day 14. Due to the inherent standardization of the kit format, ELISA kits may offer an advantage in terms of greater precision and the ability to compare results across different studies and laboratories. Oral baiting monitoring serology results are analyzed in this paper to provide useful guidance for future research.

Susannah Gold (2020) said that for thousands of years, people have been afraid of rabies because of the possibility of contracting the disease from a rabid dog. The fear of rabies stems in part from its reputation as a life-ending disease that can strike any mammalian species. There have been numerous challenges to the widely held belief that exposure to the rabies virus is always fatal. Despite the fact that only a few cases of human survival after a clinical infection have been documented, several studies have found rabies-specific antibodies in the sera of apparently healthy humans, domestic animals, and wildlife that have not been vaccinated. These seropositive people may have been exposed to the rabies virus but have not developed a fatal disease as a result of it. Although the methods used to detect these antibodies vary widely, and serology tests can be difficult to interpret, this has left a hazy picture of their significance. Nonlethal rabies exposure is defined in this review as exposure that does not result in death, and the possible consequences for rabies epidemiology are discussed. Despite the overwhelming evidence that nonlethal rabies exposure does occur, our findings show that studies involving serology that do not make use of appropriate controls and cutoffs will be unable to accurately estimate the true prevalence of nonlethal rabies exposure in humans.

Inna G. Ovsyannikov (2017) said that the population's antibody response to the measles vaccine varies widely. The inter-individual variation in antibody responses is influenced by host genes. Human leukocyte antigen (HLA) molecules are known to interact with killer cell immunoglobulin-like receptors (KIR), which may influence humoral immune responses to viral antigens. We conducted a large population-based study in 2,506 healthy immunized subjects (ages 11 to 41 years) to identify HLA and KIR associations with measles vaccine-induced neutralizing antibodies to build on and improve our previous work with HLA genes and explore the genetic contribution of KIR genes to inter-individual variability in measles

vaccine-induced antibody responses. Both HLA and KIR loci had no statistically significant associations after accounting for the numerous statistical tests of allele effects on measles-specific neutralizing antibody titers. The B*57*02 and DQB*06*02 alleles have been shown to be associated, but more research is needed in other cohorts to confirm these findings. According to the results, the B*57:01 allele (1,040 mIU/mL; p = 0.0002) was associated with a lower measles antibody titer. Alleles such as DQB1*06:02 (1,349 mIU/mL; *15:05 (2,547 mIU/mL; *4*) and DRB1*15:04 (4*) showed a link to greater levels of measles antibodies. There were strong non-significant associations with KIR genotypes, suggesting that copy number and haplotypes at KIR loci are unlikely to play a significant role in antibody response to measles vaccination. These results add to our understanding of how measles vaccine-induced immunity is mediated by HLA and KIR alleles.

Wenbo Wang (2019) said that Rabies vaccines' effectiveness could be affected by differences in street rabies virus (RABV) genetics or antigenicity. Glycoprotein (G) levels in street isolates should be monitored in the future. We retrieved and analyzed every single RABVG sequence available in the public database. The reactivities of 99 naturally occurring mutants to well-characterized neutralizing monoclonal antibodies (mAbs) and vaccine-induced antisera were examined by means of a pseudovirus system. When comparing vaccine and street isolate samples, researchers discovered a discrepancy in the G sequence of the latter, with mutant strains showing resistance to neutralizing mAbs and vaccine-induced antibodies. The majority of the antigenic variants have emerged since 2010 in a wide range of animal hosts and geographical locations. Antigenic variants have multiplied recently, necessitating more stringent surveillance of street isolates.

Eason, William (2018) says that there are lyssaviruses, most commonly the rabies virus, that cause rabies, an often-fatal encephalitic disease in humans and animals (RABV). Antibodies, antigens (proteins), and nucleic acids can all be detected in human antemortem samples and tests for rabies (genomic RNA). Neutralizing or binding antibodies in the cerebrospinal fluid (CSF) or serum samples from unimmunized individuals without prior rabies vaccination or passive immunization with purified immunoglobulins are used in the serological diagnosis of human rabies in the United States. All RABV-expressed proteins can elicit neutralizing antibodies, but these are primarily directed against the surface-expressed glycoprotein (G protein), while antibodies that bind to viral antigens target the nucleoprotein (N protein). We developed an enzyme-linked immunosorbent assay (ELISA) using purified recombinant E. coli N protein to determine CSF and serum N protein-specific antibody responses during RABV infection. In the CSF and serum of previously diagnosed human rabies cases, protein-specific IgG and IgM subtypes were found. Individual rabies cases had anti-N protein seroconversion throughout their illness, as well. As the current binding antibody assay is used in diagnosis, we compared the N protein ELISA results to those of an indirect fluorescent antibody (IFA) test. The results showed that our ELISA is in line with the IFA test. The N protein ELISA had a sensitivity and specificity range of 78.38-100 percent and a range of 75.76-96.77 percent based on the IFA findings. According to our findings, an N protein ELISA can be used to detect RABV-specific IgG and/or IgM antibodies in human CSF or serum.

