RABIES, A GLOBAL THREAT: TAKING SCIENCE A STEP FORWARD

by

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Abstract

Rabies is the most deadly disease on earth and has a 99.9% human fatality rate. Rabies kills 61,000 humans annually and results in an economic burden of $124 billion USD annually. Each day 3.3 million people live with the risk of rabies. It is estimated that 95% of human rabies cases are a result of coming in contact with an infected canine, majority of these cases being children 15 years and younger. It is estimated that 1 person every 8 minutes dies of rabies. Rabies is a highly neurotropic disease which attacks the brain and central nervous system. Once clinical symptoms are presented, death is invariably the outcome as no cure exists for rabies. Rabies is 100% preventable in humans by proper wound management and proper administration of prophylaxis. Rabies can be adequately controlled in animal populations by contraception and animal rabies vaccine efforts. Whilst it is known that rabies can be prevented in humans and controlled in animal populations, further scientific efforts are still warranted to fully understand this deadly virus so that a cure can one day be discovered. As human and animal populations continue to grow, so does the cost and burden of this horrific disease. As a result, the importance of prophylaxis and passive immunity are critical in the event of medically managing an exposure, and preventing exposures. The World Health Organization has defined global recommendations for individuals and animals who have received prophylaxis to be adequately protected. Measuring this protection is performed using a variety of approved testing methodologies, virus-neutralizing assays and antigen-binding assays. Whilst the WHO recommendations were defined from clinical studies performed with virus-neutralizing assays, the assumption that these recommendations are suitable for the antigen-binding assays is inaccurate. The testing methodologies, virus-neutralization and antigen-binding, share similarities, as they are measuring an immune response to the rabies virus. However; enough differing characteristics are presented such that exact comparisons cannot be made. Establishing the same standards and recommendations for both testing methodologies will never be sufficient.

Keywords: Rabies, WHO Recommendations, Lyssavirus, ELISA, Zoonosis, Canines

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CHAPTER 1 – INTRODUCTION TO RABIES

"Rabies, one of the world's deadliest diseases, impacts 5 billion people, causing tens of thousands of deaths every year" (Global Alliance for Rabies Control, 2013). Rabies is a global threat and can be found on every continent, excluding Antarctica (World Health Organization, 2014). Although rabies serves as a prominent global public health threat, rabies is a preventable disease in humans by means of proper vaccination and education.

History of Rabies:

Rabies, a viral disease of mammals, is one of the oldest infectious diseases known to man and can be traced back more than 4000 years (Rupprecht, 2011). In looking through historical records, the first documentation of rabies occurred in the city of Eshnunna in 2300 BC where it was written, 'If a dog is mad and the authorities have brought the fact to the knowledge of its owner; if he does not keep it in, and it bites a man and causes his death, then the owner shall pay two thirds of a mine of silver' (Baer, 2007, pg. 1). One of the first documentations of a canine rabies case comes from a Greek philosopher named Democritus in 500 BC in which descriptions of a case of canine rabies was recorded in his papers, ("A short history of rabies," 2013). The disease of rabies is also written in early poems and books; In 800 – 700 BC Homer writes about the disease in a poem titled *The Iliad* ("A short history of rabies," 2013) and in 400 BC Aristotle depicts the disease in a book titled the Natural History of Animals (Hernandez, 2009). At the time of 400 BC, Greeks identified two gods for the prevention and cure of rabies. The god to prevent the disease was known as Arisaeus and the god said to cure rabies was known as Artemis ("A short history of rabies," 2013). The word "rabies" stems from the Latin word *rabere* which is defined as rave or rage. It is believed that the word *rabere* is rooted in the Sanskrit word *rabhas* which is defined as doing violence ("A short history of rabies," 2013). By 001 – 100 AD, rabies spread across the Roman Empire and in 1271, the first large rabies outbreak is recorded, 30 people die from rabies in Germany due to an invasion of rabid wolves ("A short history of rabies," 2013). In the 1400s, rabies spread across Spain, and in the 1700s, rabies made its way through Europe. In 1703, the first case of rabies was noted in the Americas by a priest in Mexico, and in 1953, the first case of bat rabies was recorded in the United States by the Centers

for Disease Control and Prevention ("A short history of rabies," 2013).

As long as rabies has been known to man, myths about the cause, transmission and 'cures' for the disease have played an instrumental role in society and have impacted the health of animals and man. Some myths throughout history include the belief that witches cause rabies by spells and evil spirits, ambient temperatures cause rabies, or that a worm located in the base of the tongue of a canine caused rabies. It was believed that if this worm was cut and removed from the tongue, then the canine would be cured of the disease. Cauterization of wounds was also believed to cure the disease, as was having individuals physically suck the wound to remove the poisons. The same aforementioned worm was also believed to have magical powers to prevent rabies when injected. Other preventions for rabies included individuals eating cocks brains and consuming salted flesh from a rabid dog. Consuming maggots from a rabid dog were also believed to be a remedy (Baer, 2007). Furthermore, history reflects the belief of placing animal hair over the wound would cure the disease as would having the child that was bitten by a rabid animal eat the raw heart or liver of the animal that bit them (Baer, 2007). Herbal remedies thought to cure rabies throughout history included utilizing the *Angelica archangelica* plant in a powered form as an external plaster. *Ballota nigra* plant leaves were beaten with salt and then applied to the wound. The *Pelteriga canina* plant was utilized whole with black pepper and then placed in milk after which four doses were given to the individual or animal. The *Julgans regia* plant leaves would be placed in wine along with onions, honey and salt to use internally for the 'treatment' of rabies (Baer, 2007). To prevent coming in contact with a rabid animal, individuals were known to carry weasel tails as well as place the heart or tongue of a rabid dog in one's shoe (Baer, 2007). Prayer and divine intervention was another method utilized to prevent and 'cure' rabies throughout history. An example of this stems from the early centuries when Christians would travel to visit a well-known bishop by the name of St. Hubert in Europe. St. Hubert became known as the patron against rabies when it was believed that he 'cured' an individual with the disease by stating the words 'May the Lord Jesus heal you' as the individual believed to have rabies approached him (Baer, 2007). History reflects that this individual then walked away cured of the disease. This act resulted in visitations to St. Hubert from individuals around the world who believed they had the disease seeking out a cure. Individuals who owned canines suspected of having the disease from various parts of the world also visited St. Hubert to cure their canines of the 'madness'. St. Hubert utilized a metal rod in the form of a nail, known as St.

Hubert's Key to cauterize the wounds of animals and humans that were bitten (St. Hubert Club, 2014). **Figure 1.1**. The idea of heating of the nail would allow for the disease to be killed once the nail was placed into the wound (they glyptodont, 2014). The role of St. Hubert led to revolt and the speaking out against superstitions in religion ("A short history of rabies," 2013). Myths about rabies continue to play an instrumental role in societies around the world. Religious and cultural beliefs are highly influential in rabies prevention and 'treatment'. Ironically, it is also currently believed that rabies is a rare disease which is not widespread around the world, whilst some share the belief that "nothing can be done to make an impact toward rabies elimination" (Rupprecht, 2011).

Although individuals were significantly influenced by the aforementioned myths for rabies cures and preventions, not all individuals were convinced of such beliefs. "Even in the 1st century, the optimism about curing rabies was not shared by Scribonius Largus, who affirms that a rabid patient is never cured…" (Baer, 2007, pg. 2). While the source of rabies was questioned throughout history, it wasn't until the 1st century AD that the transmission of the disease from wild animals was recognized. In the New World, the first description of rabies transmission from a wild animal to man was documented in the early 16th century as a gentleman was dying from bites that were received from vampire bats. From the 1st century AD comes the 'first recorded attempt at defining the cause of rabies and prescribing treatments, as it was written in the words of a poem titled 'On Hunting' by author Grattius Faliscus (Baer, 2007). And in 1804 an individual named Zinke first demonstrated that rabies was transmitted through saliva. Zinke placed the saliva of a rabid dog onto an incision of another dog. The second dog began to fall ill on the seventh day, and by the tenth day obvious symptoms of the disease were present. This demonstration resulted in many articles being written about the pathogenesis (development of the disease) and 'treatment' of rabies throughout the 19th century. In 1879 Galitier proved that the disease could affect rabbits through an injection or by a bite of a rabid animal. Galitier's experiments and studies were followed by French chemist and microbiologist Louise Pasteur who discovered that rabies is produced in the brain and spinal cord. **Figure 1.2.** Pasteur also discovered that if rabies was injected directly into the brain, then paralysis and death were inevitably the result. Pasteur's work proved that the brain is the fundamental organ in the pathogenesis of the disease. Pasteur then proceeded to develop the first vaccine against rabies. "The initial vaccination consisted of a series of inoculations prepared from dried spinal cord

tissues from rabbits that had died from rabies, the 13th dose consisting of the most virulent preparation" (Hooper, Roy, Barkhouse, Li, Kean, 2011, pg. 60). Pasteur's vaccine was then successfully utilized on fifty canines. In 1885 Pasteur took a step forward and utilized his vaccine on a young boy the day after the boy had been repeatedly bitten by a rabid canine. After several injections of the vaccine developed by Pasteur, the boy survived.

In 1921, thirty-five years following the work of Louise Pasteur, the first national program for canine vaccinations occurred in Japan. These efforts set a model for other countries began to follow suit. The result of such endeavors lead to the World Health Organization (WHO) to set global recommendations that all canines should be vaccinated annually or every three years as approved by current vaccine labels. The WHO is an organization that "is the directing and coordinating authority for health within the United Nations system. It is responsible for providing leadership on global health matters, shaping the health research agenda, setting norms and standards, articulating evidence-based policy options, providing technical support to countries and monitoring and assessing health trends" (WHO, 2014). The WHO continues to play a leading global role in the efforts towards rabies education and elimination on a global scale to this day.

Figure 1.1

Photograph of St. Hubert's Key, utilized to cauterize wounds to 'cure' rabies (The Glyptodont, 2014).

Figure 1.2

Photograph of Dr. Louis Pasteur, French Chemist whom developed the first rabies vaccine (Bio.True Story, 2014).

Rabies Virus Introduction and Pathogenesis

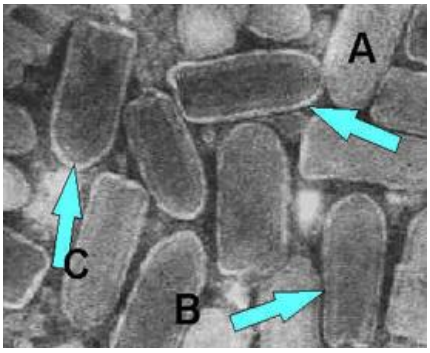
As a result of scientific advancements through time it is known that rabies is not the result of witches spells, ambient air or worms of the canine tongue as previously discussed. Rabies is an acute virus that affects the central nervous system and is almost unvaryingly fatal. Rabies is a zoonotic disease that can be transmitted from animals to humans and is primarily spread through the bite of an infected mammal through saliva. Other routes of transmission include aerosol transmission, mucosal contact (i.e., mouth, nose, eyes) licking of broken skin, transplantations of organs and corneas, and penetration of the skin from a scratch where bleeding occurs. It is important to note that non-bite transmissions rarely cause rabies in humans (Rupprecht, 1996) and that human to human transmission, aside from transplantations, has never been documented (International Travel and Health, 2014). In the United States, 5 human rabies cases out of 154 were a result of non-bite incidences from 1950 through 1980 (Rupprecht, 1996). Two cases resulted from laboratory-acquired infections and two cases were cave explorers, all of which were undoubtedly exposed to high concentrations of rabies virus (Rupprecht, 1996). The fifth human rabies case resulted from a transplant of a cornea which was received from a patient that was dying from an unsuspecting case of rabies encephalitis (Rupprecht, 1996).

The rabies virus is highly neurotropic (affects or attacks the nervous system). Once the virus enters an individual it, may replicate within the tissue (at or near the entry site) and remain sequestered (segregated or secluded) during incubation. The virus will then enter the peripheral nerves and travel to the central nervous system where it will continue to replicate, then travel out to the organs, including the salivary glands. The salivary glands are the primary exit portal in which the virus is excreted and passed on to other hosts. In humans, five general clinical stages for rabies are recognized: incubation, prodromal stage, acute neurologic phase, coma and death or very rarely, recovery (Rupprecht, 2011). In humans, the incubation period can be anywhere from ten days to ten years whilst the average incubation is three to six weeks (Rupprecht, 2014). The prodromal stage consists of nonspecific signs, such as headache, fever, nausea, sore throat, anxiety, increased sensitivity to noise and light, hallucinations, fear of air (aerophobia), fear of water (hydrophobia), etc. Additional abnormal signs can also occur during the prodromal stage, such as increased libido, nightmares, depression, insomnia, etc. The acute neurologic phase is where signs of dysfunction within the central nervous system become apparent. During the onset of the acute neurologic phase, encephalitis (inflammation of the brain) occurs in which the

central nervous system becomes dominated by the virus. At this time, the disease is then classified into one of two forms: hyperactivity, known as furious rabies, or paralysis known as paralytic rabies (also known as dumb rabies). "At the end of the acute neurologic phase, periods of rapid, irregular breathing may begin; paralysis and coma soon follow. Respiratory arrest may occur thereafter, unless the patient is receiving ventilator assistance, which may prolong survival for days, weeks, or longer, with death due to other complications" (Rupprecht, 1996). While ventilation assistance can prolong the clinical signs of rabies, it will rarely affect the outcome of the disease itself. Once the onset of symptoms occurs, rabies is nearly one hundred percent fatal. "Rabies has one of the highest case-fatality ratios of any infectious disease" (MMWR, 2011).

The rabies virus is best described as an enveloped, rod or bullet shape (bacilliform) in its appearance (morphology). **Figure 1.3.** One end of the virus particle (also known as virion) appears flat (planar) while the other end of the virion has a rounded appearance (hemispherical).

Figure 1.3

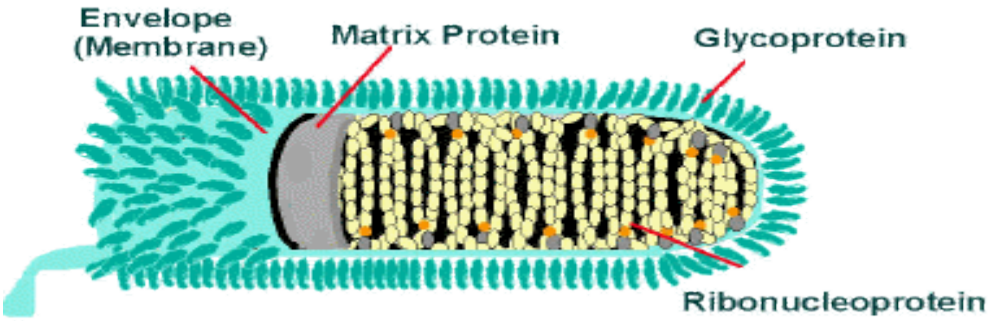


Photograph of the rabies virus. Negatively stained rabies (Rhabdovirus) as seen through an electron microscope. Notice the bullet shape of the virus (A). See the "bee hive" like striations of the RNP (B). Notice the glycoprotein spikes in the outer member bilayer (C) (Centers for Disease Control and Prevention, 2014)

The origin of the virus, is that of the Mononegavirales order (WHO, 2012). The Mononegavirales order contains numerous viruses, all of which cause harmful diseases in humans. Viruses in the Mononegavirales order are single-stranded, nonsegmented, negative-stranded ribonucleic acid (RNA) genomes (CDC, 2013). Within the Mononegavirales order, the bullet-shaped viruses are classified in the Rhabdovirdae family which consists of 12 genus species (WHO, 2012). Rabies is part of the Lyssavirus genus (WHO, 2012). To better understand how these names were identified, the Greek words rhabdos means 'rod' and lyssa

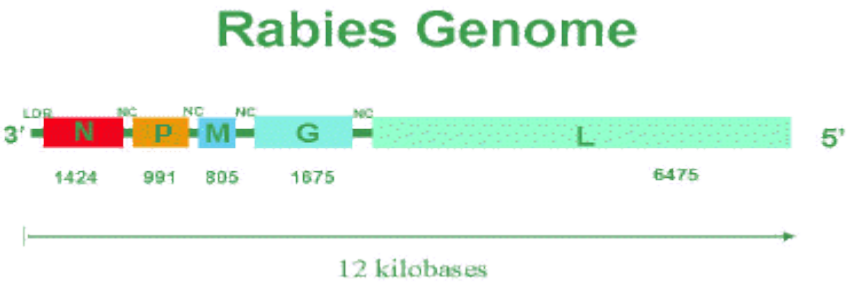
means ‘rage’ (Wunner, 2007). The rabies virion, is approximately 180 nanometers (nm) long and 75 nm wide (CDC, 2013). Rabies is an RNA virus, as previously mentioned, in which the virus particle (virion) encodes five different proteins. These proteins are nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase (L) (CDC, 2013). The basic structure of the rabies virus is depicted below. **Figure 1.4**. Relative size and order of the proteins are depicted below. **Figure 1.5.**

Figure 1.4



Rabies virion are rod or bullet-shape with spike-like glycoprotein covering the surface. The ribonucleoprotein is composed of RNA encased in nucleoprotein (Centers for Disease Control and Prevention, 2014)

Figure 1.5



The rabies virus encodes five proteins; (N) nucleoprotein, (P) phosphoprotein, (M) matrix protein, (G) glycoprotein and (L) polymerase. The order and the size of the proteins is imperative as this makes up the virus (Centers for Disease Control and Prevention, 2014).

It is important to note that the location of these five proteins and the RNA determine the structure of the rabies virus. Whilst this paper will not go into detail in describing the replication and strategies of the rabies virus, it is important to understand the virus and how it affects individuals and continues to survive and infect new individuals.

All rhabdoviruses, including the rabies virus, include two major structural components. These two components are a helical ribonucleoprotein core (RNP) and a surrounding envelope. The L and the P protein are associated with the RNP as they aid in the replication process. The G protein which is spiked, as depicted in Figure 1.4, is located on the surface of the virion as it is used to physically attach to the host cell membranes (Rupprecht, 1996). The M protein is associated with the RNP and the envelope which plays a key role in virion assembly and egress (Okumura, Harty, 2011). The M protein is also known to play an important role in giving the virion its bullet-shaped appearance (Wunner 2007). Without going into great detail, the N protein encapsidates (encloses) the genomic and antigenomic RNA. The genomic includes an individual's full DNA sequences (Web definitions, 2014).