According to a woman's perspective (2018), infected animals can carry the virus and infect humans, which causes an encephalitic disease. The prototypical member of the lyssavirus genus, rabies virus (RABV), is the most common lyssavirus that infects humans. A few weeks can sometimes turn into several months when it comes to the human incubation period for RABV. In the prodromal stage, no antibodies or viruses are present. Only after the onset of encephalitic symptoms can antibodies, antigens, and nucleic acids be

detected, and at that point, the disease is nearly 100% fatal. Tests on animals suspected of being infected with rabies have thus been used as the primary intervention in human RABV infection prevention and subsequent post-infection prophylaxis. Antigen detection in brain tissues of animals is the most widely used diagnostic test for the RABV-encoded nucleoprotein (N protein). Immuno-microscopy with anti-N protein antibodies reveals large and granular inclusions made up of N protein in infected cells' cytoplasm. An MS-based method for the detection of N proteins was investigated in this study, which did not require any special antibody reagents or microscopy. There is no need for amplification with the MS-based method described here, and it can identify any previously sequenced N protein in the database. The results show that an MS/MS-based method for the detection of N proteins and the determination of amino acid sequences in animal diagnostic samples can be used to obtain information on RABV variants. This research shows that future rabies diagnostic tests built on MS platforms have promise.

Irwin Jacobson (2018) says that Oral rabies vaccination programmes primarily targeting mesocarnivores are used in North America to control rabies in terrestrial wildlife. Rabid skunk (Mephitis mephitis) control efforts have had mixed results, and further improvements to existing bait products or new bait designs and attractants may be required. With captive striped skunks, we looked at their preference for six different flavors of the ONRAB® "Ultralite" placebo bait (Artemis Technologies, Guelph, Ontario, Canada). Using the same skunks as before, researchers tested the efficacy of various ONRAB vaccine doses and delivery methods. A strong preference for a single flavor was not observed; cheese, egg, and chicken-flavored baits were preferred over plain-flavored ones. Skunks were challenged 335 days after vaccination, and vaccine efficacy ranged from 80 to 100 percent across a log dose range tested via an oral cavity route (109.3-1010.1 median tissue culture infective doses), compared to the more limited vaccine efficacy via bait delivery. Our findings showed that ONRAB vaccine efficacy lasted longer in skunks and that the vaccine titer and volume could only be limited in novel skunk bait designs.

Rajendra Singh (2018) said that viral zoonosis rabies is a deadly disease caused by a lyssavirus. All mammals with a warm blood supply, including humans, are susceptible to it. Asia, Africa, and Latin America have the highest concentrations of it. Despite the fact that the exact scope of the disease is unknown, some studies estimate that 174 lakh people are bitten by dogs every year, with 20,000 of them dying as a result. According to the Global Alliance for Rabies Control, India suffers annual economic losses of more than $2,000 due to rabies, primarily as a result of premature deaths, vaccine costs, lost income for animal bite victims, and other expenses. Despite efforts to eradicate rabies, the disease remains the most dreaded of all incurable human diseases despite a slight decline in incidence. A neurotropic virus's variable incubation period within the host means that once pathogenesis begins with detectable clinical symptoms, death is inevitable for the host. Rapid diagnosis of suspected cases is essential for the effective treatment and management of rabies. The OIE and FAO recommend using a direct fluorescent antibody test for diagnosis (dFAT). In India, postexposure prophylaxis involves the administration of over 3 million vaccine units each year. Rabies can be controlled and prevented with both pre-exposure and post-exposure vaccines for humans and animals. Hopefully, by 2030 we will be free from dog-transmitted human rabies thanks to an increased focus on increasing public awareness, improving diagnostics, and implementing regular vaccination programmes for target species.

3.0 EXPERIMENTATION

3.1 MATERIALS & METHODOLOGY

3.1.1 SERUM SAMPLES

Serum samples were obtained from subjects in a rabies vaccine regimen clinical trial. Subjects were placed into three groups based on vaccine and regimen types: Group A and B received purified Vero cell rabies vaccine (PVRV) with either the pre-exposure regimen (Group A, n=63); or post-exposure regimen utilizing Modified Thai Red Cross Schedule (TRC) (Group B, n=63); or purified chick embryo cell vaccine (PCECV) using TRC Schedule (Group C, n=63). All vaccines were administered via the intradermal route. Subjects did not receive rabies immune globulin (RIG). Serum samples collected on day 0, day 14, day 28 or 30 (referred to as day 30 elsewhere in the report), and day 90 after initial vaccination were included in the study. All serum samples were coded to ensure that testing was conducted blindly. The study was approved by Kansas State University Institutional Review Board, protocol 7012.

3.1.2 SEROLOGICAL TESTING

All serum samples were tested with both a SN and ELISA method. The SN method, the RFFIT, was performed as previously described and validated for use with human sera at the KSU Rabies Laboratory. The challenge virus strain used was CVS-11. RVNA titer values were standardized to IU/mL values by comparison with the Standard Rabies Immune Globulin (SRIG) (WHO 1st international RIG/Lot R-3 FDA/CBER).

The indirect ELISA method, Bio-Rad Platelia Rabies Kit II ELISA (Marnes-la-Coquette, France) was performed using the Bio-Rad Evolis instrument per the manufacturer's instructions. The kit contains strips of wells coated rabies glycoprotein (G protein) for use as the antigen. The secondary (detection) system is an enzyme conjugated Staphylococcus aureus protein A/substrate colour reaction. The results were reported in EU/mL (anti-rabies glycoprotein level) calculated by comparison of the sample optical density reading against a standard curve of positive standards supplied in the kit.

Samples producing results above the upper level of quantitation (ULOQ) per each method were pre-diluted and retested to obtain an endpoint result within the range of each assay. Both assays have been validated for the purpose of measuring antibody response to rabies vaccination in human sera in the Kansas State University Rabies Laboratory.

3.1.3 STATISTICAL ANALYSIS

After serological testing was completed, the identification of the groups was unblended and the IU/mL and EU/mL results were analyzed for comparison of the two methods by the basis of percentage of each group's subjects achieving adequate vaccination levels at days 14, 30 and 90. The average IU/mL and EU/mL of each group was calculated by day and the students t-test used to determine significant difference ($p=0.05$). The kappa test was used to determine agreement between the methods using different cut-off values to determine a logical, useful cut-off value for the ELISA method. Additionally, statistical comparison of individual results, IU/mL versus EU/mL, by a paired t-test was performed, to determine if a consistent relationship between RFFIT and ELISA results could be established.

4.0 RESULTS AND DISCUSSIONS

4.1 RESULTS

Table 4.1 Median and range of rabies antibodies per group per day and by test method.

Group		RFFIT IU/mL results				ELISA EU/mL results			
		Day 0	Day 14	Day 30	Day 90	Day 0	Day 14	Day 30	Day 90
A	*PVRV Pre-exposure Regimen*								
	Median	0.05	1.00	2.50	0.80	0.063	0.571	1.120	1.253
	Range	0.05 to 8.60	0.05 to 90.0	0.20 to 28.0	0.05 to 27.00	0.063 to 7.558	0.063 to 64.29	0.184 to 65.65	0.063 to 45.66
B	*PVRV TRC Regimen*								
	Median	0.05	28.00	10.40	2.40	0.063	2.712	3.908	3.651
	Range	0.05 to 2.0	1.30 to 1930.0	0.40 to 670.0	0.50 to 370.0	0.063 to 2.43	0.637 to 200.0	0.625 to 200.0	0.817 to 200.0
C	*PCEC TRC Regimen*								
	Median	0.05	19.80	7.85	3.40	0.063	1.400	1.810	2.464
	Range	0.05 to 0.05	4.90 to 115.8	2.60 to 28.0	0.70 to 14.7	0.063 to 0.219	0.297 to 63.96	0.625 to 6.199	0.613 to 9.919
	All Subjects								
	Median	0.05	14.85	5.65	2.40	0.063	1.546	2.112	2.186
	Range	0.05 to 8.60	0.05 to 1930.0	0.20 to 670.0	0.05 to 370.0	0.063 to 7.56	0.063 to 200.0	0.184 to 200.0	0.063 to 200.0