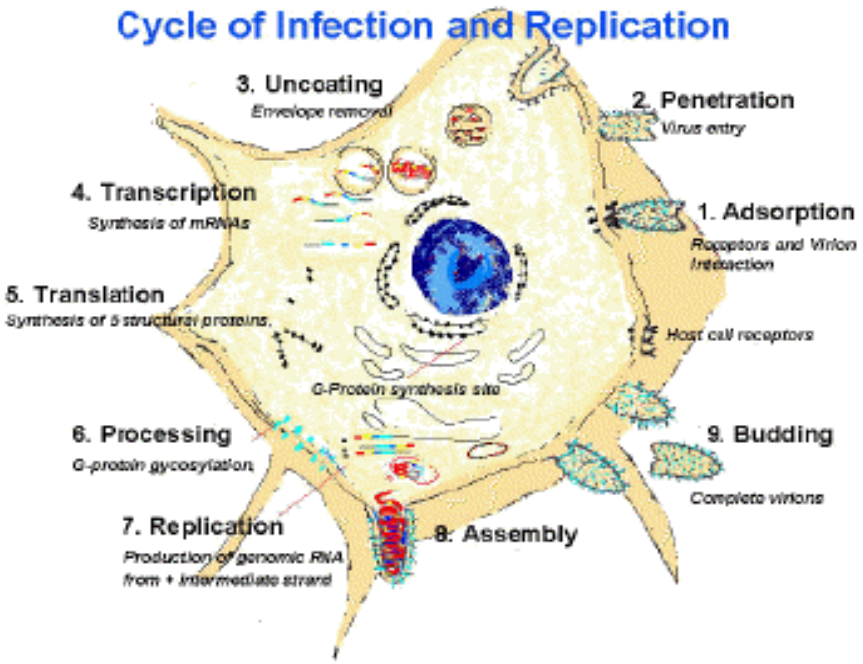
The life cycle of a rabies infection begins by the attachment of the virion to the surface of the cell membranes in which penetration of the virion is initiated. It is important to note that most often the virion attaches to a receptor molecule that will permit the virion to enter the susceptible cell membranes (Wunner, 2007). The life cycle of the rabies infection can be divided into three phases. The first phase, as discussed, includes the attachment of the virion to susceptible cell membranes by which the virion then enters the host cell. This phase initiates the infection process. This phase of the life cycle is considered to be the most difficult phase (Wunner, 2007). The second phase of the life cycle includes replication of the virion in the host cell. The third phase consists of final replication of new virions which are then released (budding) from the cell to attach to new host cells, starting the life cycle process over again.

Figure 1.6.

"Rabies is a fatal disease. Clinical management of rabies patients should include adequate sedation and care in an appropriate medical facility with suitable emotional and physical support" (WHO, 2012). If intensive care is not provided, death normally occurs within two weeks of the onset of clinical signs, although typically, death results in seven to ten days after the first clinical sign (WHO, 2012). It is important to note that there currently is no cure for rabies after the onset of clinical symptoms. An experimental approach, known as the Milwaukee

protocol, has been utilized around the world on approximately 40 patients. This protocol involves inducing the patient into a coma state and administering antiviral drug treatment. Out of the 40 patients around the world, there are 4 reported survivors (Centers for Disease Control and Prevention, 2013). "Despite these observations, rabies must still be considered 100% fatal for practical purposes, and preventive measures remain the only way to guarantee survival after a bite by a rabid animal" (Centers for Disease Control and Prevention, 2013, pg. 1).

Figure 1.6



Steps of the life cycle of the rabies virus/virion for infection and replication in a susceptible host cell (Centers for Disease Control and Prevention, 2014).

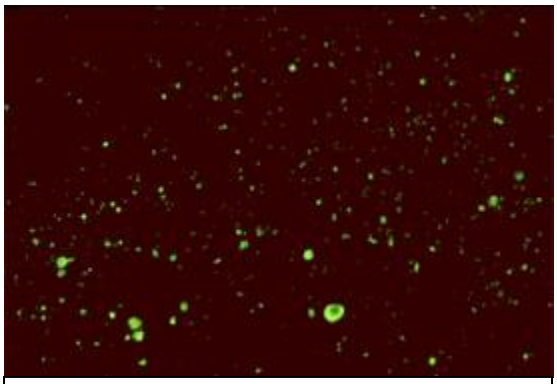
Rabies Diagnosis

As aforementioned, rabies is part of the Rhabdoviridae family and is caused by a Lyssavirus which is an acute progressive encephalitic disease that can affect all mammals. It has also been discussed that "rabies has one of the highest case-fatality ratios of any infectious disease" (Compendium of Animal Rabies Prevention and Control, 2011). Rabies is a difficult

disease to diagnose as it mimics the signs of other diseases and is often misdiagnosed in the early stages when clinical signs first appear (World Organization for Animal Health, 2009). Although a variety of testing methods exist, it is important to note that a negative test does not rule out a rabies infection.

In animals (all mammals excluding humans) rabies can be diagnosed, however, the animal must first be euthanized (put to death) as tissues from the brain must be collected and tested. Two tissues from the brain are required for testing and in order to successfully rule out a rabies diagnosis, the cerebellum and the brain stem must be test negative. Brain tissue is required as the rabies virus resides in the nervous tissue and not blood as other viruses (Centers for Disease Control and Prevention, 2014). The testing method utilized is the Direct Fluorescent Antibody test known as the DFA test. While other testing methods exist, the DFA test is the gold standard for rabies diagnosis and must be performed according to standard protocol (Compendium of Animal Rabies Prevention and Control, 2011). The DFA test utilizes antibodies that attach to the rabies virus (antigen) in the brain samples if the animal is positive for rabies. When the animal is positive for rabies the samples will show a green-apple fluorescent color when viewed under a fluorescent microscope. **Figure 1.7**. This green-apple fluorescent will not be viewed for a negative sample. **Figure 1.8**. While the DFA test and other methodologies are beneficial in diagnosing rabies, it has been proven through public health surveillance and pathogenic studies that euthanizing an animal is not always necessary. For animals that have a low probability of being rabid after biting an individual, the animal may be quarantined for 10 days to rule out rabies. If the animal survives on and after the 10^{th} day than the rabies virus was not present in the saliva at the time of the bite. This quarantine is effective as the salivary gland is an exit route and the animal will not survive the virus longer than 10 days once the virus is shedding in the saliva (Compendium of Animal Rabies Prevention and Control, 2011). For humans, a test does not exist for diagnosing rabies infection prior to the onset of clinical disease, and unless the rabies-specific signs of hydrophobia or aerophobia are present, the clinical diagnosis may be difficult (World Health Organization, 2013). Once clinical symptoms are present, several tests are available as no single test is sufficient in diagnosing human rabies ante-mortem (before death). Ante-mortem diagnosis includes detecting the antigen or nucleic acids of the rabies virus or by virus isolation.

Figure 1.7



DFA test viewed through a fluorescent microscope that is positive for rabies, (Centers for Disease Control and Prevention, 2014).

Figure 1.8

DFA test viewed through a fluorescent microscope that is negative for rabies, (Centers for Disease Control and Prevention, 2014).

Samples utilized to detect rabies from humans include saliva, corneal impressions, eye wash fluid, central spinal fluid (CSF), tears, and skin biopsies (nuchal). The nuchal biopsies are taken from the nape of the neck as the rabies virus is found in cutaneous nerves, which are located at the base of the hair follicle (World Organization for Animal Health, 2009). Serum may also be tested to look for antibody production against the rabies virus. Antibody production is also tested for when testing the CSF. It is important to note that antibody production in the CSF can result from individuals that have previously been vaccinated against rabies. It is also noteworthy that antibody response from a true rabies infection occurs in the late stages of the illness and the individual may die prior to this occurrence, resulting in a negative CSF test for circulating antibodies at the time of death. These samples may also be utilized for post-mortem (after death) diagnosis in humans along with samples of the brain from the infected individual. As a result, "postmortem diagnosis is usually by immunofluorescence to detect viral antigens in the brain" (WHO, 2013, pg. 3).

Rabies Vaccine, Prophylaxis and Immunoglobulin

Several scientific and technological advancements have progressed since following the days of Louis Pasteur when the first rabies vaccine was developed in the 1880s. Injectable animal rabies vaccine is currently approved for dogs, cats, ferrets, horses, sheep and cows. The vaccines provide a variation of duration of immunity from 1 to 4 years (Compendium of Animal Rabies Prevention and Control, 2011). In reference to domesticated animals, local governments are tasked with ensuring that effective vaccine programs are maintained. Animal vaccines may be administered intramuscularly (in the muscle) or subcutaneous (under the skin).

Controlling rabies in wildlife is extremely difficult (Compendium of Animal Rabies Prevention and Control, 2011); however, the vaccination of free ranging wildlife or selected wildlife populations can be beneficial. The oral rabies vaccine (ORV) was developed and designed to vaccinate certain wildlife species in the form of a sachet. **Figure 1.9**. The sachets are approximately the size of a matchbox and along with the vaccine the sachet can include fishmeal to attract certain animal species. **Figure 1.10**. The sachets are distributed in the animal's environment by air and ground personnel. Once the animal finds and bites into the sachet the animal then swallows the vaccine and is then vaccinated for rabies. ORVs have been utilized in Europe since 1980, Canada since 1985 and the United States since 1990 (United States Department of Agriculture, 2012).

ORVs have been successfully utilized to vaccinate raccoons, foxes, and coyotes in the United States. The attempt to successfully vaccinate skunks with ORVs in the United States was attempted in the state of Texas during the routine ORV baiting in February 2014 (Forsyth, 2014). It is important to note that translocation of wildlife has made a significant impact in the spread of rabies. As a result, the translocation of known rabies carrier species should be prohibited (Compendium of Animal Rabies Prevention and Control, 2011). In respect to human rabies vaccines (prophylaxis), two areas of emphasis exists 1) pre-exposure prophylaxis and 2) post-exposure prophylaxis. Pre-exposure prophylaxis is aimed at vaccinating individuals that have a risk of being exposed to the rabies virus. Pre-exposure prophylaxis is used as a preventive measure. Individuals in this category include veterinarians, individuals traveling to an area with increased risks to rabies (endemic), animal control officers, wildlife officers or laboratory personnel working with the rabies virus (WHO, 2014).

Figure 1.9

Photograph of coated sachet baits containing rabies vaccine only utilized for ORVs, (United States Department of Agriculture, 2012).

Figure 1.10

Photograph of ORV baits containing fishmeal and rabies vaccine, (United States Department of Agriculture, 2012).

Individuals in this group are considered high risk or continuous. Pre-exposure prophylaxis should be considered for all individuals that consistently have the potential to be exposed to the rabies virus or have frequent potential contact with animals that can carry the rabies virus. These individuals are considered moderate and low risk or frequent and infrequent. **Table 1.1**. Pre-exposure prophylaxis consist of three doses of vaccine administered intramuscularly on day 0 (day of first vaccine), 7 and 21 or 28 (Centers for Disease Control and Prevention, 2014).

Table 1.1

Rabies Pre-exposure Prophylaxis Guide

Risk Category	Nature of Risk	Typical Population	Pre-exposure Recommendations
Continuous	Virus present continuously, often in high concentrations. Specific exposures likely to go unrecognized. Bite, nonbite, or aerosol exposure.	Rabies research laboratory workers; rabies biologics production workers.	Primary course. Serologic testing every 6 months; booster vaccination if antibody titer is below acceptable level.
Frequent	Exposure usually episodic, with source recognized, but exposure also might be unrecognized. Bite, nonbite, or aerosol exposure.	Rabies diagnostic lab workers, spelunkers, veterinarians and staff, and animal-control and wildlife workers in rabies-enzootic areas. All persons who frequently handle bats.	Primary course. Serologic testing every 2 years; booster vaccination if antibody titer is below acceptable level.
Infrequent	Exposure nearly always episodic with source recognized. Bite or nonbite exposure.	Veterinarians and terrestrial animal-control workers in areas where rabies is uncommon to rare. Veterinary students. Travelers visiting areas where rabies is enzootic and immediate access to appropriate medical care including biologics is limited.	Primary course. No serologic testing or booster vaccination.
Rare (population at large)	Exposure always episodic with source recognized. Bite or nonbite exposure.	U.S. population at large, including persons in rabies-epizootic areas.	No vaccination necessary.

Rabies Pre-exposure Prophylaxis Guide, (Centers for Disease Control and Prevention, 2014).

Table 1.2

Rabies Post-exposure Prophylaxis Guide

Type of vaccination	Route	Regimen
Primary	Intramuscular	Human diploid cell vaccine (HDCV) or purified chick embryo cell vaccine (PCECV); 1.0 mL (deltoid area), one each on days 0,* 3, 7, and 14
Booster†	Intramuscular	HDCV or PCECV; 1.0 mL (deltoid area), day 0 and 3

*Day 0 is the day the first dose of vaccine is administered.

†Persons in the continuous-risk category should have a serum sample tested for rabies virus neutralizing antibody every 6 months, and persons in the frequent-risk category should be tested every 2 years. An intramuscular booster dose of vaccine should be administered if the serum titer falls to maintain a value of at least complete neutralization at a 1:5 serum dilution by Rapid Fluorescent Focus Inhibition Test.

Rabies Post-exposure Prophylaxis Guide, (Kentucky Public Health Practice Reference Section, 2010).

Post-exposure prophylaxis is aimed at vaccinating individuals that have possibly been exposed to the rabies virus. 'Have possibly' been exposed is stated as individuals may not be aware they have been exposed or bitten. An example is when a bat is found in the same area such as; a bedroom or tent, of an infant, adult or child (includes intoxicated or mentally disabled individuals). Bats have small sharp teeth and often times the bite is unnoticed. Bite marks from a bat can go unnoticed and often disappear in a short time frame. Post-exposure prophylaxis for individuals that have not been previously immunized for rabies include four intramuscular doses on days 0 (day of first vaccine), 3, 7 and 14 (Centers for Disease Control and Prevention, 2011). Individuals that have previously been immunized and have documentation of virus neutralizing antibody production in response to the initial rabies immunizations will receive two booster doses at days 0 (day of first vaccine) and 3. **Table 1.2**. Rabies immune globulin (RIG) is also administered with post-exposure prophylaxis for individuals that have never previously received rabies prophylaxis. Two types of RIG exist that are administered around the world, human rabies immune globulin (HRIG) and equine rabies immune globulin (ERIG). RIG is an injection that is administered on around the wound as RIG should be infiltrated where the teeth and saliva penetrated into the skin (Centers for Disease Control and Prevention, 2014). "Rabies immune globulin is a sterile solution of antibodies that provides individuals with immediate short-term protection against rabies" (Government of Saskatchewan, 2014, pg. 1). RIG is administered on day 0, the same day the first dose of post-exposure prophylaxis is administered. RIG is administered as the antibodies provide short passive immunity to protect against the rabies virus while the individual's body is in process of producing antibodies from the prophylaxis (Mayo Clinic, 2014). RIG is administered according to body weight, 20 IU/kg (Centers for Disease Control and Prevention, 2013). Outlined below are the three categories that exist to determine if an individual is to receive RIG along with the 4 dose post-exposure prophylaxis. **Table 1.3.**

Immunocompromised individuals receive a 5 dose regimen for post-exposure prophylaxis, administered on days O (day of first vaccine), 3, 7, 14 and 28 (Centers for Disease Control and Prevention, 2011). Immunocompromised individuals also receive one dose of RIG on day 0. For infants and children post-exposure prophylaxis and RIG is the same regimen as adults (Centers for Disease Control and Prevention, 2013). Pregnant women, unless immunocompromised, are directed to follow the 4 dose post-exposure and RIG regimen (Center for Disease Control and Prevention, 2010).

Table 1.3

Categorization of exposure and indications for post-exposure rabies prophylaxis†

Category of Exposure	Type of exposure to a domestic or wild[1] animal suspected or confirmed to be rabid, or animal unavailable for testing	Recommended Post-exposure Prophylaxis
I	Touching or feeding animals Licks on intact skin Contact of intact skin with secretions or excretions of a rabid animal or human case	None, if reliable case history is available
II	Nibbling of uncovered skin Minor scratches or abrasions without bleeding	Administer vaccine immediately[2]. Stop treatment if animal remains healthy throughout an observation period of 10 days[3] or is proven to be negative for rabies by a reliable laboratory using appropriate diagnostic techniques.
III	Single or multiple transdermal bites[4] or scratches, licks on broken skin Contamination of mucous membrane with saliva (i.e. licks) Exposure to bats[5]	Administer rabies vaccine immediately and rabies immunoglobulin, preferable as soon as possible after initiation of post-exposure prophylaxis. Rabies immunoglobulin can be injected up to 7 days after first vaccine dose administration. Stop treatment if animal remains healthy throughout an observation period of 10 days or is proven to be negative for rabies by a reliable laboratory using appropriate diagnostic techniques.

1 Exposure to rodents, rabbits or hares does not routinely require rabies post-exposure prophylaxis.
2 If an n apparently healthy dog or cat in or from a low-risk area is placed under observation, treatment may be delayed.
3 This observation period applies only to dogs and cats. Except for threatened or endangered species, other domestic and wild animals suspected of being rabid should be euthanized and their tissues examined for the presence of rabies antigen by appropriate laboratory techniques.
4 Bites especially on the head, neck, face, hands and genitals are category III exposures because of the rich innervation of these areas.
5 Post-exposure prophylaxis should be considered when contact between a human and a bat.

The first step of defense when bitten or scratched by any animal is proper wound care and management. At the time of the bite or scratch, immediately wash the wound aggressively for several minutes with an abundant amount of soap and water. This action can significantly decrease the risk for rabies as it physically decreases the amount of saliva and rabies virus from the wound (WHO, 2013). Medical consultation immediately follows this crucial first-aid step of washing of the wound for determining the appropriate action. "It is important to remember that rabies is a medical urgency but not an emergency. Decisions should not be delayed" (Centers for Disease Control and Prevention, 2011, pg. 1).

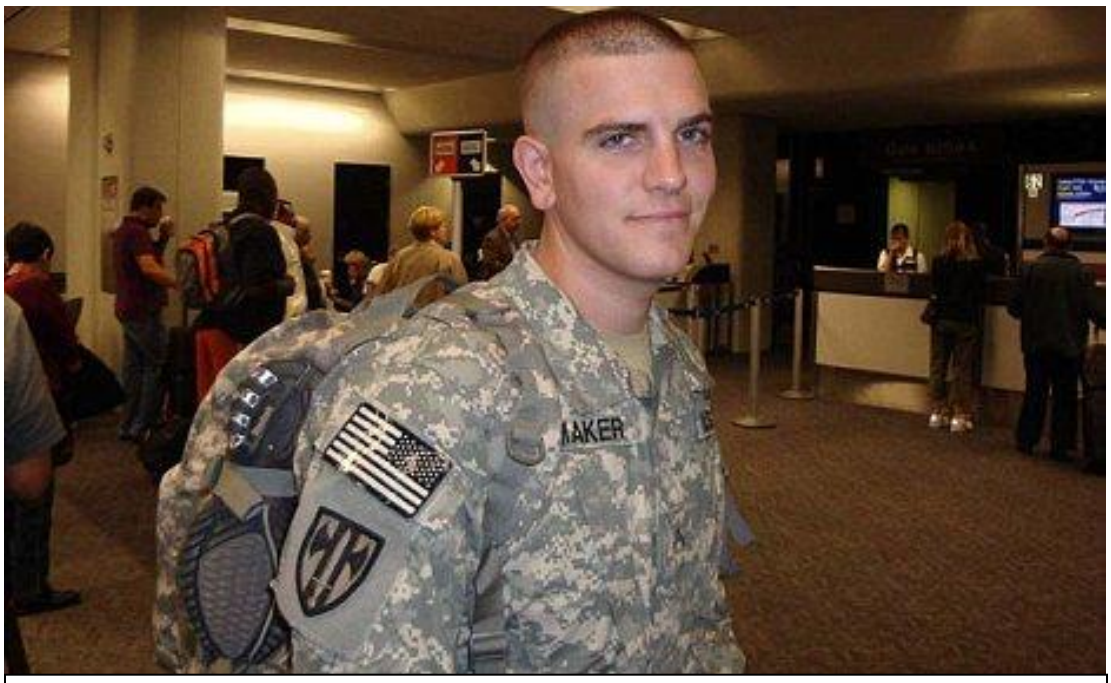
Human Rabies Case

When discussing the rabies virus, pathogenesis, diagnosis and prophylaxis; it is often hard to put it into perceptive the events that take place and how an individual is affected from the rabies virus and why rabies is such an important public health concern. To better understand, a human case from 2011 will illustrate the timeline from exposure to death disclosing the physical characteristics and final impact of the disease for one individual.

In January 2011 Private First Class (Pfc.) Kevin Shumaker, 24 years old, received a bite to his right hand from a feral/community dog while deployed in Afghanistan. **(Figure 1.11)**. As Pfc. Shumaker was preparing for a new military assignment, on August 14th, 2011 he experienced shoulder pain and paresthesia (unexplained burning, tingling or pricking on the skin) during his travel to Fort Drum, New York from Grafenwöhr Germany (Morbidity and Mortality Weekly Report, 2012). Prior to August 14th, 2011 Pfc. Shumaker was medically listed as a healthy individual. Pfc. Shumaker began to have symptoms of nausea, vomiting and fever. August 15th, 2011 Pfc. Shumaker visited an emergency room where he was released (Morbidity and Mortality Weekly Report, 2012). August 15th and 16th, 2011 Pfc. Shumaker visited a chiropractor for the reasons of pain and then August 17th, 2011 Pfc. Shumaker made a second emergency room visit and again was released (Morbidity and Mortality Weekly Report, 2012). The diagnosis for both emergency room visits were listed as gastritis and neck tendonitis. August 18th, 2011 Pfc. Shumaker was having difficulties swallowing and on August 19th, 2011 he was evaluated by the medical team at Fort Drum (Morbidity and Mortality Weekly Report, 2012). At this time Pfc. Shumaker's symptoms were ataxia (lack of muscle control) and syncope

(partial or complete loss of consciousness) in which the medical team at Fort Drum sent him to the same emergency room that he previously visited on two occasions the few days prior (Morbidity and Mortality Weekly Report, 2012). At the time of his arrival Pfc. Shumaker was dehydrated, lucid (easily understood) and hydrophobic and showing signs of aerophobia (Morbidity and Mortality Weekly Report, 2012). Pfc. Shumaker advised he received a bite from a dog while in Afghanistan. As a result of this knowledge and the aforementioned recent medical history, rabies was suspected. The New York State Department of Health (NYSDOH) and the Centers for Disease Control and Prevention (CDC) were then immediately notified. On that same day, August 19th, 2011, Pfc. Shumaker was transported to a second hospital (Morbidity and Mortality Weekly Report, 2012). At the time he arrived he was becoming agitated and combative.

Figure 1.11



Private First Class Kevin Shumaker. Photo taken October 28, 2010 (Mail Online, 2012).

Upon his arrival, serum, saliva, CSF and a nuchal biopsy samples were taken and submitted for rabies analysis. On August 20th and 21st, 2011 the test results revealed rabies virus antigen in the hair follicles from the nuchal biopsy and viral RNA was revealed in the saliva and CSF

(Morbidity and Mortality Weekly Report, 2012). The CDC confirmed that rabies virus antibodies were detected in the serum and CSF. On August 19th, 2011 the Milwaukee Protocol was implemented as a life saving measure for Pfc. Shumaker (Morbidity and Mortality Weekly Report, 2012). Pfc. Shumaker was intubated, his pupils were dilated and his nervous system was not functioning properly. Although no brain abnormalities were present a pacemaker was inserted due to complete heart blockage. On August 20th, 2011 intracranial pressure was monitored by the placement of and external ventricular drain (Morbidity and Mortality Weekly Report, 2012). August 21st, 2011 he suffered from a severe form of diabetes and on August 22nd there was severe brain swelling and acute respiratory distress (Morbidity and Mortality Weekly Report, 2012). Small brain hemorrhaging began on August 28th and on August 30th severe brain hemorrhaging was documented (Morbidity and Mortality Weekly Report, 2012). Family was advised that Pfc. Shumaker would unlikely recover and life support was ceased on August 31st, 2011 (Morbidity and Mortality Weekly Report, 2012).

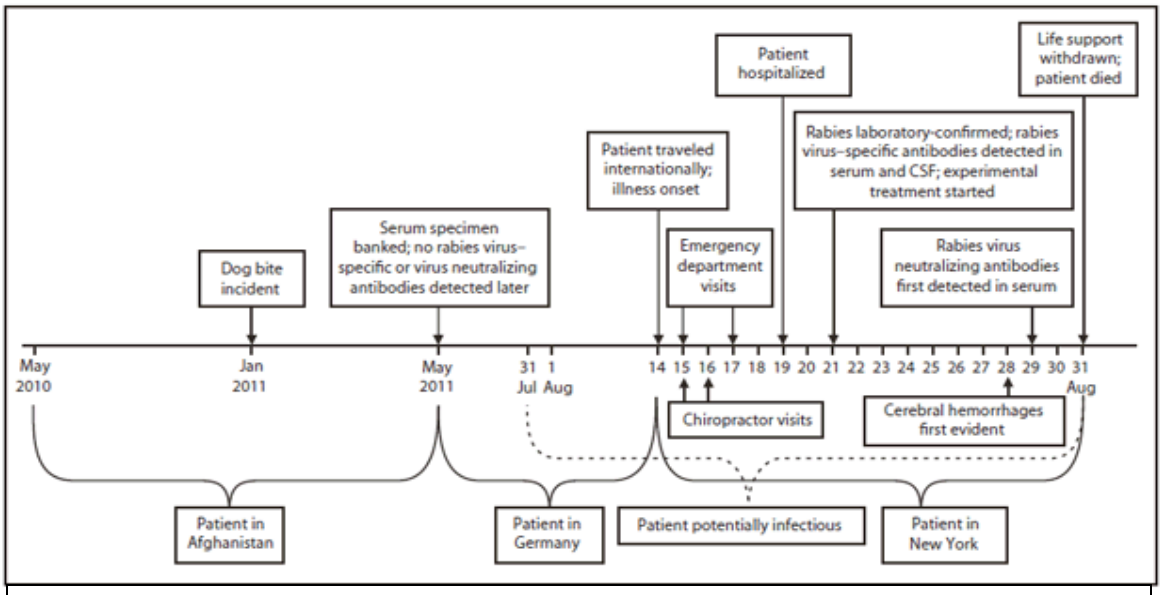
It is noteworthy to mention that a serum sample of Pfc. Shumaker from May 2011 was tested at the CDC in August 2011 and no specific rabies antibodies were found in this sample (Morbidity and Mortality Weekly Report, 2012). This indicates that Pfc. Shumaker did not receive post-exposure prophylaxis or RIG following the dog bite to the right hand.

The timeline of the incidents that occurred from the time Pfc. Shumaker was in Afghanistan to the time of his unfortunate death from the rabies virus is represented below. **Table 1.4.**

Global Burden of Rabies

As stated by the World Organization for Animal Health (OIE), rabies is found on 6 of the 7 world continents, Antarctica is the exception (OIE, 2014). Globally, dogs are the main source of rabies as over 95% of human deaths result from infected dogs (Briggs, 2013). According to current statistics provided by the Partners for Rabies Prevention an average of 61,000 human deaths occur annually from rabies (WHO, 2012). Of those deaths, up to 60% are children under the age of 15 years old (Global Alliance for Rabies Control, 2013). It is known that 84% of the 61,000 human deaths occur in rural areas (WHO, 2012). These rural areas are in "poor, remote regions where healthcare provision is minimal, and reporting even more so, plus awareness among the general population almost non-existent" (Briggs, 2013).

Table 1.4



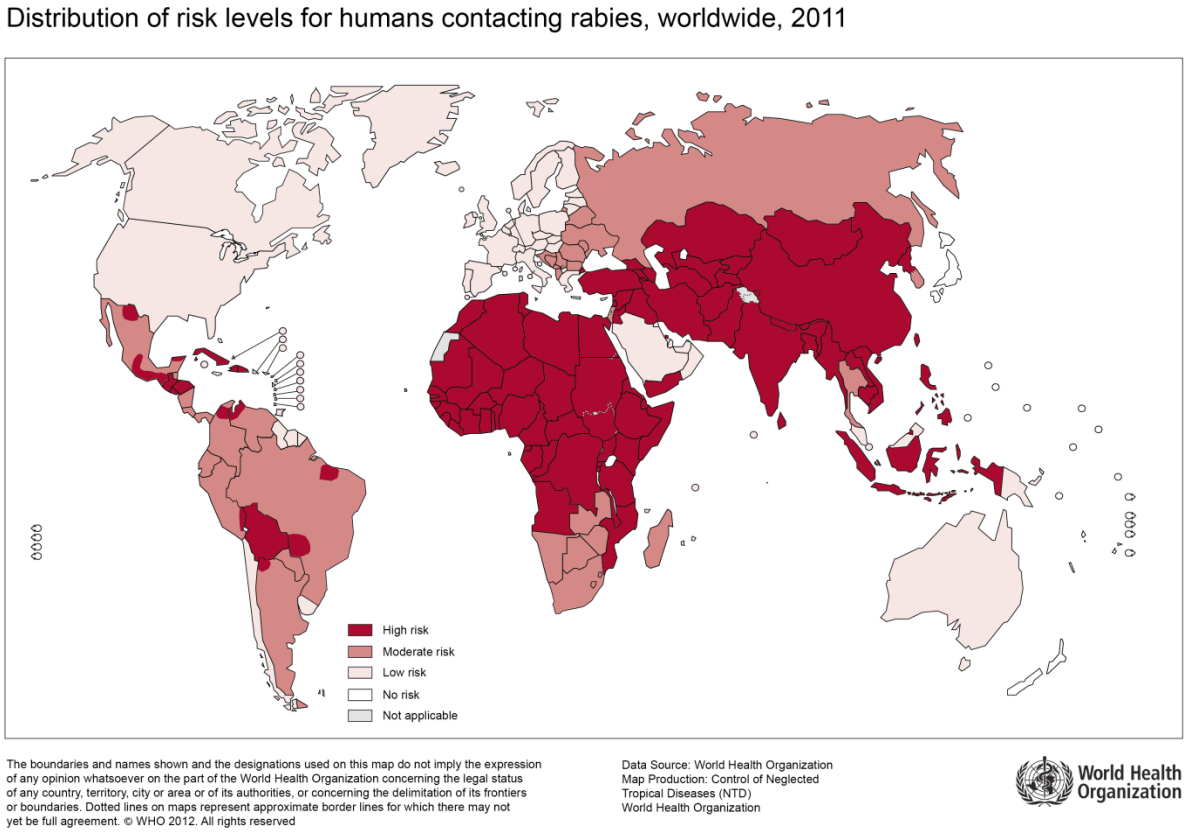
Timeline of events that involved the human rabies case of Pfc. Kevin Shumaker (Morbidity and Mortality Weekly Report - *MMWR*, 2012)

"And because most cases tend to be isolated, the statisticians tend to miss the big picture worldwide, which is that one person dies very eight minutes" (Briggs, 2013). Underreporting, misdiagnosis, poor surveillance and the lack of infrastructure result in an underestimation of knowing the true burden of the disease on a global scale (WHO, 2012). Over 95% of the human deaths from rabies occur in Asia and Africa (Global Alliance for Rabies Control, 2013) with India reporting the highest incidence globally (WHO, 2012). The global distribution of risk for human rabies is depicted below (WHO, 2012). **Figure 1.12.** It is estimated that 3.3 million people live with the risk of rabies every day (Global Alliance for Rabies Control, 2014).

Whilst it is known that rabies impacts life on a physical, social and psychological level, rabies also impacts the world on an economic level. In 2013 it was assessed that canine rabies results in $124 billion USD annually for global economic output (Global Alliance for Rabies Control 2013). Whilst loss of human life is the most devastating impact of rabies, loss of human life also results in the highest financial cost (Global Alliance for Rabies Control, 2013). The financial cost takes into account the deaths and the future lost earnings of the individuals that have died, which is known to directly impact the communities (Global Alliance for Rabies Control, 2013). The next highest financial cost of rabies is the cost of rabies prophylaxis (Global

Alliance for Rabies Control, 2013). In reviewing specific costs around the world, we find that in the United States the CDC estimates that $300 million are spent on rabies prevention annually (WHO, 2012). Although fox rabies has been eliminated from Western Europe, $6.5 billion USD is spent annually for a control program for fox rabies along the eastern boarder of the European Union (WHO, 2012). In Asia, approximately $1.5 billion USD is spent annually on post-exposure prophylaxis (WHO, 2012). In taking a direct look at post-exposure prophylaxis, in Asia and individual pays 3.87% of their gross national income for a full series of post-exposure prophylaxis while and individual in Africa will pay 5.80% (WHO, 2012). To put this into perspective, this constitutes the pay of working 51 days for the individual from Africa and 31 days for the average individual in Asia (WHO, 2012).

Figure 1.12



Global representation of the four risk categories for human rabies by countries or areas. The four risk categories are high, moderate, low, none (World Health Organization, 2012).

In looking back to the year 1985, it is documented that Latin America and the Caribbean had an annual cost of $30 million USD from loss of livestock directly resulting from vampire bat rabies.

The economic cost of rabies is substantial. "Rabies is the deadliest disease on earth with a 99.9% fatality rate" (Global Alliance for Rabies Control, 2014), and due to the result of growing populations of humans and canines around the world the economic cost of rabies along with the burden of human deaths will also continue to grow (WHO, 2012).

Rabies-Free/Controlled Countries and Political Units

Rabies directly impacts the world both physically and economically. Whilst the disease can detrimentally affect certain regions of the world, some areas of the world have successfully eradicated canine rabies, whereas other regions have obtained a status of rabies free, or rabies controlled. In order for a country to be identified as rabies free, or rabies controlled, per the OIE the following provisions must be achieved:

- ✓ The disease is notifiable (reported to appropriate authorities).
- ✓ Effective disease surveillance has been implemented and is in operation.
- ✓ Regulatory measures for control and prevention have been implemented. This is to also include effective importation procedures.
- ✓ Two years free from having an indigenous case of rabies in animals and man. It is noteworthy to mention that isolating Australian or European Bat Lyssavirus would not affect this status.
- ✓ No imported case of carnivores outside of the quarantine station has been confirmed with rabies for the previous six months.

Regions of the world that have achieved and are currently maintaining a rabies-free status are listed below. **Table 1.5.** As aforementioned, the physical and economic cost of rabies is projected at $124 billion USD annually. Do to the severity of the disease, physically and economically, once a region achieves rabies-free status, maintaining such status is vital. The OIE specifically outlines the following recommendations in order to ship dogs and cats into rabies-free regions from countries that are considered infected with rabies:

- ✓ Veterinary authorities must provide an international veterinary certificate that confirms the animal has not shown clinical signs of rabies within the past 48 hours of shipment.

- ✓ The animals must be permanently identified (such as tattoo or microchip) and that identifier must be listed on said certificate.
- ✓ The animal must be vaccinated for rabies, and received not less than six months or more than one year prior to shipment. The initial rabies vaccine should have been administered while the animal was at least 3 months old in age. The vaccine is to be from an inactivated virus vaccine or from a recombinant vaccine that expresses the rabies virus glycoprotein.
- ✓ The animal must be subjected to an antibody test not less than three months or more than twenty four months prior to shipment. The antibody test must reflect that the animal is carrying an antibody response of at least 0.5 IU/mL, which meets WHO recommendations.
- ✓ Animals that have not been vaccinated following the above noted criteria, the importing country can require the animal be placed in quarantine at a quarantine location within that countries territory for six months.

Table 1.5

Regions of the world that are currently rabies-free (Centers for Disease Control and Prevention, 2013)

Region	Countries/Localities
Africa	Cape Verde, Mauritius, Reunion, Sao Tome, and Principe, Seychelles
Americas	North: Bermuda, Saint Pierre and Miquelon Caribbean: Antigua and Barbuda, Aruba The Bahamas, Barbados, Cayman Islands, Dominica, Guadeloupe, Jamaica, Martinique, Montserrat, Netherlands, Antilles, Saint Kitts (Saint Christopher) and Nevis, Saint Lucia, Saint Martin, Saint Vincent and Grenadines, Turks and Caicos, Virgin Islands (UK and US)
Asia and the Middle East	Hong Kong, Japan, Kuwait, Malaysia (Sabah), Qatar, Singapore, United Arab Emirates
Europe[1]	Albania, Austria, Belgium, Corsica, Cyprus, Czech Republic, Denmark, Finland, France, Germany, Gibraltar, Hungary, Iceland, Ireland, Isle of Man, Liechtenstein, Luxembourg, Monaco, Netherlands, Norway (except Svalbard), Portugal, Slovakia, Slovenia, Spain (except Ceuta and Melilla), Sweden, Switzerland, United Kingdom
Oceania[2]	Australia[2], Cook Islands, Fiji, French Polynesia, Guam Hawaii, Kiribati, Micronesia, New Caledonia, New Zealand, Northern Mariana Islands, Palau, Papua New Guinea, Samoa, Vanuatu

1. Bat lyssaviruses have been reported throughout Europe, including areas that are reportedly free of rabies in other wild mammals.
2. Most of Pacific Oceania is reportedly "rabies-free", with the exception of Australia, where lyssaviruses in bats have been reported, as well as fatal human rabies cases.

These recommendation and provisions are strictly enforced and followed throughout the world by governing bodies. In the event an individual is choosing to ship a dog or cat to a rabies-free region these guidelines must be adhered to. Failure to adhere to these guidelines can, and has, resulted in euthanasia of the animal.

CHAPTER 2 – CAPSTONE EXPERIENCE

Rabies Virus Testing Methodologies

Rabies is a public health concern for both, countries that are considered infected with rabies and rabies-free regions. In rabies-free regions, new or previously identified rabies serotypes can be introduced or re-introduced at any time. Until a day comes where rabies is eliminated on a global scale in canines and wildlife, "public health management of humans at risk of exposure to rabies will continue to depend heavily on rabies immunization, including both pre-exposure and post exposure prophylaxis, to prevent infection and death" (Rabies, 2nd Edition, 2007, pg. 548). Continuous efforts of animal rabies vaccination programs are also critical in public health management for rabies prevention and control in which herd immunity is essential.