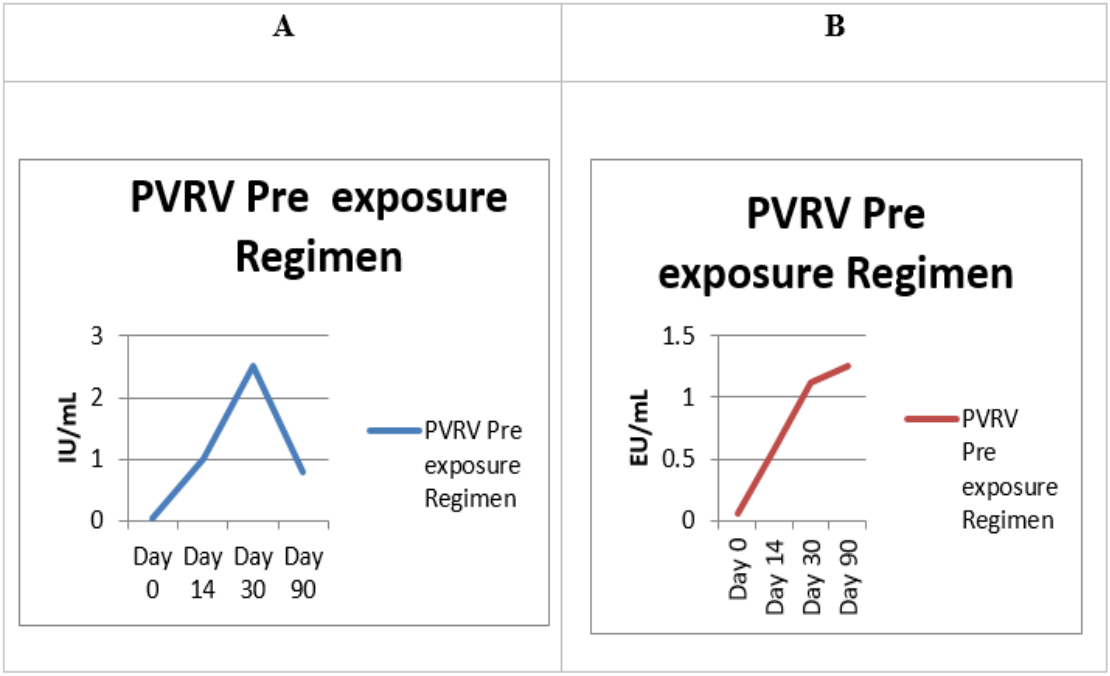

Figure 4.1 Kinetics of rabies vaccine response as measured by RFFIT (RVNA IU/mL) in panel A and as measured by indirect ELISA (anti-rabies glycoprotein EU/mL) in panel B.

The anti-rabies glycoprotein levels (EU/mL) as measured by ELISA peaked at day 30 on average though there was a lesser peak at day 14 as compared to day 30 while the peak IU/mL values as measured by RFFIT was at day 14. The anti-rabies glycoprotein levels were consistently lower than the RVNA levels on day 14 and day 30 for all groups (to greater extent in groups B and C) and nearly equal to the RVNA levels at day 90. The range of IU/mL (RFFIT) and EU/mL (ELISA) were similarly wide in each group and at each day with the widest ranges occurring at days 14 and 30. Subjects with the highest levels of response were identified by both methods; two subjects in Group B both had RFFIT and ELISA results of >/=200 IU/mL or EU/mL at day 14.

The relationship between RFFIT and ELISA results per individual as examined in all groups/subjects was variable and particularly affected by day of blood collection. At 14 days' post-vaccination, the majority of subjects had RFFIT results more than 50% higher than their ELISA result. On day 30 the results were of a mixed relationship with some subjects having higher RFFIT than ELISA results, others with results within 50%, and a smaller portion of subjects with higher ELISA than RFFIT results. By day 90, the majority of subjects had ELISA and RFFIT results that were comparable within 50%; the next largest group having ELISA results than their RFFIT results; and the smallest group had higher RFFIT results than their ELISA results. When each vaccine group was examined separately the same trend was observed but differing proportions in each category. For example, in Group A there are approximately equal numbers of subjects with RFFIT and ELISA results within 50% as there are subjects with higher RFFIT/lower ELISA result whereas for Group C, nearly all the subjects have higher RFFIT than ELISA results. The higher the RVNA level reached by a group the more discrepant the results between the two methods were at day 14 and day 30.

Table 4.2 The percentage of subjects with adequate response to rabies vaccination using 0.5 IU/mL for RFFIT and 0.5 EU/mL for ELISA as the definition. Comparison of groups A and B represent comparison of vaccine regimen and groups C and B, vaccine type.

	Group A vs Group B				Group C vs Group B			
	RFFIT		ELISA		RFFIT		ELISA	
Day	Group A	Group B	Group A	Group B	Group C	Group B	Group C	Group B
Day 14	76%	100%	34%	100%	100%	100%	92%	100%
Day 30	61%	98%	54%	100%	100%	98%	100%	100%
Day 90	48%	100%	55%	100%	100%	100%	100%	100%

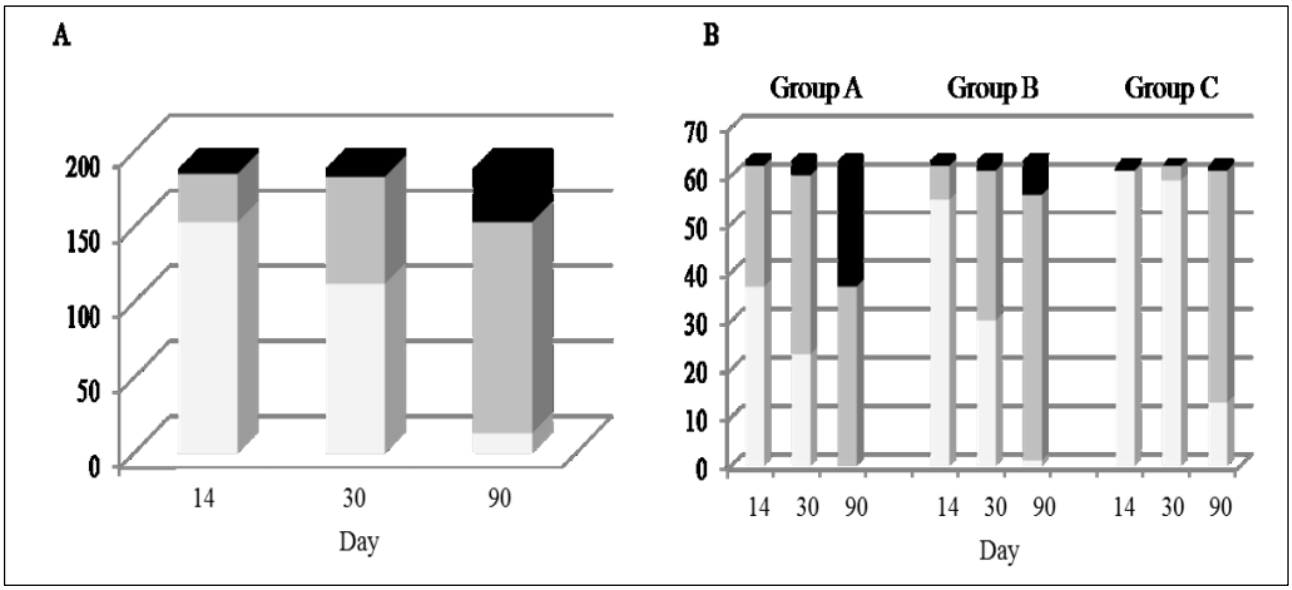

Figure 4.2 The relationship of RFFIT IU/mL to ELISA EU/mL at different time points from vaccination overall (panel A) and by group (panel B).

The number of subjects with RFFIT IU/mL greater than their ELISA EU/mL is in light grey, the number of subjects with RFFIT IU/mL with 50% of their ELISA EU/mL values is in grey, and the number of subjects with RFFIT IU/mL less than their ELISA EU/mL is in black.

Using 0.5 IU/mL for RFFIT and 0.5 EU/mL for ELISA as the cut-off values, the number of subjects achieving an adequate response (percentage of subjects with results equal to or above cut-off value at day 14) between groups leads to different conclusions by method. Using ELISA results, Group A's response is inferior to Group B's at (34% versus 100% achieving adequate response level, respectively); using RFFIT results the two groups are more similar in response with 76% Group A and 100% Group B subjects reaching the cut-off levels. However, in the comparison of Group C to Group B, the groups appear to produce nearly the same percentages above the cut-off values by both test methods, 92% versus 100% by ELISA, and 100% versus 100% by RFFIT. The groups by day were analyzed using the student's t-test to determine whether use of method would affect the assessment of group differences. The vaccine responses as measured by ELISA for Group A compared to Group B and for Group C compared to Group B are determined to be significantly different (p=0.05) at both day 14 and day 30, while the response as measured by RFFIT determined only Group A compared to Group B at day 14 were significantly different.