In order to successfully monitor such management programs, utilizing the appropriate testing methodologies to ascertain antibody response is vital. As demonstrated throughout this paper, a variety of scenarios exists in which precisely measuring a rabies immunological response is critical in pre and post mortem subjects. Recall, this is to include evaluating a humoral response to a vaccine, as well as, for diagnosing subjects suspected of having rabies. Throughout the decades, various testing methodologies have been developed. These testing methodologies differ in how they detect rabies virus antibodies along with the ease and practicality at which they are performed (Rabies, 2nd Edition, 2007). "Methods available for the detection and measurement of rabies virus-specific antibodies are either antigen-binding assays or virus-neutralization assays" (Moore, Hanlon, 2010, Pg. 2). The virus neutralizing assays consist of the Rapid Fluorescent Focus Inhibition Test (RFFIT) and the Fluorescent Antibody Virus Neutralization (FAVN) test. Antigen-binding assays consist of Enzyme-Linked Immunosorbent Assay (ELISAs) and Indirect Immunofluorescence Assay (IFAs). It is important to understand how these testing methodologies differ. RFFIT and FAVN identify and measure rabies neutralizing antibodies (virus-neutralizing assay) in a sample. **Figure 2.1**. Whilst ELISAs and IFAs identify and measure antigen-binding antibodies (antigen-binding assay). **Figure 2.2**. To further explain, "the outcome of a virus-neutralizing assay is based on a measurement of virus growth in cell culture, i.e., defining whether virus escapes neutralization or not" (Moore, Hanlon,

2010, Pg. 2). "Antigen-binding assays…are detected, quantified, and characterized by their ability to bind to various rabies virus antigens" (Moore, Hanlon, 2010, Pg. 2). As these two testing methodologies; virus-neutralizing assays and antigen-binding assays; differ in their identifications and measurements, the results (measured in IU/mL) of each assay should not be equally comparable to one another. For further understanding, IU/mL is defined as a substance (i.e., antibodies), that are detected (in this scenario, serum or CSF), which result in a biological response (i.e., adhering to the rabies virus) (Dictionary.com, 2014).

In reference to determining sufficient levels of immune response to the rabies virus, the WHO has set global standards that any subject with a result of less than 0.5 IU/mL is not adequately protected against rabies (WHO, 2012). This global standard of 0.5 IU/mL is currently utilized for sample results for both virus-neutralizing assays and antigen-binding assays. The 0.5 IU/mL standard was set by a working group in 1978 at the Joint WHO/IABS Symposium after reviewing several clinical trials (Moore, 2013). The clinical trials that were reviewed to determine the 0.5 IU/mL standard were tested utilizing virus-neutralizing assays, and the clinical trials were a 'point-in-time' reading, as the samples tested were collected 1 month following vaccination (Moore, 2013). As a result of utilizing the virus-neutralizing assay to set the 0.5 IU/mL standard; along with a point-in-time aspect, the idea of accepting a set global standard of 0.5 IU/mL for other testing methodologies (i.e., ELISAs and IFAs) is inaccurate.

ELISAs have been compared to virus-neutralization assays in which results were found to be similar, and noted as fair to good (Moore, 2013). These findings are not astonishing as each testing methodology measures specific antibody responses. It is important to note that due to the difference between the testing methodologies, results will not be consistent between subjects for reasons of Ig subclasses, various affinities, neutralizing abilities and unique polyclonal responses (Moore, 2013). The Kansas State University Rabies Laboratory previously completed and in-house investigation on comparing a commercial ELISA kit (Bio-Rad Platella Rabies Kit II) with the RFFIT (Moore, 2013). The KSU Rabies Laboratory utilized clinical trial samples, over set periods of time, just as the working group identified the 0.5 IU/mL for global standards (Moore, 2013). The result of this in-house investigation echoes the concept that utilizing the 0.5 IU/mL standard for the two assays (virus-neutralization and antigen-binding) "…will never result in agreement for all individuals" (Moore, 2013, Poster). It was further stated that "evaluating the kinetics of the RVNA/anti-glycoprotein response, combined with laboratory validation of the

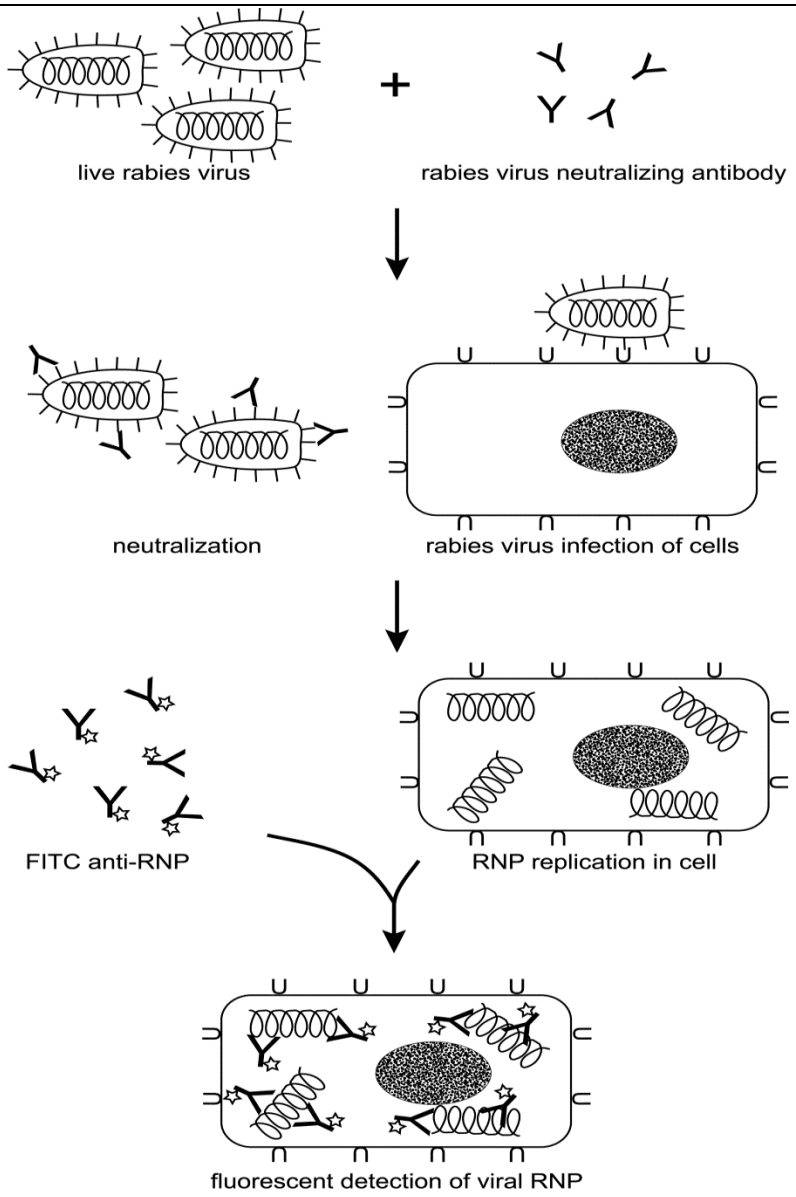
specific test method and consideration of the use of the results (vaccine efficacy, determination of booster, detection of vaccine bait-uptake) is a logical approach for setting a useful cut-off for *both* RFFIT and ELISA methods" (Moore, 2013, Poster). Once more, evidence supports that the two assay testing methodologies(virus-neutralizing and antigen-binding), although similar, consist of substantial differences to warrant further investigation on determining appropriate standards for each assay as one global standard is not adequate.

Investigating the Performance of a Rabies ELISA

In looking further into the analysis of appropriate cut-off values for *both* virus-neutralization assays and antigen-binding assays, I had the unique opportunity to partner up with Zoetis to investigate their commercial Indirect ELISA. Zoetis, a global leading animal health company, brought forward their commercial Indirect ELISA kit (SERELISA® Rabies Ab Mono Indirect) to investigate the performance of a rabies ELISA testing methodology to determine adequate immune response levels to rabies vaccines. This investigation also allows for further exploration of the differing characteristics of the testing methodologies and to explore potential differing cut-off values, as aforementioned.

The Zoetis Indirect ELISA is a commercially available ELISA kit designed to detect anti-rabies antibodies in canine and feline serum samples (Zoetis, 2014). **(Figure 2.3)**. The indirect ELISA is quantitative, in which results are interpreted in comparison to a standard curve to precisely calculate the antibody concentration in each sample well. Quantitative analysis is interpreted versus qualitative, qualitative simply indicates by color change if the antibody is present (at a pre-determined statistical level) in the sample well. This kit also adheres to the WHO global standard in which a minimum of 0.5 IU/mL result is required to determine adequate protection per sample, as aforementioned. You shall recall that the WHO standards were determined by results from virus-neutralizing assays, and this ELISA is an antigen-binding assay. To date, the WHO does not have adequate recommendations for immune response levels for ELISAs (antigen-binding assays). The kit insert outlines the contents included, steps and dilutions required to perform the ELISA along with how to interpret results (Appendix A) (Zoetis, 2014).

Figure 2.1



Rabies neutralization assays involve mixing of fixed amount of live rabies virus with serum containing RVNA antibodies (Y shapes) and allowing neutralization to occur through interaction of rabies virus neutralizing antibodies with the rabies virus glycoprotein (spikes). Non-neutralized rabies virus infects cells added to the mixture. Rabies virus in the infected cells in detected with fluorescence (Y shape with star attached) (Moore, 2007).

Figure 2.2

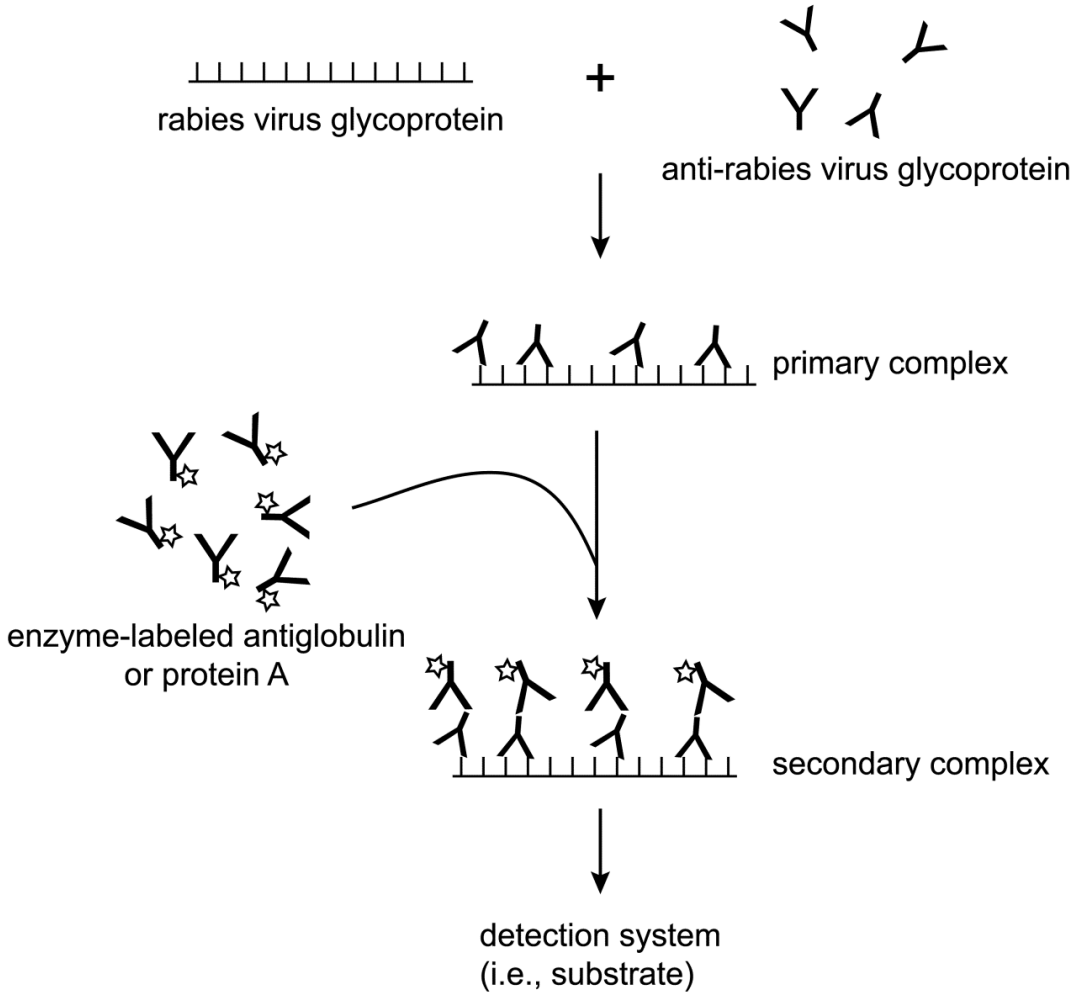


Illustration of the binding assay in an indirect ELISA using rabies virus glycoprotein (short straight vertical lines) bound to microtiter wells (bottom horizontal line). Presence of rabies virus glycoprotein binding antibodies (Y-shaped symbols) is detected by an enzyme-labeled antiglobulin or *Staphlococcus aureus* protein A (Y shapes with star attached). A detection system involving the addition of an enzyme substrate leads to a color change (Moore, 2007).

The long term objective for the investigation of the SERELISA® Rabies Ab Mono Indirect ELISA is dual in nature. The first objective will investigate the performances of this ELISA on human sample origin for the appreciation of the protection status obtained after Rabies vaccination. The second objective is to establish standardization curves and appreciate the

robustness of the test when using different reference sera (WHO-1, WHO-2, OIE and Kansas State University). The capstone portion of this paper will focus on the initial phases of testing the different reference sera (WHO-1, WHO-2, OIE and KSU). The investigating of the performances of this ELISA on human samples will directly follow; however, will not fall within the timeline of this paper. Investigation of the SERELISA® Rabies Ab Mono Indirect ELISA was performed at the Kansas State Veterinary Diagnostic Rabies Laboratory in Manhattan, KS. To being the process of working with Zoetis, an IRB (Research Involving Human Subjects) application was required for approval through Kansas State University (KSU). The IRB application (Appendix B) was submitted, reviewed, investigated and approved (IRB #7012) by the KSU Committee for Research Involving Human Subjects on January 27, 2014. In conjunction with the IRB application process a USDA (United States Department of Agriculture) Veterinary Biological Product Permit was pursued. The USDA Veterinary Biological Product Permit (Appendix C) for Research and Evaluation was approved on November 22, 2013. The approved IRB and USDA permits were forwarded to Zoetis. Immediately following, I then began to draft a protocol for this project, as required by KSU and Zoetis. Title of the protocol is "Validating And Standardization Of The Synbiotics Rabies ELISA Test, SERELISA® Rabies Ab MONO Indirect, For Detection Of Anti-Rabies Antibodies In Individual Human Serum". The drafted protocol (Appendix D) was submitted to the KSU Rabies Laboratory Director and Zoetis Director of Global Marketing for review. Upon acceptance of the drafted protocol Zoetis then moved forward internally to secure a set number of the ELISA kits to be shipped directly to the KSU Rabies Laboratory. The laboratory received the ELISA kits Monday morning, March 31st. A conference call between me and the KSU Rabies Laboratory Director and Zoetis Director took place that Monday afternoon. After some 'tweaking' of the outlined microplates in the protocol, testing of the reference sera on the ELISA plates began that same afternoon. Testing of the reference sera on the Indirect ELISA continued beyond the time of writing this paper. The preparations and results that can be shared up to the end point of this paper are discussed.

Figure 2.3

Photograph of the Zoetis SERELISA® Rabies Ab Mono Indirect Kit and its contents per kit (Zoetis, 2014).

The initial physical preparation for the first test, and all consecutive tests to follow; consisted of attaining stock samples of the reference sera (WHO-1, WHO-2, OIE, and KSU). The stock reference sera were provided, as were all additional supplies not provided within the contents of the Indirect ELISA kit, by the KSU Rabies Laboratory. For documentation purposes, the titration and label of each reference sera (WHO-1, WHO-2, OIE, and KSU) are identified. **Table 2.1.** Each stock reference sera (WHO-1, WHO-2, OIE, and KSU) was reconstituted and diluted to the serial dilutions listed below. **Table 2.2.** The reference sera and their serial dilutions, as illustrated in Table 2.2, will be utilized to validate the Indirect ELISA and determine which reference sera's will be utilized alongside the human serum samples when tested.
The microplate set up for the first ELISA run (identified as plate 10 – 1st half) was set up as follows, with the expected run results below. **Table 2.3.**

Table 2.1

Reference Sera	**IU/mL**	**Label**
OIE	6.7	Batch 3
WHO-1	59.0	Lot R3 US Standard
WHO-2	30.0	RAI
KSU	59.0	TRP-1

Titration and label identifier for each reference sera utilized for the SERELISA® Rabies Ab MONO Indirect ELISA (Moore, 2014).

Table 2.2

	OIE	**WHO-1**	**WHO-2**	**KSU**
IU/mL	6.7	59.0	30.0	59.0 → 17.0
Dilution 1	6.7	5.9	6.0	6.0
Dilution 2	2.23	1.97	2.0	2.0
Dilution 3	0.67	0.59	0.6	0.6
Dilution 4	0.447	0.39	0.4	0.4
Dilution 5	0.223	0.197	0.2	0.2
Dilution 6	0.067	0.059	0.06	0.06
Dilution 7	0.0223	0.0197	0.02	0.02

Reconstitution and serial dilutions of each reference sera utilized for the SERELISA® Rabies Ab MONO Indirect ELISA (Moore, 2014).

Table 2.3

	1	2	3	4	5	6
A	N 1:10	N 1:10	WHO1 1:300,000	WHO1 1:300,000	KSU Ref #1 1:100	KSU Ref #1 1:100
B	P 1:10	P 1:10	OIE 1:100	OIE 1:300	KSU Ref #2 1:100	KSU Ref #2 1:100
C	WHO1 1:1,000	WHO1 1:1,000	OIE 1:1,000	OIE 1:1,500	KSU Ref #3 1:100	KSU Ref #3 1:100
D	WHO1 1:3,000	WHO1 1:3,000	OIE 1:3,000	OIE 1:10,000	KSU Ref #4 1:100	KSU Ref #4 1:100
E	WHO1 1:10,000	WHO1 1:10,000	OIE 1:30,000	WHO2 1:500	KSU Ref #1 1:200	KSU Ref #1 1:200
F	WHO1 1:15,000	WHO1 1:15,000	WHO2 1:1,500	WHO2 1:5,000	KSU Ref #2 1:200	KSU Ref #2 1:200
G	WHO1 1:30,000	WHO1 1:30,000	WHO2 1:7,500	WHO2 1:15,000	KSU Ref #3 1:200	KSU Ref #3 1:200
H	WHO1 1:100,000	WHO1 1:100,000	WHO2 1:50,000	WHO2 1:150,000	KSU Ref #4 1:200	KSU Ref #4 1:200

	1	2	3	4	5	6
A	N 1:10	N 1:10	0.0197 IU/ml	0.0197 IU/ml	0.15 IU/ml	0.15 IU/ml
B	P 1:10	P 1:10	6.7 IU/ml	2.23 IU/ml	2.8 IU/ml	2.8 IU/ml
C	5.9 IU/ml	5.9 IU/ml	0.67 IU/ml	0.447 IU/ml	0.5 IU/ml	0.5 IU/ml
D	1.97 IU/ml	1.97 IU/ml	0.223 IU/ml	0.067 IU/ml	0.1 IU/ml	0.1 IU/ml
E	0.59 IU/ml	0.59 IU/ml	0.0223 IU/ml	6 IU/ml	7.5 IU/ml	7.5 IU/ml
F	0.39 IU/ml	0.39 IU/ml	0.2 U/ml	0.6 IU/ml	1.4 IU/ml	1.4 IU/ml
G	0.197 IU/ml	0.197 IU/ml	0.4 IU/ml	0.2 IU/ml	0.25 IU/ml	0.25 IU/ml
H	0.059 IU/ml	0.059 IU/ml	0.06 IU/ml	0.02 IU/ml	0.05 IU/ml	0.05 IU/ml

Plate 10, 1st Half, Set up and expected results.

Upon completion of the test procedures, each plate is placed into an automated plate reader, in which is the optical density (OD) is measured bichromatically (450 and 630 nm) (Zoetis, 2014). The data from the automated plate reader was submitted directly to Zoetis to incorporate into their program for interpretation and review. Upon interpretation and review, it was noted that the end results of the plate was not as expected. It was discussed per telephone that the WHO-1

and WHO-2 serial dilutions were too similar in their results; no other detailed points were noted. This outcome resulted in making changes to the design of plate 1 listed in the protocol. Plate 1 was now to be set up as with updated serial dilution for the WHO-1 reference sera. **Table 2.4.** It was determined that Plate 2 would remain as projected in the protocol. **Table 2.5**. The new plate scheme for Plate 1 and the original plate scheme for Plate 2 and their expected results are:

Table 2.4

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1:10	N 1:10	OIEa 1:30,000	OIEa 1:30,000	WHO1 1:150,000	WHO1 1:300,000	WHO2 1:75,000	WHO2 1:150,000	KSU 1:150,000	KSU 1:300,000	OIEa 1:30,000	OIEa 1:30,000
B	P 1:10	P 1:10	KSU Ref#1 1:100	KSU Ref#1 1:100	KSU Ref#2 1:100	KSU Ref#2 1:100	KSU Ref#3 1:100	KSU Ref#3 1:100	KSU Ref#4 1:100	KSU Ref#4 1:100	WHO1 1:150,000	WHO1 1:300,000
C	OIEa 1:100	OIEa 1:100	WHO1 1:500	WHO1 1:1,000	WHO2 1:250	WHO2 1:500	KSU 1:500	KSU 1:1,000	OIEa 1:100	OIEa 1:100	WHO1 1:500	WHO1 1:1,000
D	OIEa 1:300	OIEa 1:300	WHO1 1:1,500	WHO1 1:3,000	WHO2 1:750	WHO2 1:1,500	KSU 1:1,500	KSU 1:3,000	OIEa 1:300	OIEa 1:300	WHO1 1:1,500	WHO1 1:3,000
E	OIEa 1:1,000	OIEa 1:1,000	WHO1 1:5,000	WHO1 1:10,000	WHO2 1:2,500	WHO2 1:5,000	KSU 1:5,000	KSU 1:10,000	OIEa 1:1,000	OIEa 1:1,000	WHO1 1:5,000	WHO1 1:10,000
F	OIEa 1:1,500	OIEa 1:1,500	WHO1 1:7,500	WHO1 1:15,000	WHO2 1:3,750	WHO2 1:7,500	KSU 1:7,500	KSU 1:15,000	OIEa 1:1,500	OIEa 1:1,500	WHO1 1:7,500	WHO1 1:15,000
G	OIEa 1:3,000	OIEa 1:3,000	WHO1 1:15,000	WHO1 1:30,000	WHO2 1:7,500	WHO2 1:15,000	KSU 1:15,000	KSU 1:30,000	OIEa 1:3,000	OIEa 1:3,000	WHO1 1:15,000	WHO1 1:30,000
H	OIEa 1:10,000	OIEa 1:10,000	WHO1 1:50,000	WHO1 1:100,000	WHO2 1:25,000	WHO2 1:50,000	KSU 1:50,000	KSU 1:100,000	OIEa 1:10,000	OIEa 1:10,000	WHO1 1:50,000	WHO1 1:100,000

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1:10	N 1:10	0.0223 IU/ml	0.0223 IU/ml	0.0197 IU/ml	0.0197 IU/ml	0.02 IU/ml	0.02 IU/ml	0.0197 IU/ml	0.0197 IU/ml	0.0223 IU/ml	0.0223 IU/ml
B	P 1:10	P 1:10	15.0 IU/ml	15.0 IU/ml	2.8 IU/ml	2.8 IU/ml	0.5 IU/ml	0.5 IU/ml	0.1 IU/ml	0.1 IU/ml	0.0197 IU/ml	0.0197 IU/ml
C	6.7 IU/ml	6.7 IU/ml	5.9 IU/ml	5.9 IU/ml	6 IU/ml	6 IU/ml	5.9 IU/ml	5.9 IU/ml	6.7 IU/ml	6.7 IU/ml	5.9 IU/ml	5.9 IU/ml
D	2.23 IU/ml	2.23 IU/ml	1.97 IU/ml	1.97 IU/ml	2 IU/ml	2 IU/ml	1.97 IU/ml	1.97 IU/ml	2.23 IU/ml	2.23 IU/ml	1.97 IU/ml	1.97 IU/ml
E	0.67 IU/ml	0.67 IU/ml	0.59 IU/ml	0.59 IU/ml	0.6 IU/ml	0.6 IU/ml	0.59 IU/ml	0.59 IU/ml	0.67 IU/ml	0.67 IU/ml	0.59 IU/ml	0.59 IU/ml
F	0.447 IU/ml	0.447 IU/ml	0.39 IU/ml	0.39 IU/ml	0.4 IU/ml	0.4 IU/ml	0.39 IU/ml	0.39 IU/ml	0.447 IU/ml	0.447 IU/ml	0.39 IU/ml	0.39 IU/ml
G	0.223 IU/ml	0.223 IU/ml	0.197 IU/ml	0.197 IU/ml	0.2 IU/ml	0.2 IU/ml	0.197 IU/ml	0.197 IU/ml	0.223 IU/ml	0.223 IU/ml	0.197 IU/ml	0.197 IU/ml
H	0.067 IU/ml	0.067 IU/ml	0.059 IU/ml	0.059 IU/ml	0.06 IU/ml	0.06 IU/ml	0.059 IU/ml	0.059 IU/ml	0.067 IU/ml	0.067 IU/ml	0.059 IU/ml	0.059 IU/ml

Plate 1 – Set Up & Expected Results

Table 2.5

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1:10	N 1:10	OIEa 1:30,000	OIEa 1:30,000	WHO1 1:300,000	WHO1 1:300,000	WHO2 1:150,000	WHO2 1:150,000	KSU 1:300,000	KSU 1:300,000	WHO2 1:150,000	WHO2 1:150,000
B	P 1:10	P 1:10	KSU Ref#1 1:100	KSU Ref#1 1:100	KSU Ref#2 1:100	KSU Ref#2 1:100	KSU Ref#3 1:100	KSU Ref#3 1:100	KSU Ref#4 1:100	KSU Ref#4 1:100	KSU 1:300,000	KSU 1:300,000
C	OIEa 1:100	OIEa 1:100	WHO1 1:1,000	WHO1 1:1,000	WHO2 1:500	WHO2 1:500	KSU 1:1,000	KSU 1:1,000	WHO2 1:500	WHO2 1:500	KSU 1:1,000	KSU 1:1,000
D	OIEa 1:300	OIEa 1:300	WHO1 1:3,000	WHO1 1:3,000	WHO2 1:1,500	WHO2 1:1,500	KSU 1:3,000	KSU 1:3,000	WHO2 1:1,500	WHO2 1:1,500	KSU 1:3,000	KSU 1:3,000
E	OIEa 1:1,000	OIEa 1:1,000	WHO1 1:10,000	WHO1 1:10,000	WHO2 1:5,000	WHO2 1:5,000	KSU 1:10,000	KSU 1:10,000	WHO2 1:5,000	WHO2 1:5,000	KSU 1:10,000	KSU 1:10,000
F	OIEa 1:1,500	OIEa 1:1,500	WHO1 1:15,000	WHO1 1:15,000	WHO2 1:7,500	WHO2 1:7,500	KSU 1:15,000	KSU 1:15,000	WHO2 1:7,500	WHO2 1:7,500	KSU 1:15,000	KSU 1:15,000
G	OIEa 1:3,000	OIEa 1:3,000	WHO1 1:30,000	WHO1 1:30,000	WHO2 1:15,000	WHO2 1:15,000	KSU 1:30,000	KSU 1:30,000	WHO2 1:15,000	WHO2 1:15,000	KSU 1:30,000	KSU 1:30,000
H	OIEa 1:10,000	OIEa 1:10,000	WHO1 1:100,000	WHO1 1:100,000	WHO2 1:50,000	WHO2 1:50,000	KSU 1:100,000	KSU 1:100,000	WHO2 1:50,000	WHO2 1:50,000	KSU 1:100,000	KSU 1:100,000

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1:10	N 1:10	0.0223 IU/ml	0.0223 IU/ml	0.0197 IU/ml	0.0197 IU/ml	0.02 IU/ml	0.02 IU/ml	0.0197 IU/ml	0.0197 IU/ml	0.02 IU/ml	0.02 IU/ml
B	P 1:10	P 1:10	15.0 IU/ml	15.0 IU/ml	2.8 IU/ml	2.8 IU/ml	0.5 IU/ml	0.5 IU/ml	0.1 IU/ml	0.1 IU/ml	0.0197 IU/ml	0.0197 IU/ml
C	6.7 IU/ml	6.7 IU/ml	5.9 IU/ml	5.9 IU/ml	6 IU/ml	6 IU/ml	5.9 IU/ml	5.9 IU/ml	6 IU/ml	6 IU/ml	5.9 IU/ml	5.9 IU/ml
D	2.23 IU/ml	2.23 IU/ml	1.97 IU/ml	1.97 IU/ml	2 IU/ml	2 IU/ml	1.97 IU/ml	1.97 IU/ml	2 IU/ml	2 IU/ml	1.97 IU/ml	1.97 IU/ml
E	0.67 IU/ml	0.67 IU/ml	0.59 IU/ml	0.59 IU/ml	0.6 IU/ml	0.6 IU/ml	0.59 IU/ml	0.59 IU/ml	0.6 IU/ml	0.6 IU/ml	0.59 IU/ml	0.59 IU/ml
F	0.447 IU/ml	0.447 IU/ml	0.39 IU/ml	0.39 IU/ml	0.4 IU/ml	0.4 IU/ml	0.39 IU/ml	0.39 IU/ml	0.4 IU/ml	0.4 IU/ml	0.39 IU/ml	0.39 IU/ml
G	0.223 IU/ml	0.223 IU/ml	0.197 IU/ml	0.197 IU/ml	0.2 IU/ml	0.2 IU/ml	0.197 IU/ml	0.197 IU/ml	0.2 IU/ml	0.2 IU/ml	0.197 IU/ml	0.197 IU/ml
H	0.067 IU/ml	0.067 IU/ml	0.059 IU/ml	0.059 IU/ml	0.06 IU/ml	0.06 IU/ml	0.059 IU/ml	0.059 IU/ml	0.06 IU/ml	0.06 IU/ml	0.059 IU/ml	0.059 IU/ml

Plate 2 – Set Up & Expected Results

Upon completion of the test performance of Plate 1 and 2, the data identified (OD readings) from the automated plate reader was again forwarded to Zoetis for interpretation and review. Consultation revealed that while the plates consisted of good, consistent and clean results throughout, an excess of variation was detected between a few of the duplicated serial dilutions than projected. This resulted in one validation between the reference sera. In order for any of the reference sera to be validated on the microplate, a >.0.95 of the correlation coefficient between the Neperian logarithm (ln) ODs and in ln Rabies Ab concentrations for the reference sera is needed. The values attained were identified at 86.2, 97.2, 94.8, 83.8, 91.7 and 88.3 for Plate 1 and 92.6, 94.3, 87.9, 81.1, 85.1 and -19.5 for Plate 2. Several values were in reach of the >.0.95 needed for validation, with one reference sera reaching the validation point. These results led to further discussion, in which it was decided that these two microplates would be re-run whilst removing some of the reference sera and changing the WHO-1 and WHO-2 dilutions,

cutting the dilutions in half. The second run for Plate 1 and Plate 2 were now identified with the following reference sera and dilutions for the WHO-1 and WHO-2. **Table 2.6.**

Table 2.6

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1:10	N 1:10	OIEa 1:30,000	OIEa 1:30,000	WHO1 1:150,000	WHO1 1:150,000	WHO2 1:75,000	WHO2 1:75,000	KSU 1:150,000	KSU 1:50,000	OIEa 1:30,000	OIEa 1:30,000
B	P 1:10	P 1:10	KSU Ref#1 1:100	KSU Ref#1 1:100	KSU Ref#2 1:100	KSU Ref#2 1:100	KSU Ref#3 1:100	KSU Ref#3 1:100	KSU Ref#4 1:100	KSU Ref#4 1:100	WHO1 1:150,000	WHO1 1:150,000
C	OIEa 1:100	OIEa 1:100	WHO1 1:500	WHO1 1:500	WHO2 1:250	WHO2 1:250	KSU 1:500	KSU 1:500	OIEa 1:100	OIEa 1:100	WHO1 1:500	WHO1 1:500
D	OIEa 1:300	OIEa 1:300	WHO1 1:1,500	WHO1 1:1,500	WHO2 1:750	WHO2 1:750	KSU 1:1,500	KSU 1:1,500	OIEa 1:300	OIEa 1:300	WHO1 1:1,500	WHO1 1:1,500
E	OIEa 1:1,000	OIEa 1:1,000	WHO1 1:5,000	WHO1 1:5,000	WHO2 1:2,500	WHO2 1:2,500	KSU 1:5,000	KSU 1:5,000	OIEa 1:1,000	OIEa 1:1,000	WHO1 1:5,000	WHO1 1:5,000
F	OIEa 1:1,500	OIEa 1:1,500	WHO1 1:7,500	WHO1 1:7,500	WHO2 1:3,750	WHO2 1:3,750	KSU 1:7,500	KSU 1:7,500	OIEa 1:1,500	OIEa 1:1,500	WHO1 1:7,500	WHO1 1:7,500
G	OIEa 1:3,000	OIEa 1:3,000	WHO1 1:15,000	WHO1 1:15,000	WHO2 1:7,500	WHO2 1:7,500	KSU 1:15,000	KSU 1:15,000	OIEa 1:3,000	OIEa 1:3,000	WHO1 1:15,000	WHO1 1:15,000
H	OIEa 1:10,000	OIEa 1:10,000	WHO1 1:50,000	WHO1 1:50,000	WHO2 1:25,000	WHO2 1:25,000	KSU 1:50,000	KSU 1:50,000	OIEa 1:10,000	OIEa 1:10,000	WHO1 1:50,000	WHO1 1:50,000

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1:10	N 1:10	OIEa 1:30,000	OIEa 1:30,000	WHO1 1:150,000	WHO1 1:150,000	WHO2 1:75,000	WHO2 1:75,000	KSU 1:150,000	KSU 1:50,000	WHO2 1:75,000	WHO2 1:75,000
B	P 1:10	P 1:10	KSU Ref#1 1:100	KSU Ref#1 1:100	KSU Ref#2 1:100	KSU Ref#2 1:100	KSU Ref#3 1:100	KSU Ref#3 1:100	KSU Ref#4 1:100	KSU Ref#4 1:100	KSU 1:500	KSU 1:500
C	OIEa 1:100	OIEa 1:100	WHO1 1:500	WHO1 1:500	WHO2 1:250	WHO2 1:250	KSU 1:500	KSU 1:500	WHO2 1:250	WHO2 1:250	KSU 1:1,500	KSU 1:1,500
D	OIEa 1:300	OIEa 1:300	WHO1 1:1,500	WHO1 1:1,500	WHO2 1:750	WHO2 1:750	KSU 1:1,500	KSU 1:1,500	WHO2 1:750	WHO2 1:750	KSU 1:5,000	KSU 1:5,000
E	OIEa 1:1,000	OIEa 1:1,000	WHO1 1:5,000	WHO1 1:5,000	WHO2 1:2,500	WHO2 1:2,500	KSU 1:5,000	KSU 1:5,000	WHO2 1:2,500	WHO2 1:2,500	KSU 1:7,500	KSU 1:7,500
F	OIEa 1:1,500	OIEa 1:1,500	WHO1 1:7,500	WHO1 1:7,500	WHO2 1:3,750	WHO2 1:3,750	KSU 1:7,500	KSU 1:7,500	WHO2 1:3,750	WHO2 1:3,750	KSU 1:15,000	KSU 1:15,000
G	OIEa 1:3,000	OIEa 1:3,000	WHO1 1:15,000	WHO1 1:15,000	WHO2 1:7,500	WHO2 1:7,500	KSU 1:15,000	KSU 1:15,000	WHO2 1:7,500	WHO2 1:7,500	KSU 1:50,000	KSU 1:50,000
H	OIEa 1:10,000	OIEa 1:10,000	WHO1 1:50,000	WHO1 1:50,000	WHO2 1:25,000	WHO2 1:25,000	KSU 1:50,000	KSU 1:50,000	WHO2 1:25,000	WHO2 1:25,000	KSU 1:150,000	KSU 1:150,000

New Scheme for plate number 1 and plate number 2

Upon the completion of test performance for Plate 1 and Plate 2, second run, the OD values from the automated plate reader was submitted to Zoetis for interpretation and review. The overall results of this run reflected that the WHO-1 and WHO-2 reference sera gave improved end results. Recall, that in order for any of the reference sera to be validated on the microplate, a > 0.95 of the correlation coefficient between the Neperian logarithm (ln) ODs and in ln Rabies Ab concentrations for the reference sera is needed. The ending values attained in this run were identified at 93.2, 94.6, 93.1, 86.9, and 93.5 for Plate 1 and 96.9, 94.8, 89.0, 84.4, 90.9 and -13.6 for Plate 2. Concern did not weigh heavily on the -13.6 result as this reference sera has been

performing in the 80 and 90th percentile in all consecutive runs. Also note, one of the reference sera was above the 0.95 in Plate 1, resulting in a second validation result. In discussing this run per telephone, Zoetis, KSU and I discussed some possible methods to slightly alter the next run to obtain ideal results. Items such as performing plates in parallel with multiple technicians, additional plate washings, and running the duplicate reference sera in triplicate were discussed. As discussions pursued, it was identified that the plate washing steps were performed manually, verses by automated machine. This reflection led to further discussion of the differences in the end results that might be attained by utilizing the automated plate washer. It is believed that the automated plate washer would remove additional excess 'loose' antibodies, which would result in a more accurate reading of the OD values. The decision was then unanimously made to run a microplate with the duplicate reference sera (WHO-1 and WHO-2) in a series of three with both manual and electronic wash cycles. The first duplicate set of WHO-1 and WHO-2 would be washed manually, the second duplicate set of WHO-1 and WHO-2 would be washed by the automated plate washer, and the third duplicate set of WHO-1 and WHO-2 would be washed manually. It is noteworthy to mention that the Indirect ELISA calls for two wash cycles. Thus the second duplicate set of WHO-1 and WHO-2 was to receive two washes from the automated plate washer. As a result of the pate washer not functioning properly, this duplicate set was washed manually for the first wash and then by the automated plate washer for the second wash as the error was resolved. The plate scheme for this run was to be performed as follows. **Table 2.7.**

Table 2.7

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1:10	N 1:10	WHO1 1:150,000	WHO1 1:150,000	WHO1 1:500	WHO1 1:500	WHO2 1:750	WHO2 1:750	WHO1 1:500	WHO1 1:500	WHO2 1:250	WHO2 1:250
B	P 1:10	P 1:10	WHO2 1:250	WHO2 1:250	WHO1 1:1,500	WHO1 1:1,500	WHO2 1:2,500	WHO2 1:2,500	WHO1 1:1,500	WHO1 1:1,500	WHO2 1:750	WHO2 1:750
C	WHO1 1:500	WHO1 1:500	WHO2 1:750	WHO2 1:750	WHO1 1:5,000	WHO1 1:5,000	WHO2 1:3,750	WHO2 1:3,750	WHO1 1:5,000	WHO1 1:5,000	WHO2 1:2,500	WHO2 1:2,500
D	WHO1 1:1,500	WHO1 1:1,500	WHO2 1:2,500	WHO2 1:2,500	WHO1 1:7,500	WHO1 1:7,500	WHO2 1:7,500	WHO2 1:7,500	WHO1 1:7,500	WHO1 1:7,500	WHO2 1:3,750	WHO2 1:3,750
E	WHO1 1:5,000	WHO1 1:5,000	WHO2 1:3,750	WHO2 1:3,750	WHO1 1:15,000	WHO1 1:15,000	WHO2 1:25,000	WHO2 1:25,000	WHO1 1:15,000	WHO1 1:15,000	WHO2 1:7,500	WHO2 1:7,500
F	WHO1 1:7,500	WHO1 1:7,500	WHO2 1:7,500	WHO2 1:7,500	WHO1 1:50,000	WHO1 1:50,000	WHO2 1:75,000	WHO2 1:75,000	WHO1 1:50,000	WHO1 1:50,000	WHO2 1:25,000	WHO2 1:25,000
G	WHO1 1:15,000	WHO1 1:15,000	WHO2 1:25,000	WHO2 1:25,000	WHO1 1:150,000	WHO1 1:150,000			WHO1 1:150,000	WHO1 1:150,000	WHO2 1:75,000	WHO2 1:75,000
H	WHO1 1:50,000	WHO1 1:50,000	WHO2 1:75,000	WHO2 1:75,000	WHO2 1:250	WHO2 1:250						

New plate scheme for the next scheduled run.