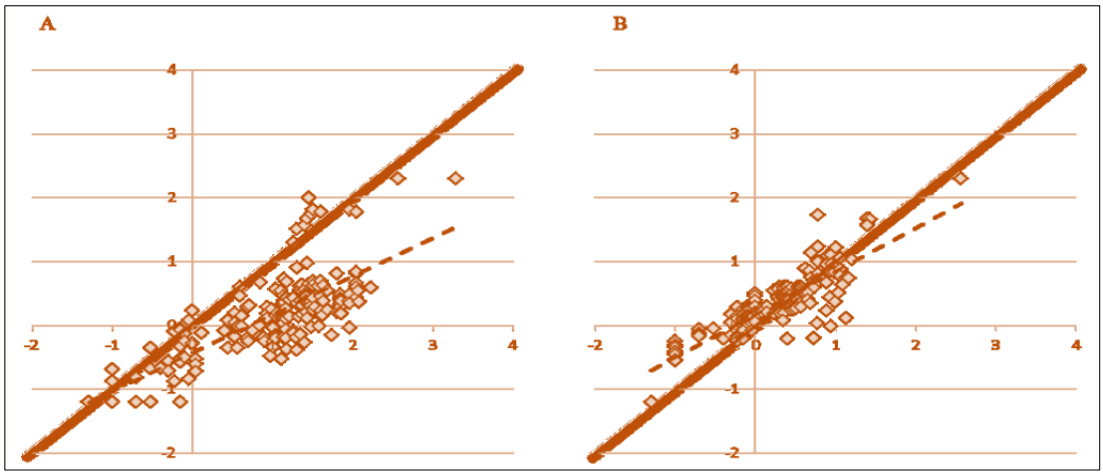

Figure 4.3 Correlation of RFFIT IU/mL to ELISA EU/mL results at day 14 (panel A) and at day 90 (panel B). Result values were log transformed and are displayed with the RFFIT results on the x-axis and the ELISA results on the y-axis. The regression line is the black dashed and the line of identity is the solid black.

Head to head RFFIT/ELISA comparison of individual results demonstrated a wider discrepancy at day 14 than at day 90 (see Figure 4.3). For example, graphing the Group C individual responses clearly shows the varying kinetics of RVNA and anti-rabies glycoprotein response by both time (days from vaccination) and magnitude (level) due in large part to the inability of the ELISA method to detect IgM (only IgG anti-rabies glycoprotein binding measured) while RFFIT detect neutralizing antibody of both IgG and IgM class. The variation of response by individual is demonstrated by selected subjects; some of the largest and smallest discrepant ELISA versus RFFIT results are noted. The best agreement between RFFIT and ELISA result values for individuals was found at day 90 (with the majority of values within 50%) and yet

large variation in results (both in value and in which method produced higher values) from the two methods were present at all the time points.

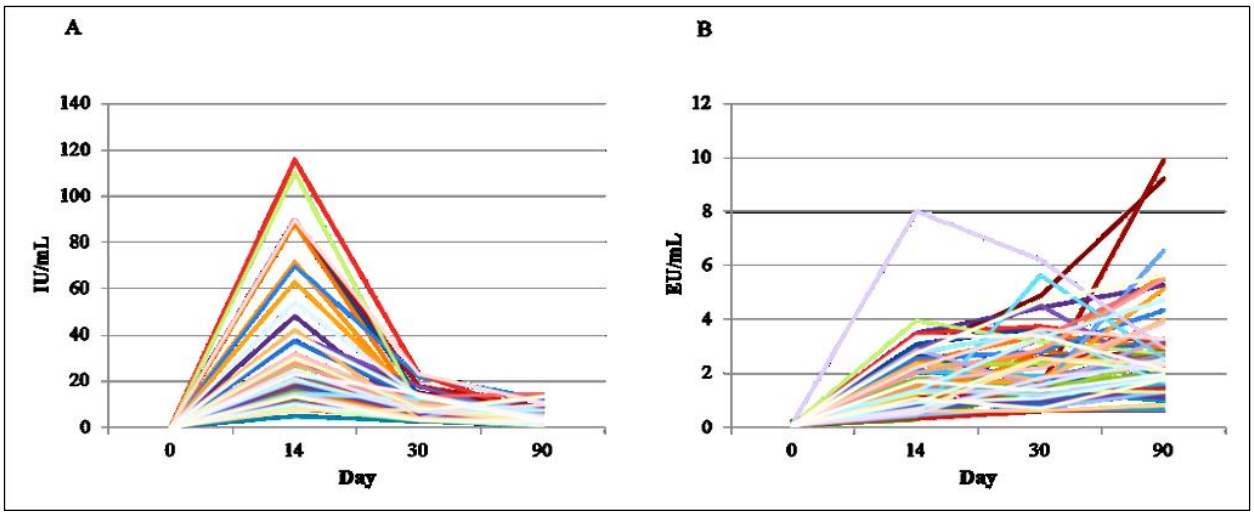

Figure 4.4 Individual results, RFFIT IU/mL in panel A and ELISA EU/mL in panel B, for subjects in group C. The results of one subject with very high ELISA (63.96 EU/mL) and moderately high RFFIT (32.0 IU/mL) results at day 14 were not plotted to allow illustration of the peak of the majority of subjects results.

Table 4.3 RFFIT and ELISA result comparison of selected group C subjects at day 14 and day 90. Subjects with nearly equal RFFIT (IU/mL) and ELISA (EU/mL) results are highlighted in red.