The end results of this run and analysis and interpretation by Zoetis were still pending at the time this paper was due to the committee. As I continue to move forward in this project with Zoetis and Kansas State University, we are all optimistic that the final goals and objectives will result in multiple publications.

CHAPTER 3 – CONCLUSIONS & RECOMMENDATIONS

Conclusion

Rabies is a neurological disease that has affected the lives of animals and man for centuries. Rabies is known as the deadliest disease to man with a 99.9% fatality rate (GARC, 2014) and is found on every continent excluding Antarctica (WHO, 2014). This disease cost lives and impacts culture, religion and economics, devastating communities globally. Annually, rabies claims the lives of 61,000 (WHO, 2012) humans, which is grossly under reported as 84% (WHO, 2012) of the deaths occur in rural areas in the poorest countries. Majority of these human deaths, up to 60%, are children ages 15 and younger (GARC, 2013). Asia and Africa result in majority of the human annual deaths, 95% (WHO, 2012) with India having the highest fatality rate (GARC, 2013). Human and animal prophylaxis, in conjunction with proper wound management, have proven to prevent and control the virus, however, limiting factors prevent successful distribution and administration. These factors include lack of government infrastructure, culture and religious beliefs, lack of funding for the vaccines, location of vaccine administration and more. Whilst rabies can occur in all mammals, 95% of human deaths result from infected canines (Briggs, 2013). Rabies is 100% preventable in humans and can be eradicated in canines through successful vaccination programs. Individuals and organizations around the world spend tireless efforts on building and establishing educational rabies programs and vaccine incentives. Although there has been great success in these efforts, it is still not enough as people continue to die from rabies. Rabies cost the world an astonishing $124 billion USD annually (GARC, 2013) and as long as populations continue to grow for humans and canines, the cost and economic burden will also continue to grow (WHO, 2012). As a result of this horrific disease, the importance of prophylaxis and passive immunity are critical in the event of medically managing an exposure, and preventing exposures. Utilizing the appropriate testing methodologies with rigorously defined recommendations to ensure adequate protection against the rabies virus is vital.

Recommendations

As human and canine populations continue to grow, the cost and economic burden will also continue to grow (WHO, 2012). In order to reduce the burden of rabies there is a need for further solid foundation for prevention and control. Canine rabies can be eradicated with proper animal vaccination distribution and administration in conjunction with an ORV program. As obstacles exist for free roaming canines, additional efforts with ORV must be implemented. ORV would provide crucial benefits for saving lives and decreasing the economic burden in the poorest areas. Contraception initiatives with vaccine programs and ORV also promise to enhance the control of rabies globally (Rupprecht, 2011). Minimizing human exposures to infected animals is also essential. Ensuring prompt wound management and proper administration of prophylaxis (and RIG) for exposed individuals is critical. Further use of effective blueprints and international advocacy for prevention and control is needed on a global scale. In reference to research, additional studies are necessary for development of recommendations that are science based. Well-designed studies are needed for additional data in the following areas; potential shedding of virus in milk, PEP protocols for domestic animals, earliest age at which rabies vaccine is most effective, viral shedding periods for domestic livestock and lagomorphs and the ecology of rabies in wildlife species (Compendium of Animal Rabies Prevention and Control, 2011). Further investigation into the differing testing methodologies is also warranted to identify suitable global standards and recommendations for each assay, to ensure immune response levels are adequate for protection against the rabies virus. Rabies is 100% preventable in humans and the eradication of canine rabies is obtainable through vaccine and contraceptive campaigns. Implementing national programs with effective surveillance and control efforts are imperative as is obtaining political commitments globally and implementing additional global standards and recommendations for measuring immune responses.

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APPENDIX A – ZOETIS ELISA KIT INSERT

SYNBIOTICS

SERELISA® Rabies Ab Mono Indirect

KIT FOR THE DETECTION OF ANTI-RABIES ANTIBODIES IN DOG OR CAT INDIVIDUAL SERUM

INDIRECT IMMUNOENZYMATIC TECHNIQUE

192 single well reactions

I. PRINCIPLE OF THE TEST

The Serelisa® Rabies Ab mono indirect kit allows a quantitative detection of Rabies antibodies in individual dog and cat serum samples. A minimum of 0.5 IU/ml Rabies antibodies is required to protect against Rabies infection, according to the World Health Organisation recommendations (WHO, 1992. Expert Committee on Rabies, 8th Report. World Health Organisation, Geneva, Technical Report Series n° 824). The reaction is composed of three steps:

1. Each serum sample is placed in a well sensitised with inactivated Rabies viral antigens. Antibodies present in the sample bind to the viral antigens coated at the bottom of the well.

2. After a wash step, Protein A / peroxidase conjugate is added. It fixes to the immunoglobulins (antibodies) previously captured, forming a complex:

(Rabies Ag) - (Ab anti-Rabies) - (Protein A/ peroxidase)

3. Excess conjugate is eliminated by a wash step. The enzyme linked to the complex is revealed by addition of a substrate which is transformed into a coloured product. After stopping the reaction, the optical densities are measured. The presence or absence of antibodies is determined by using threshold values obtained from the positive control.

II. KIT COMPOSITION AND CONSERVATION

REAGENT NATURE	RECONSTITUTION AND CONSERVATION
Microplate containing six 16-well strips sensitized with Rabies antigens	Use within 4 weeks after opening of the sachet which must be closed after use.
Conjugate : Protein A / peroxidase (CJ) (10X concentrated)	Dilute 10 times in the conjugate diluent and use within 24 hrs following dilution.
Buffered peroxidase substrate (PS)	Ready-to-use.
Negative control (N) (10X concentrated)	Dilute 10 times in the sample diluent and use within 24 hrs following dilution.
Positive control (P) (10X concentrated)	Dilute 10 times in the sample diluent and use within 24 hrs following dilution.
Sample diluent (SD)	Ready-to-use.
Wash solution (W) (10X concentrated)	Dilute 10 times in distilled or demineralized water. Use within 48 hrs following dilution.
Conjugate diluent (CD)	Ready-to-use.
Stop solution (S)	Ready-to-use.
Adhesive film	6 films

Note: Store diluted reagents at +5 ± 3°C and use as mentioned above.

III. MATERIALS AND REAGENTS REQUIRED (NOT SUPPLIED)

- *WHO* reference serum
- Distilled or demineralized water.
- Adjustable or set pipettes to measure and deliver between 0 to 1000 µl. Measurement deviation must be ≤ 10% for volumes ≤10 µl and ≤ 5% for all other volumes.
- Graduated cylinders (100 ml and 1000 ml).
- Manual, automatic or semi-automatic washing device for microtitration plates.
- Microplate reader, fitted with filters for bichromatic reading at 450 and 630 nm. It is also possible to use a monochromatic reader fitted with a 450 nm filter.
- Incubator at +37 ± 3°C.

IV. PRECAUTIONS FOR USE

The quality of the results depends on the respect of good laboratory practices and the procedure (see paragraph VI).

1. Do not mix or associate reagents from kits with different batch numbers
2. Do not use reagents after the expiry date.
3. Place all reagents at laboratory temperature for at least 1 hour prior to use.
4. Handle all reagents and samples as biohazardous material.
5. Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
6. Never pipette by mouth.
7. Avoid inter sample contamination during sample collection, storage or transport. Use separate disposable pipette tips for each sample.
8. Avoid contamination of the substrate solution with metallic ions, oxidizing agents or detergents. Make sure that all containers are clean. Do not use the same container or the same pipette tip for the conjugate and the substrate.
9. It is recommended to dispose reagents and contaminated material according to the applicable regulations. The safety data sheets for the product are available upon request.

Risk and safety phrases:
R23/25: Toxic by inhalation and if swallowed.
R35: Causes severe burns.
R36/37/38: Irritating to eyes, respiratory system and skin.
R41: Risk of serious damage to eyes.
R 42/43: May cause sensitization by inhalation and skin contact.
S7: Keep container tightly closed.
S24: Avoid contact with skin.
S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
S30: Never add water to the product.
S45: In case of accident or if you feel unwell, seek medical advice immediately.

V. SAMPLES

The reaction is performed on heat-inactivated (30 min. +56°C) individual serum diluted at 1:100. Testing the appropriate set of dilutions for the *WHO* standard serum containing 6.7 IU/ml is necessary.
This standard serum is supplied by the *national laboratory of standards NISBC United.*
Serum samples should be stored as follows:

Samples	Cold (+5°C)	Freeze (-20°C)	Lab Temperature (+23°C)
Serum	max. 7 days	Yes	No

VI. PROCEDURE

Strictly comply with the procedure indicated below. Use negative and positive controls in duplicate for each test run, or at least for each plate.

A. **PRELIMINARY STEPS.**

1. Carefully set up the distribution and identification of controls and samples.

2. Prepare the sera to be tested. Dilutions are performed in the kit sample diluent (SD). Dilutions should be performed as follows:
the samples are first pre-diluted at 1:10 in a blank microplate (10 µl of sample in 90 µl of SD)
3. For serum titration, a set of seven dilutions of the WHO standard serum should be performed either in tubes or in microplates with an initial dilution of 1:10 and 1:100 then 1:25, 1:60, 1:80, 1:170, 1:400 and 1:800.
The preparation of he WHO standard serum range of dilutions must be carried out as follows: 1:100 (not deposite in the test plate) : 10 µl WHO 1:10 + 90 µl SD.

WHO dilution	Preparation
1:10	25 µl of WHO + 225 µl of sample diluent SD
1:25	40 µl of 1:10 dil + 60 µl of SD
1:60	25 µl of 1:10 dil + 125 µl of SD
1:80	20 µl of 1:10 dil + 140 µl of SD
1:170	10 µl of 1:10 dil + 160 µl of SD
1:400	25 µl of 1:100 dil + 75 µl of SD
1:800	20 µl of 1:100 dil + 140 µl of SD

This range of dilution of *WHO* standard serum shall be present in every serial and plates.

B. TEST PROCEDURE

I - CONTROLS AND SAMPLES DISTRIBUTION

1. Control distribution:
Controls are not ready-to-use and should be diluted at 1:10.
Dispense 90 µl of sample diluent, and add 10 µl of the kit negative control into wells A1 and A2, and 10 µl of the positive control to wells B1 and B2.

2. Distribution of samples and *WHO* serum dilutions:
Dispense 90 µl of sample diluent, add 10 µl of either 1:10 sample pre-dilution or each WHO serum dilution from 1:10 to 1:800 into the test wells and mix gently.
- Strips should always be placed on the frame so that both washer and reader can be used.
- Cover the wells with adhesive film, cut to the necessary length by the number of strips used.
- Mix the plate manually by gentle shaking or by using a plate agitator.

	1	2	3	4
A	N 1:10	N 1:10	WHO 1:8000	WHO 1:8000
B	P 1:10	P 1:10	S1 1:100	S1 1:100
C	WHO 1:100	WHO 1:100	S2 1:100	S2 1:100
D	WHO 1:250	WHO 1:250	S3 1:100	S3 1:100
E	WHO 1:600	WHO 1:600	S4 1:100	S4 1:100
F	WHO 1:800	WHO 1:800	S5 1:100	S5 1:100
G	WHO 1:1700	WHO 1:1700	S6 1:100	S6 1:100
H	WHO 1:4000	WHO 1:4000	S7 1:100	S7 1:100

Antibody quantification (final dilution)

Over OD values may be observed for the 1:100 WHO dilution. In this case, use the 6 following WHO dilutions to perform the regression curve.

3. Incubation of the plate
1 hour ± 5 min. at +37 ± 3°C.

WASHING:
Wash buffer: dilute the concentrated washing solution (W) 1:10 in distilled or demineralized water.
Carefully remove the adhesive film and wash 4 times.

II - ADDITION OF THE CONJUGATE

1. Preparation of the conjugate:
Dilute the concentrate (CJ) 1:10 in the conjugate diluent (CD). 2 ml are needed for one strip, meaning 200 µl of CJ in 1.8 ml of CD.

2. Distribution of the conjugate:
Add 100 µl of diluted conjugate to all the wells and cover with a new piece of adhesive film.

3. Incubation of conjugate:
1 hour ± 5 min. at +37 ± 3°C.

WASHING:
Carefully remove the adhesive film and wash 4 times.

III - REVELATION

1. Addition of the substrate:
Add 100 µl of buffered peroxidase substrate (PS) per well. Do not cover with adhesive film at this stage. Mix by gentle shaking the plate manually or use a plate agitator to ensure correct homogenisation.

2. Incubation of substrate:
Incubate for 30 ± 5 min. at laboratory temperature (+23 ± 5°C), shielded from light.

3. Addition of the Stop Solution:
Add 50 µl of stop solution (S) per well.
Mix by gentle shaking the plate manually or by using a plate agitator. Make sure that no bubbles occur in the wells.

4. Measure of the optical density:
Measure the optical density (OD) bichromatically at 450 and 630 nm or monochromatically at 450 nm (in the yellow band).
Reading bichromatically is strongly recommended. Should a monochromatic reader be used, ensure the cleanliness of the bottom of the wells prior to reading.

VII. TEST VALIDATION

The results of each test run (or for each plate) are valid:
- when the optical density (OD) obtained with the positive control is ≥ 0.300, and
- when the optical density (OD) obtained with the negative control is < 0.50 x OD P, and
- when the correlation coefficient between the Neperian logarithm (ln) ODs and ln Rabies Ab concentrations for the *WHO* standard serum is > 0.95.

VIII. EXPRESSION AND INTERPRETATION OF THE RESULTS

Method: TITER CALCULATION USING THE REGRESSION CURVE
(We recommend the use of an Excel spreadsheet. Synbiotics will provide you with a ready-to-use file upon request)
- Calculate the average OD value for each sample tested and each WHO serum dilution.

- Calculate the Neperian logarithm (ln) value for each average OD and the ln value of the Rabies Ab concentration for each WHO dilution (from 6.7 to 0.0223 IU/ml, without taking into account the 1:100 testing dilution factor).

- Plot the ln (OD) (Y-axis) as a function of the ln (Rabies Ab concentration) (X-axis) in order to draw the reference curve for the *WHO* standard serum.

- Using all individual results obtained for the *WHO* standard serum dilutions, perform a linear regression curve between ln Rabies Ab concentrations (expressed in EU/ml) (equivalent units per ml) and ln (OD), to establish the corresponding mathematics model:

$$\ln[\text{Rabies Ab concentration (EU/ml)}] = a + b \cdot \ln \text{OD}$$

- For each tested sample, calculate the average OD value and then the Rabies antibody concentration of the sample expressed as «equivalent units per ml» (EU/ml), from the established model:

$$\text{Sample Rabies Ab concentration (EU/ml)} = e^{(a + b \cdot \ln \text{OD})}$$

- If the calculated titre > 0.6, the animal is considered as protected.
- If the calculated titre is < 0.6, the animal is considered as not protected (a confirmation using FAVN may be performed).

Should you have any question, please contact us:
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FOR VETERINARY USE ONLY /
FOR IN VITRO USE ONLY

Manufacturer:
DELPHARM BIOTECH
2 rue Alexander Fleming
69366 Lyon Cedex 07
France

EXAMPLE

* Positive control:

well B1 = 0.610; well B2 = 0.690 → $\overline{OD}$ P = 0.650

* Negative control:

well A1 = 0.190; well A2 = 0.210 → $\overline{OD}$ N = 0.200

* Sample 1: OD 1 = 1.790 OD 2 = 1.750 → $\overline{OD}$ = 1.770

* Sample 2: OD 1 = 0.350 OD 2 = 0.390 → $\overline{OD}$ = 0.370

* Test validation: $\overline{OD}$ P = 0.650 > 0.300 et $\overline{OD}$ N = 0.200 < 0.50 x 0.650 = 0.325 , therefore valid test.

Samples (final dilution)	Ab UI/ml	OD 1	OD 2	average OD	ln [conc. Ab]	ln (average OD)
WHO 1:100	6.7	over	over	over	1.9021	-
WHO 1:250	2.233	1.280	1.237	1.259	0.8033	0.2299
WHO 1:600	0.67	0.809	0.751	0.780	-0.4005	-0.2485
WHO 1:800	0.447	0.600	0.620	0.610	-0.8052	-0.4943
WHO 1:1700	0.2233	0.406	0.425	0.416	-1.4992	-0.8783
WHO 1:4000	0.067	0.214	0.217	0.216	-2.7031	-1.5348
WHO 1:8000	0.0223	0.148	0.154	0.151	-3.8032	-1.8905
Sample 1 1:100	unknown	1.790	1.750	1.770	unknown	0,5710
Sample 2 1:100	unknown	0.350	0.390	0.370	unknown	-0,9943

Mathematics Model:

ln [Rabies Ab] = 0.255 + 2.063 * ln OD

* Test validation: correlation coefficient r = 0.996 > 0.95, therefore valid test

* Rabies Ab concentration in Sample 1:

$e^{(0.255 + 2.063 * \ln OD)} = e^{(0.255 + 2.063 * 0.5710)}$ = 4.19 EU /ml → protected

* Ab Concentration in sample 2:

$e^{(0.255 + 2.063 * \ln OD)} = e^{(0.255 + 2.063 * -0.9943)}$ = 0.17 EU /ml → not protected (FAVN confirmation).