Group C – Day 14			Group C – Day 90		
Subject #	**IU/mL**	**EU/mL**	**Subject #**	**IU/mL**	**EU/mL**
9	413	0.297	75	11.3	11.295
11	4.9	0.625	88	2.4	2.623
17	13.5	2.16	105	6	53.18
23	10	0.554	165	2.5	2.559
39	110	3.981	171	7.7	1.678
42	42.4	2.374	180	11	3.256

4.2 DISCUSSION

In a broad view, both SN and ELISA methods for rabies serology measure the presence of antibody through a specific antibody-antigen interaction. Although other components of the immune system are involved, protection from clinical rabies after infection relies heavily on the presence of RVNA. Because SN rabies serological methods detect the neutralization activity of RVNAs *in vitro*, mimicking the protective action of these antibodies *in vivo*, they are the best methods to quantify the level of immunity after rabies vaccination and subsequently the need for booster administration. The technical performance of SN methods, such as the RFFIT, requiring high levels of biosafety facilities and expertise makes the use of these methods a difficult proposition in some areas of the world. Antigen binding assays such as

indirect and competitive ELISA methods are rapid, simple, and do not require manipulation of live rabies virus which makes them a practical alternative to SN methods. Simply replacing the method used for rabies antibody measurement and using the same cut-off value for "positive" and "negative" for purposes of determination of adequate response to rabies vaccination is problematic due to the inherent differences in how and what the different type of methods measure. SN methods measure neutralizing function without differentiation of the contribution of immunoglobulin classes (IgM, IgG, and IgA) present in the sera. The usefulness *in vivo* of high levels of IgM neutralization activity is not as critical for inhibition of rabies infection as IgG, due to the inability of IgM to reach the interstitial areas of tissue with high levels of virus (typically from saliva in the bite of a rabid animal). Conversely, ELISA methods measure the level rabies specific binding antibodies regardless of the antibodies ability to neutralize the virus, and, depending on the secondary (or detection) antibody, may only detect IgG or certain subclasses of IgG. In response rabies vaccination, the humoral immune response will be primarily IgM at early days developing to mostly IgG after antibody maturation and class switching at later days. In addition, the response is polyclonal with the proportion of neutralizing to non-neutralizing varying per individual genetics as a major factor. For the study presented here, the comparison of SN to ELISA only applies to the RFFIT method as performed at KSU Rabies Laboratory against an indirect ELISA with rabies G protein as the antigen and protein A as the secondary. Other indirect ELISA methods (using other proteins or whole virus and secondary antibodies) or competitive ELISAs may produce various correlation and agreement to SN methods. Modification of either basic method, SN or ELISA, would require method validation including assignment of cut-off value for the purpose of testing.

The results of this study indicate there is no consistent relationship between the two measures of rabies antibody response to vaccination (SN/RFFIT and indirect ELISA/Bio-Rad kit) either in degree of agreement or in direction of response (one always higher than the other) for individual subjects. Even though there is a larger numerical difference between the values produced by the two assays at day 14 and day 30, there are still individuals with significantly different responses at day 90 where the primary class of antibody is expected to be IgG, demonstrating that antibody class alone is not the sole source of difference in the measures.

The agreement of the two methods results from all subjects at all-time points tested was determined by the kappa test. Using the 0.5 value for both methods gives an agreement value of 94.0%, kappa statistic 0.56 for determination of adequate vaccine response. To attempt the establishment of a cut-off level that ensures all "positive" ELISA results include only those subjects with a RVNA level of 0.5 IU/mL or above for measures on day 14, 30, and day 90, a level of 1.0 EU/mL was identified as meeting this criterion. Nonetheless using the 1.0 EU/mL for the ELISA cut-off level and 0.5 IU/mL for RFFIT reduces the overall agreement of results to 81.2%, kappa statistic 0.32 and as a consequence 106 discrepant results, all of which are >/=0.5 IU/mL by RFFIT and <1.0 EU/mL by ELISA. Of these 106, 76% are from day 14 and 30 and 40% are below 1.0 IU/mL by RFFIT. This conservative cut-off level is designed for no overestimation of the neutralizing antibody level by ELISA testing in this set of subjects, for both early and late measures of antibody response and allows the use of the ELISA method as a screening test. Used in this manner, the ELISA would identify adequately vaccinated individuals (individuals that would be expected to have RVNA equal to or above 0.5 IU/mL); and individuals with ELISA results below 1.0 EU/mL would require testing by RFFIT to identify those with levels at or above 0.5 IU/mL. Based on this study, an estimated retest rate would be 18.8% for samples drawn between 14 and 90 days after

vaccination. Alternatively, if a less conservative level of 0.25 EU/mL for ELISA is used for comparison of with the 0.5 IU/mL RFFIT level, an overall agreement of results is 96.1%, but it allows 16 discrepant ELISA "positive"/RFFIT "negative" findings, hence labelling those subjects with antibody responses as adequate based solely on antibody binding levels. Use of this cut-off value scheme allows an improved agreement of the comparison of "percentage above cut-off level" between Groups A and B for the ELISA results, with Group A at 70% and Group B at 100% subjects above cut-off at day 14. If the lower limit of quantitation (LLOQ) for both methods are used as cut-off levels (0.2 IU/mL for RFFIT at KSU and 0.125 EU/mL for the Bio-Rad ELISA), the agreement of methods becomes 97.2% for this set of subjects at time points of days 14, 30, and 90.

If each time point is considered independently for assignment of ELISA result cut-off for best agreement and kappa statistic with the 0.5 IU/mL cut-off for RFFIT results, at day 14 it was 0.25 EU/mL, and at day 90 it was 0.5 EU/mL; whereas on day 30 the use of the LLOQ for both methods as the cut-off value resulted in the highest agreement and kappa statistic values. Comparing individual results by the t-test pairwise indicates that only at day 90 was there no significant difference between the measures by RFFIT and ELISA.

Table 4.4 Each of the subject's results were categorized as 'positive' (having a result at or above the assigned cut-off value for the method) or 'negative' (having a result below the assigned cut-off value for the method) and the kappa test used to determine agreement between the RFFIT and ELISA methods for determination of adequate response per day and overall for different cut-off schemes.

Cut-off value scheme RFFIT/ELISA		**Day 14**	**Day 30**	**Day 90**	**Overall**
0.5IU/mL/0.5EU/mL	Agreement %	89.9	95.7	96.3	94.0
	kappa	0.56	0.32	0.68	0.560
0.5IU/mL/1.0EU/mL	Agreement %	70.7	81.4	91.5	81.2
	kappa	0.26	0.12	0.61	0.320
0.5IU/mL/0.25EU/mL	Agreement %	96.3	99.5	92.5	96.1
	kappa	0.78	0.80	0.12	0.590
0.2IU/mL/0.125EU/mL	Agreement %	95.7	100.0	95.7	97.2
	kappa	0.48	1	0.19	0.37

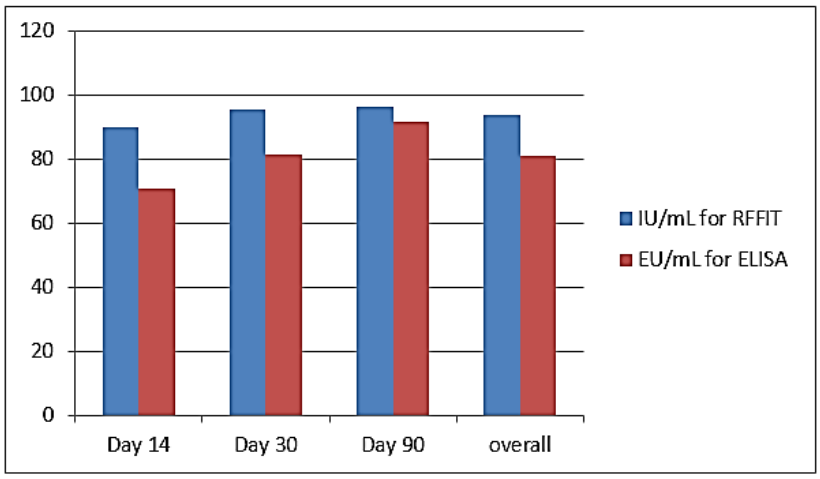

Figure 4.5 Average result values, IU/mL for RFFIT (skyblue bars) and EU/mL for ELISA (red bars). Significant difference ($p<0.05$) between method results were noted at days 14 and 30 as well as overall (*).

5.0 CONCLUSION

Rabies vaccine response can be measured by both antigen-binding and serum neutralization methods, but these measures are not the same due to differences in what and how each test measures. It is not expected that RFFIT and ELISA results have a consistent relationship since RFFIT measures neutralizing function of the rabies specific antibodies (IgG and IgM), which is not proportional in a defined degree to the binding ability of the rabies specific IgG antibodies as detected by the Bio-Rad ELISA kit. Assigning the same cut-off level for both test methods will never result in agreement for all individuals. The relationship of RFFIT and ELISA results over various time points post vaccination, as illustrated in this study, demonstrates further that one cut-off is not appropriate, though good agreement can be achieved by assigning logical cut-offs considering time point of blood draw post-vaccination for groups of subjects if not on an individual basis. Evaluating the kinetics of the RVNA/anti-glycoprotein response, combined with laboratory validation of the specific test method and consideration of the use of the results (vaccine efficacy, determination of booster, detection of vaccine bait-uptake) is a logical approach for setting a useful cut-off for both RFFIT and ELISA methods. The importance of choosing the method 'fit for purpose' AND ensuring that the testing procedure is conducted appropriately with adequate quality assurance procedures in place cannot be overstated for a measure as important as the RVNA level—the most important immune component for the prevention of clinical rabies.

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