APPENDIX B – KSU IRB APPROVED APPLICATION

FOR OFFICE USE ONLY: IRB Protocol # _____ Application Received: _____
Routed: _____ Training Complete: _____

Committee for Research Involving Human Subjects (IRB)

Application for Approval Form
Last revised on January 2011

ADMINISTRATIVE INFORMATION:

- **Title of Project:** (if applicable, use the exact title listed in the grant/contract application)
  Cut-off Values for Rabies Serology Methods - Are They Interchangeable?

- **Type of Application:**
  New/Renewal Revision (to a pending new application)
  Modification (to an existing #_____ approved application)

- **Principal Investigator:** (must be a KSU faculty member)

Name:	**Dr. M. M. Chengappa**	**Degree/Title:**	**Department Head, University Distinguished Professor**
Department:	**Diagnostic Medicine and Pathobiology**	**Campus Phone:**	**785-532-4403**
Campus Address:	**Mosier Hall K-222 Manhattan, KS 66506**	**Fax #:**	**785-532-4039**
E-mail	**chengapa@vet.k-state.edu**		

- **Contact Name/Email/Phone for Questions/Problems with Form:** **Mylissia Smith Stukey / mstukcy@vet.k-state.edu / 785-532-4621 or Susan Moore / smoore@vet.k-state.edu / 785-532- 4472**

- **Does this project involve any collaborators not part of the faculty/staff at KSU?** (projects with non-KSU collaborators may require additional coordination and approvals)**:**
  No
  Yes

- **Project Classification** (Is this project part of one of the following?)**:**
  Thesis
  Dissertation
  Faculty Research
  Other: MPH Capstone
  Note: Class Projects should use the short form application for class projects.

- **Please attach a copy of the Consent Form:**
  Copy attached
  Consent form not used

- **Funding Source: Internal External (identify source and attach a copy of the sponsor's grant application or contract as submitted to the funding agency)**
  Copy attached Not applicable

- **Based upon criteria found in 45 CFR 46 – and the overview of projects that may qualify for exemption explained at** http://www.hhs.gov/ohrp/policy/checklists/decisioncharts.html **, I believe that my project using human subjects should be determined by the IRB to be exempt from IRB review:**

  No

  Yes **(If yes, please complete application <u>including</u> Section XII. C. 'Exempt Projects'; remember that only the IRB has the authority to determine that a project is exempt from IRB review)**

If you have questions, please call the University Research Compliance Office (URCO) at 532-3224, or comply@ksu.edu

Human Subjects Research Protocol Application Form

The KSU IRB is required by law to ensure that all research involving human subjects is adequately reviewed for specific information and is approved prior to inception of any proposed activity. Consequently, it is important that you answer all questions accurately. If you need help or have questions about how to complete this application, please call the Research Compliance Office at 532-3224, or e-mail us at **comply@ksu.edu**.

Please provide the requested information in the shaded text boxes. The shaded text boxes are designed to accommodate responses within the body of the application. As you type your answers, the text boxes will expand as needed. After completion, print the form and send the original and one photocopy to the Institutional Review Board, Room 203, Fairchild Hall.

Principal Investigator:	**Dr. M. M. Chengapp**
Project Title:	**Cut-off Values for Rabies Serology Methods - Are They Interchangeable?**
Date:	**January 2014**

MODIFICATION

Is this a modification of an approved protocol? Yes No If yes, please comply with the following:

If you are requesting a modification or a change to an IRB approved protocol, please provide a concise description of all of the changes that you are proposing in the following block. Additionally, please highlight or bold the proposed changes in the body of the protocol where appropriate, so that it is clearly discernable to the IRB reviewers what and where the proposed changes are. This will greatly help the committee and facilitate the review.

NON-TECHNICAL SYNOPSIS (brief narrative description of proposal easily understood by nonscientists):

Vaccine equivalency is determined partly by seroconversion levels. Seroconversion is a process of developing antibodies in the serum to combat infection. Medical decisions and advice are based on such seroconversion levels for vaccines, for example, vaccine booster administration. In reference to rabies vaccines, this seroconversion level is recognized to be 0.5 IU/mL by the World Health Organization (WHO). The IU/mL is the potency level of antibodies per mL recognized as an effective response to the rabies vaccine. This project investigates weather or not the 0.5 IU/mL level (recognized by the WHO) can be used interchangeably between testing methodologies for vaccine response interpretations. To determine sufficient rabies antibody levels from serum, the testing methodologies measure different ascepts of the antibody response leading to different testing interpretations. For example, one testing methodology measures the antibody neutralizing function from the vaccine whilst another testing methodology measures the binding function of the rabies antibodies to a rabies viral protein. As a result of these differences between testing methodologies, the result of the IU/mL (0.5) are not expected to be appropriate for all tests. As a result, individual testing methodologies need to determine an independent value that represents an effective response to rabies vaccination. This study will investigate and possibly determine the appropriate levels for each testing methodology. This will allow for further education and information to medical professionals for improved decision making of when a vaccine booster is indeed warranted.

I.

BACKGROUND (concise narrative review of the literature and basis for the study)**:**

The 0.5 IU/mL level is recognized globally as indication of adequate response to vaccination in humans. Often forgotten are the circumstances of the origin of this determination. Reports from the WHO Expert Committee on Rabies (the 3rd/1957 through the 6th/1973) state that vaccination response be verified in the serum 1 month after vaccination by detection of RVNA, but no specific level is named. A Working Group convened during the 1978 Joint WHO/IABS symposium defined a cut-off after review of several clinical trial studies; concluding "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 in 1984 the 0.5 IU/mL cut-off noted as the level required 1 month after

vaccination and that booster vaccinations are required until that level is reached. The important points are: the level was determined based on results from serum neutralization methods (MNT and RFFIT); it was for specific time point (1 month after vaccination); and it was for a specified purpose (demonstrate seroconversion). To extrapolate this value to other methods, time points, and purposes is incorrect. Serum neutralization and ELISA rabies serology methods have been compared many times with similar findings: correlation is fair to good. This is not surprising as both are measuring, in different ways, the specific rabies antibody response to rabies vaccination. The reasons the comparison cannot be "good" for all samples are: the methods measure different characteristics of rabies antibodies (neutralizing function for SNs and binding function for ELISA); the normal response to rabies vaccination is polyclonal with various affinities, Ig subclasses, and neutralizing abilities, a unique polyclonal response per individual. This means the relationship of binding antibody measurement to neutralizing function will not be constant between individuals.

II. PROJECT/STUDY DESCRIPTION (please provide a concise narrative description of the proposed activity in terms that will allow the IRB or other interested parties to clearly understand what it is that you propose to do that involves human subjects. This description must be in enough detail so that IRB members can make an informed decision about proposal).

This project is a comparative study to compare the safety and immunogenicity between two human rabies vaccines. It is an open study involving the serum of 189 healthy volunteers. The serum will be divided into three groups (A, B and C). Each group will consist of 63 serum samples that will have received one of the vaccines with a specific vaccine regimen. Group A and B serum will been vaccinated with the same human vaccine with differing vaccine regimen days. Group C serum will have been vaccinated with the second vaccine for the same vaccine regimen days as group B.

The samples received will consist of 5 blood draws from each individual. The first blood draw will be on day 0 to determine a baseline. The following blood draws will be utilized to monitor antibody production. All samples will be blinded and blood samples will have the rabies antibody concentrations evaluated by utilizing the Rapid Fluorescent Focus Inhibition Test (RFFIT) and an Enzyme Linked Immunoassay (ELISA).

III. OBJECTIVE (briefly state the objective of the research – what you hope to learn from the study)**:**

To determine whether 0.5 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), just as the 0.5 IU/mL was established for SN methods.
The objectives of this study are:
1) To characterize the rabies antibody response to vaccination using an ELISA method that detects anti-rabies glycoprotein IgG and to modify the method to detect anti-rabies glycoprotein IgM for additional characterization of the response.
2) To evaluate the adequate response by comparing antibody levels (EU/mL vs. IU/mL).
3) To determine if a correlation between ELISA results and RFFIT results can be made in the determination of adequate response for individuals and for a group.

IV. DESIGN AND PROCEDURES (succinctly outline formal plan for study)**:**

A.	Location of study:	**Kansas State University KSVDL Rabies Laboratory Manhattan, KS**
B.	Variables to be studied:	**Human Blood Serum**
C.	Data collection methods: (surveys, instruments, etc – **PLEASE ATTACH**)	**Receive serum samples by courier, RFFIT and ELISA testing methodologies, Reed and Muench calculation chart, International Unit calculation formula, Bio-Rad EU/mL calculation, Zoetis EU/mL calculation.**
D.	List any factors that might lead to a subject dropping out or withdrawing from a study. These might include, but are not limited to emotional or physical stress, pain, inconvenience, etc.:	**NA**
E.	List all biological samples taken: (if any)	**Human Blood**

. **RESEARCH SUBJECTS:**

A. Source: **PHARMA-SURREY INTERNATIONAL, INC. #46 Bulusan St. Sta. Mesa Heights, Quezon City, Philippines.**

B. Number: **189 Serum Samples**

C. Characteristics: (list any unique qualifiers desirable for research subject participation) **NA**

D. Recruitment procedures: (Explain how do you plan to recruit your subjects? Attach any fliers, posters, etc. used in recruitment. If you plan to use any inducements, ie. cash, gifts, prizes, etc., please list them here.) **NA**

VI. RISK – PROTECTION – BENEFITS: The answers for the three questions below are central to human subjects research. You must demonstrate a reasonable balance between anticipated risks to research participants, protection strategies, and anticipated benefits to participants or others.

A. **Risks for Subjects:** (Identify any reasonably foreseeable physical, psychological, or social risks for participants. State that there are "no known risks" if appropriate.)
NA

B. **Minimizing Risk:** (Describe specific measures used to minimize or protect subjects from anticipated risks.)
NA

C. **Benefits:** (Describe any reasonably expected benefits for research participants, a class of participants, or to society as a whole.)
NA

In your opinion, does the research involve **more than minimal risk** to subjects? ("Minimal risk" means that "the risks of harm anticipated in the proposed research are not greater, considering probability and magnitude, than those ordinarily encountered in daily life or during the performance of routine physical or psychological examinations or tests.")

Yes **No**

VII. CONFIDENTIALITY: Confidentiality is the formal treatment of information that an individual has disclosed to you in a relationship of trust and with the expectation that it will not be divulged to others without permission in ways that are inconsistent with the understanding of the original disclosure. Consequently, it is your responsibility to protect information that you gather from human research subjects in a way that is consistent with your agreement with the volunteer and with their expectations. If possible, it is best if research subjects' identity and linkage to information or data remains unknown.

Explain how you are going to protect confidentiality of research subjects and/or data or records. Include plans for maintaining records after completion.

Samples are labeled with a ID number blinded to the laboratory. The laboratory does not receive or have access to personal information about the subjects providing the serum samples.

VIII. INFORMED CONSENT: Informed consent is a critical component of human subjects research – it is your responsibility to make sure that any potential subject knows exactly what the project that you are planning is about, and what his/her potential role is. (There may be projects where some forms of "deception" of the subject is necessary for the execution of the study, but it must be carefully justified to and approved by the IRB). A schematic for determining when a waiver or alteration of informed consent may be considered by the IRB is found at http://www.hhs.gov/ohrp/policy/consentckls.html Even if your proposed activity does qualify for a waiver of informed consent, you must still provide potential participants with basic information that informs them of their rights as subjects, i.e. explanation that the project is research and the purpose of the research, length of study, study procedures, debriefing issues to include anticipated benefits,

study and administrative contact information, confidentiality strategy, and the fact that participation is entirely voluntary and can be terminated at any time without penalty, etc. Even if your potential subjects are completely anonymous, you are obliged to provide them (and the IRB) with basic information about your project. See informed consent example on the URCO website. It is a federal requirement to maintain informed consent forms for 3 years after the study completion.

Yes No Answer the following questions about the informed consent procedures.

A. Are you using a written informed consent form? If "yes," include a copy with this application. If "no" see b.

B. In accordance with guidance in 45 CFR 46, I am requesting a waiver or alteration of informed consent elements (See Section VII above). If "yes," provide a basis and/or justification for your request.

NA

C. Are you using the online Consent Form Template provided by the URCO? If "no," does your Informed Consent document has all the minimum required elements of informed consent found in the Consent Form Template? (Please explain)

As per discussion with Heath Ritter per telephone 1-14-14, 1:50 PM CST: The clinical trial is being performed and managed by Pharma-Surrey International, Inc. in the Philippines. Pharma-Surrey is submitting the blood samples to KSU for analysis for research purposes only. As a result, the consent form template submitted here is the form Pharma-Surrey has on file signed by all healthy volunteers for this clinical trial. In this specific situation the KSU IRB template is not needed for use.

D. Are your research subjects anonymous? If they are anonymous, you will not have access to any information that will allow you to determine the identity of the research subjects in your study, or to link research data to a specific individual in any way. Anonymity is a powerful protection for potential research subjects. (An anonymous subject is one whose identity is unknown even to the researcher, or the data or information collected cannot be linked in any way to a specific person).

NA

E. Are subjects debriefed about the purposes, consequences, and benefits of the research? Debriefing refers to a mechanism for informing the research subjects of the results or conclusions, after the data is collected and analyzed, and the study is over. (If "no" explain why.) Attach copy of debriefing statement to be utilized.

NA

***It is a requirement that you maintain all signed copies of informed consent documents for at least 3 years following the completion of your study. These documents must be available for examination and review by federal compliance officials.**

IX. <u>PROJECT INFORMATION</u>: (If you answer yes to any of the questions below, you should explain them in one of the paragraphs above)

Yes No Does the project involve any of the following?

a. Deception of subjects
b. Shock or other forms of punishment
c. Sexually explicit materials or questions about sexual orientation, sexual experience or sexual abuse
d. Handling of money or other valuable commodities
e. Extraction or use of blood, other bodily fluids, or tissues
f. Questions about any kind of illegal or illicit activity
g. Purposeful creation of anxiety
h. Any procedure that might be viewed as invasion of privacy
i. Physical exercise or stress
j. Administration of substances (food, drugs, etc.) to subjects
k. Any procedure that might place subjects at risk

l. Any form of potential abuse; i.e., psychological, physical, sexual
m. Is there potential for the data from this project to be published in a journal, presented at a conference, etc?
n. Use of surveys or questionnaires for data collection
IF YES, PLEASE ATTACH!!

X. SUBJECT INFORMATION: (If you answer yes to any of the questions below, you should explain them in one of the paragraphs above)

Yes No Does the research involve subjects from any of the following categories?

a. Under 18 years of age (these subjects require parental or guardian consent)
b. Over 65 years of age
c. Physically or mentally disabled
d. Economically or educationally disadvantaged
e. Unable to provide their own legal informed consent
f. Pregnant females as target population
g. Victims
h. Subjects in institutions (e.g., prisons, nursing homes, halfway houses)
i. Are research subjects in this activity students recruited from university classes or volunteer pools? If so, do you have a reasonable alternative(s) to participation as a research subject in your project, i.e., another activity such as writing or reading that would serve to protect students from unfair pressure or coercion to participate in this project? If you answered this question "Yes," explain any alternatives options for class credit for potential human subject volunteers in your study. (It is also important to remember that: Students must be free to choose **not** to participate in research that they have signed up for **at any time** without penalty. Communication of their decision can be conveyed in any manner, to include **simply not showing up** for the research.)
NA
j. Are research subjects **audio** taped? If yes, how do you plan to protect the recorded information and mitigate any additional risks?
NA
k. Are research subjects' images being recorded (video taped, photographed)? If yes, how do you plan to protect the recorded information and mitigate any additional risks?
NA

XI. **CONFLICT OF INTEREST:** Concerns have been growing that financial interests in research may threaten the safety and rights of human research subjects. Financial interests are not in themselves prohibited and may well be appropriate and legitimate. Not all financial interests cause Conflict of Interest (COI) or harm to human subjects. However, to the extent that financial interests may affect the welfare of human subjects in research, IRB's, institutions, and investigators must consider what actions regarding financial interests may be necessary to protect human subjects. Please answer the following questions:

Yes No

a. Do you or the institution have any proprietary interest in a potential product of this research, including patents, trademarks, copyrights, or licensing agreements?
b. Do you have an equity interest in the research sponsor (publicly held or a non-publicly held company)?
c. Do you receive significant payments of other sorts, eg., grants, equipment, retainers for consultation and/or honoraria from the sponsor of this research?
d. Do you receive payment per participant or incentive payments?
e. If you answered yes on any of the above questions, please provide adequate explanatory information so the IRB can assess any potential COI indicated above.

XII. PROJECT COLLABORATORS:

A. KSU Collaborators – list anyone affiliated with KSU who is collecting or analyzing data: (list all collaborators on the project, including co-principal investigators, undergraduate and graduate students)

Name:	Department:	Campus Phone:	Campus Email:
Susan Moore	**KSVDL**	**785-532-4472**	**smoore@vet.k-state.edu**
Mylissia Smith Stukey	**DMP**	**785-532-4621**	**mstukey@vet.k-state.edu**
Leslie Engelman	**KSVDL**	**785-532-4483**	**leslieae@vet.k-state.edu**
Sami Pralle	**KSVDL**	**785-532-4483**	**sjbernha@k-state.edu**
Hattie Hartschuh	**KSVDL**	**785-532-4483**	**hattie@k-state.edu**
Rolan Davis	**KSVDL**	**785-532-4483**	**rdavis@vet.k-state.edu**

B. Non-KSU Collaborators: (List all collaborators on your human subjects research project not affiliated with KSU in the spaces below. KSU has negotiated an Assurance with the Office for Human Research Protections (OHRP), the federal office responsible for oversight of research involving human subjects. When research involving human subjects includes collaborators who are not employees or agents of KSU the activities of those unaffiliated individuals may be covered under the KSU Assurance only in accordance with a formal, written agreement of commitment to relevant human subject protection policies and IRB oversight. The Unaffiliated Investigators Agreement can be found and downloaded at http://www.k-state.edu/research/comply/irb/forms/Unaffiliated%20Investigator%20Agreement.doc

C.

The URCO must have a copy of the Unaffiliated Investigator Agreement on file for each non-KSU collaborator who is not covered by their own IRB and assurance with OHRP. Consequently, it is critical that you identify non-KSU collaborators, and initiate any coordination and/or approval process early, to minimize delays caused by administrative requirements.)

Name:	Organization:	Phone:	Institutional Email:
NA			

Does your non-KSU collaborator's organization have an Assurance with OHRP? (for Federalwide Assurance and Multiple Project Assurance (MPA) listings of other institutions, please reference the OHRP website under Assurance Information at: http://ohrp.cit.nih.gov/search).

No

Yes **If yes, Collaborator's FWA or MPA #** _____

Is your non-KSU collaborator's IRB reviewing this proposal?

No

Yes **If yes, IRB approval #** _____

C. Exempt Projects: 45 CFR 46 identifies six categories of research involving human subjects that may be exempt from IRB review. The categories for exemption are listed here: http://www.hhs.gov/ohrp/policy/checklists/decisioncharts.html. If you believe that your project qualifies for exemption, please indicate which exemption category applies (1-6). Please remember that only the IRB can make the final determination whether a project is exempt from IRB review, or not.

Exemption Category: _____

XIII. CLINICAL TRIAL Yes No
(If so, please give product.)

Export Controls Training:
-The Provost has mandated that all KSU faculty/staff with a full-time appointment participate in the Export Control Program.
-If you are not in our database as having completed the Export Control training, this proposal will not be approved until your participation is verified.
-To complete the Export Control training, follow the instructions below:
Click on:

http://www.k-state.edu/research/comply/ecp/index.htm

1. After signing into K-State Online, you will be taken to the Export Control Homepage
2. Read the directions and click on the video link to begin the program
3. Make sure you enter your name / email when prompted so that participation is verified

If you click on the link and are not taken to K-State Online, this means that you have already completed the Export Control training and have been removed from the roster. If this is the case, no further action is required.

-Can't recall if you have completed this training? Contact the URCO at 785-532-3224 or comply@ksu.edu and we will be happy to look it up for you.

Post Approval Monitoring: The URCO has a Post-Approval Monitoring (PAM) program to help assure that activities are performed in accordance with provisions or procedures approved by the IRB. Accordingly, the URCO staff will arrange a PAM visit as appropriate; to assess compliance with approved activities.

If you have questions, please call the University Research Compliance Office (URCO) at 532-3224, or comply@ksu.edu

INVESTIGATOR ASSURANCE FOR RESEARCH INVOLVING HUMAN SUBJECTS

(Print this page separately because it requires a signature by the PI.)

P.I. Name: **Dr. M. M. Chengappa**

Title of Project: **Cut-off Values for Rabies Serology Methods - Are They Interchangeable?**

XIV. ASSURANCES: As the Principal Investigator on this protocol, I provide assurances for the following:

A. **Research Involving Human Subjects: This project will be performed in the manner described in this proposal, and in accordance with the Federalwide Assurance FWA00000865 approved for Kansas State University available at http://ohrp.osophs.dhhs.gov/polasur.htm#FWA, applicable laws, regulations, and guidelines. Any proposed deviation or modification from the procedures detailed herein must be submitted to the IRB, and be approved by the Committee for Research Involving Human Subjects (IRB) prior to implementation.**

B. **Training: I assure that all personnel working with human subjects described in this protocol are technically competent for the role described for them, and have completed the required IRB training modules found on the URCO website at: http://www.k-state.edu/research/comply/irb/training/index.htm. I understand that no proposals will receive final IRB approval until the URCO has documentation of completion of training by all appropriate personnel.**

C. **Extramural Funding: If funded by an extramural source, I assure that this application accurately reflects all procedures involving human subjects as described in the grant/contract proposal to the funding agency. I also assure that I will notify the IRB/URCO, the KSU PreAward Services, and the funding/contract entity if there are modifications or changes made to the protocol after the initial submission to the funding agency.**

D. **Study Duration: I understand that it is the responsibility of the Committee for Research Involving Human Subjects (IRB) to perform continuing reviews of human subjects research as necessary. I also understand that as continuing reviews are conducted, it is my responsibility to provide timely and accurate review or update information when requested, to include notification of the IRB/URCO when my study is changed or completed.**

E. **Conflict of Interest: I assure that I have accurately described (in this application) any potential Conflict of Interest that my collaborators, the University, or I may have in association with this proposed research activity.**

F. **Adverse Event Reporting: I assure that I will promptly report to the IRB / URCO any unanticipated problems involving risks to subjects or others that involve the protocol as approved. Unanticipated or Adverse Event Form is located on the URCO website at: http://www.k-state.edu/research/comply/irb/forms/index.htm. In the case of a serious event, the Unanticipated or Adverse Events Form may follow a phone call or email contact with the URCO.**

G. **Accuracy: I assure that the information herein provided to the Committee for Human Subjects Research is to the best of my knowledge complete and accurate.**

(Principal Investigator Signature) **(date)**

APPENDIX C – USDA BIOLOGICAL PRODUCT PERMIT

United States
Department of Agriculture

UNITED STATES VETERINARY BIOLOGICAL PRODUCT PERMIT

RESEARCH AND EVALUATION

NO. ***VB-139848***

Issued at Washington, D.C. on ***11/22/2013***

Expires: ***11/22/2014***

This permit is issued pursuant to the terms of the Act of Congress approved March 4, 1913 (37 State. 832), governing the preparation, sale, barter, exchange, shipment, and importation of veterinary biological products. So far as the jurisdiction of the U.S. Department of Agriculture is concerned,

Susan Moore
Kansas State University
2005 Research Park Circle
Manhattan, KS 66502

is authorized to import

SERELISA Rabies Ab Mono Indirect (Kit for the detection of anti -rabies antibodies in dog or cat individual serum)

prepared by

Synbiotics Europe
Lyon, France

into the United States through the port of ***Any U.S. Port***

Importation shall be made subject to the following special conditions:

This importation is authorized in accordance with Title 9, Code of Federal Regulations, Part 104 for evaluation at the research/containment facility or diagnostic laboratory specified in the application filed with the Animal and Plant Health Inspection Service. All unused portions of imported materials must be autoclaved or incinerated before disposal.

A COPY OF THIS PERMIT MUST BE INCLUDED WITH THE SHIPMENT FOR PORT INSPECTION

This permit may be revoked if the permittee violates or fails to comply with said Act, the regulations made thereunder, or the conditions specified herein.

11/22/2013
Date

Donna L. Malloy

Donna L. Malloy for Director, Center for Veterinary Biologics
Animal and Plant Health Inspection Service

APHIS FORM 2006 (APR 2001)

APPENDIX D – PROJECT PROTOCOL: KSU/ZOETIS

Kansas State University
College of Veterinary Medicine
Department of Diagnostic Medicine/Pathobiology
Manhattan, KS

VALIDATING AND STANDARDIZATION OF THE SYNBIOTICS RABIES ELISA TEST, SERELISA® RABIES Ab MONO INDIRECT, FOR DETECTION OF ANTI-RABIES ANTIBODIES IN INDIVIDUAL HUMAN SERUM

Prepared By: _____ Date: _____
Mylissia Smith, MPH Candidate Kansas State University

Approved By: _____Date: _____
Susan Moore, Managing Director of the KSVDL Rabies Laboratory

Approved By: _____Date: _____
Dr. Stephane Guillossou, Zoetis - Director, Global Diagnostics Marketing – US

Appendix A - 1. INTRODUCTION

Rabies testing methodologies allow for the evaluation of vaccine response levels to determine if individuals have adequate protection against the rabies virus. The WHO recommends animal samples for export requirements and human samples previously vaccinated for rabies have a continuous antibody level response of 0.5 IU/mL to be considered adequate for an immune response to rabies (WHO, 2013). The WHO recommendations are based off of the RFFIT or MNT serology testing methodology as these methodologies measure the level of rabies virus neutralizing antibodies (RVNA) (Moore, Hanlon, 2010). This protocol is aimed to investigate the performance of a rabies ELISA testing methodology to determine adequate immune response levels to rabies vaccines. The ELISA measures quantitative antibody levels in individual serum samples. To date, the WHO does not have adequate recommendations for immune response levels for the ELISA testing methodology.

Appendix B - 2. OBJECTIVE AND ACCEPTANCE CRITERIA

2.1. Objective

The objective of this protocol is dual. The first objective will investigate the performances of this ELISA on human sample origin for the appreciation of the protection status obtained after Rabies vaccination. The second parallel objective is to establish standardization curves and appreciate the robustness of the test when using different international reference sera (WHO-1, WHO-2, OIE and Kansas State University).

2.2. Acceptance Criteria

The ELISA allows for quantitative detection of rabies antibodies in individual serum. Results of the ELISA samples are most precise when you obtain the results noted in section 6.2, Test Procedures, D of this document.

3. STUDY RATIONALE AND BACKGROUND INFORMATION

The purpose of this study is to run a series of reference samples to validate the ELISA for appreciating the performances of the test and investigating if a different cut off is mandated for assessment of the antibody immune response status in human samples. As aforementioned, the WHO 0.5 IU/mL recommended continuous antibody level for an immune response is based from the RFFIT testing methodology that measures RVNA. More specifically an RVNA test measures only neutralizing antibody in the sample due to the nature of the test itself (Moore, Gordon, Briggs, 2007). The ELISA testing methodology measures the binding of antibodies to the rabies virus antigen independently from the seroneutralizing property of the antibodies. As a result, applying the WHO 0.5 IU/mL recommendation established upon seroneutralizing detection method to binding methods such as the ELISA might lead to different performances and therefore should be investigated.

4. STUDY METHODOLOGY

The Zoetis ELISA will be compared and evaluated again reference standards from the WHO, OIE and KSU. Section 6.1 C outlines the titration and dilutions of each standard that will be utilized. The first two ELISA microplates displayed in Section 6.1 E displays the layout of how each ELISA microplate will be prepared and tested. Microplate three four displays the layout of how the microplates will be tested, which will consist of three series of human serum samples that will consist of the same titration and dilutions as specified in section 6.1 C. Upon completion of each microplate, validation and expression of each test will be interpreted.

3.1 Test Validation

The results of each test run (or for each plate) are validate:

A. When the OD obtained with the positive control is ≥ 0.3000, and

B. When the OD obtained with the negative control is < 0.50 x OD P, and

C. When the correlation coefficient between the Neperian logarithm (ln) ODs and in Rabies Ab concentrations for the *WHO* standard serum is > 0.95.

3.2 Expression and Interpretation of the Results

The method of titer calculation will be completed using the Regression Curve. The calculations will be accomplished by utilizing an Excel spreadsheet obtained by Zoetis specifically designed for this ELISA. Calculations will include:

A. Calculate the average OD value for each sample tested and each WHO serum dilution. Also calculate OIE and KSU reference serum from the WHO standard curve. Also compare results against each other.

B. Calculate the Neperian Logarithm (ln) value for each average OD and the ln Value of the Rabies Ab concentration for each WHO, KSU and OIE dilution.

C. Plot the ln (OD) (Y axis) as a function of the ln (Rabies Ab concentration) (X axis) in order to draw the reference curve for the WHO, KSU and OIE standard serum.

D. Use all individual results obtained for the WHO, KSU and OIE standard serum dilutions, perform a linear regression curve between ln Rabies Ab concentrations (expressed in EU/mL) (equivalent units per mL) and ln (OD), to establish the corresponding mathematics model:

$$\ln[\text{Rabies Ab concentration (EU/mL)}] = a + b * \ln OD$$

For each tested sample, calculate the average OD value and then the Rabies antibody concentration of the sample expressed as equivalent units per ml» (EU/mL), from the established model: Sample Rabies Ab concentration

$$(\text{EU/mL}) = e^{(a + \overset{*}{b} \ln OD)}$$

E. If the calculated titer is > 0.6, the sample is considered as protected,

F. If the calculated titer is < 0.6, the sample is considered as not protected (a confirmation utilizing FAVN testing methodology can be performed).

5. MATERIALS

5.1 Reagents

Note: for all reagents record date opened and initials on the container upon first use. Label secondary container for reagent dilution with Name, Concentration (Dilution), Date Made,

Expiration date (per original container or kit instructions), storage temperature, and initials.

Conjugate: Protein A / peroxidase (CJ) (10X concentrated)
Buffered peroxidase substrate (PS) – Ready-to-use
Negative control (N) (10X concentrated)
Positive control (P) (10X concentrated)
Sample diluent (SD) – Ready-to-use
Wash solution (W) (10X concentrated)
Conjugate diluent (CD) – Ready-to-use
Stop solution (S) – Ready-to-use

5.2 Supplies Included In Kit

Microplate containing six 16-well strips sensitized with Rabies Antigens
Adhesive film – 6 films

5.3 Supplies Required & Not Included In Kit

5.3.1 Reference Sera

WHO Reference Serum – Obtained from NIBSC
WHO #1 – 59 IU/mL
WHO #2 – 30 IU/mL

OIE – 6.7 IU/mL, Batch #3
KSU Reference Serum
KSU from WHO #1 (59 IU/mL)
KSU Internal #1- 15.0 (Pooled from positive RVNA samples from a RFFIT set)
KSU Internal #2 – 2.8 (Pooled from positive RVNA samples from a RFFIT set)
KSU Internal #3 – 0.5 (Pooled from positive RVNA samples from a RFFIT set)
KSU Internal #4 – 0.1 (Pooled from positive RVNA samples from a RFFIT set)

5.3.2 Laboratory equipment

Distilled or demineralized water

Adjustable or set pipettes to measure and deliver between 0-1000µl.

Graduated cylinders (100mL and 1000mL)

Manual, automatic or semi-automatic washing device

Microplate reader, bichromatic reading at 450 & 630 nm.

Incubator at +37 ± 3°C

6. METHOD SYNBIOTICS ELISA

6.1 Preliminary Procedure

A. Set up distribution and identification of controls and samples.

B. Prepare the sera to be tested. Dilutions are performed in the kit sample diluent (SD). Dilutions should be performed as indicated in the microplate tables below (microplates numbered 1-10).

C. For serum titration, a set of seven dilutions in duplicate of the WHO, OIE and KSU should be performed in the microplates as indicated in the microplate tables below (microplates numbered 1-10).

D. For serum titration, a set of four KSU standard reference serums in duplicate should be performed in the microplates as indicated in the microplate tables below (microplates numbered 1-10).

Microplate 1:

A	N 1:10	N 1:10	OIEa 1:30,000	OIEa 1:30,000	WHO1 1:300,000	WHO1 1:300,000	WHO2 1:150,000	WHO2 1:150,000	KSU 1:300,000	KSU 1:300,000	OIEa 1:30,000	OIEa 1:30,000
B	P 1:10	P 1:10	KSU Ref#1 1:100	KSU Ref#1 1:100	KSU Ref#2 1:100	KSU Ref#2 1:100	KSU Ref#3 1:100	KSU Ref#3 1:100	KSU Ref#4 1:100	KSU Ref#4 1:100	WHO1 1:300,000	WHO1 1:300,000
C	OIEa 1:100	OIEa 1:100	WHO1 1:1,000	WHO1 1:1,000	WHO2 1:500	WHO2 1:500	KSU 1:1,000	KSU 1:1,000	OIEa 1:100	OIEa 1:100	WHO1 1:1,000	WHO1 1:1,000
D	OIEa 1:300	OIEa 1:300	WHO1 1:3,000	WHO1 1:3,000	WHO2 1:1,500	WHO2 1:1,500	KSU 1:3,000	KSU 1:3,000	OIEa 1:300	OIEa 1:300	WHO1 1:3,000	WHO1 1:3,000
E	OIEa 1:1,000	OIEa 1:1,000	WHO1 1:10,000	WHO1 1:10,000	WHO2 1:5,000	WHO2 1:5,000	KSU 1:10,000	KSU 1:10,000	OIEa 1:1,000	OIEa 1:1,000	WHO1 1:10,000	WHO1 1:10,000
F	OIEa 1:1,500	OIEa 1:1,500	WHO1 1:15,000	WHO1 1:15,000	WHO2 1:7,500	WHO2 1:7,500	KSU 1:15,000	KSU 1:15,000	OIEa 1:1,500	OIEa 1:1,500	WHO1 1:15,000	WHO1 1:15,000
G	OIEa 1:3,000	OIEa 1:3,000	WHO1 1:30,000	WHO1 1:30,000	WHO2 1:15,000	WHO2 1:15,000	KSU 1:30,000	KSU 1:30,000	OIEa 1:3,000	OIEa 1:3,000	WHO1 1:30,000	WHO1 1:30,000
H	OIEa 1:10,000	OIEa 1:10,000	WHO1 1:100,000	WHO1 1:100,000	WHO2 1:50,000	WHO2 1:50,000	KSU 1:100,000	KSU 1:100,000	OIEa 1:10,000	OIEa 1:10,000	WHO1 1:100,000	WHO1 1:100,000

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1:10	N 1:10	0.0223 IU/ml	0.0223 IU/ml	0.0197 IU/ml	0.0197 IU/ml	0.02 IU/ml	0.02 IU/ml	0.0197 IU/ml	0.0197 IU/ml	0.0223 IU/ml	0.0223 IU/ml
B	P 1:10	P 1:10	15.0 IU/ml	15.0 IU/ml	2.8 IU/ml	2.8 IU/ml	0.5 IU/ml	0.5 IU/ml	0.1 IU/ml	0.1 IU/ml	0.0197 IU/ml	0.0197 IU/ml
C	6.7 IU/ml	6.7 IU/ml	5.9 IU/ml	5.9 IU/ml	6 IU/ml	6 IU/ml	5.9 IU/ml	5.9 IU/ml	6.7 IU/ml	6.7 IU/ml	5.9 IU/ml	5.9 IU/ml
D	2.23 IU/ml	2.23 IU/ml	1.97 IU/ml	1.97 IU/ml	2 IU/ml	2 IU/ml	1.97 IU/ml	1.97 IU/ml	2.23 IU/ml	2.23 IU/ml	1.97 IU/ml	1.97 IU/ml
E	0.67 IU/ml	0.67 IU/ml	0.59 IU/ml	0.59 IU/ml	0.6 IU/ml	0.6 IU/ml	0.59 IU/ml	0.59 IU/ml	0.67 IU/ml	0.67 IU/ml	0.59 IU/ml	0.59 IU/ml
F	0.447 IU/ml	0.447 IU/ml	0.39 IU/ml	0.39 IU/ml	0.4 IU/ml	0.4 IU/ml	0.39 IU/ml	0.39 IU/ml	0.447 IU/ml	0.447 IU/ml	0.39 IU/ml	0.39 IU/ml
G	0.223 IU/ml	0.223 IU/ml	0.197 IU/ml	0.197 IU/ml	0.2 IU/ml	0.2 IU/ml	0.197 IU/ml	0.197 IU/ml	0.223 IU/ml	0.223 IU/ml	0.197 IU/ml	0.197 IU/ml
H	0.067 IU/ml	0.067 IU/ml	0.059 IU/ml	0.059 IU/ml	0.06 IU/ml	0.06 IU/ml	0.059 IU/ml	0.059 IU/ml	0.067 IU/ml	0.067 IU/ml	0.059 IU/ml	0.059 IU/ml

Microplate 2:

A	N 1:10	N 1:10	OIEa 1:30,000	OIEa 1:30,000	WHO1 1:300,000	WHO1 1:300,000	WHO2 1:150,000	WHO2 1:150,000	KSU 1:300,000	KSU 1:300,000	WHO2 1:150,000	WHO2 1:150,000
B	P 1:10	P 1:10	KSU Ref#1 1:100	KSU Ref#1 1:100	KSU Ref#2 1:100	KSU Ref#2 1:100	KSU Ref#3 1:100	KSU Ref#3 1:100	KSU Ref#4 1:100	KSU Ref#4 1:100	KSU 1:300,000	KSU 1:300,000
C	OIEa 1:100	OIEa 1:100	WHO1 1:1,000	WHO1 1:1,000	WHO2 1:500	WHO2 1:500	KSU 1:1,000	KSU 1:1,000	WHO2 1:500	WHO2 1:500	KSU 1:1,000	KSU 1:1,000
D	OIEa 1:300	OIEa 1:300	WHO1 1:3,000	WHO1 1:3,000	WHO2 1:1,500	WHO2 1:1,500	KSU 1:3,000	KSU 1:3,000	WHO2 1:1,500	WHO2 1:1,500	KSU 1:3,000	KSU 1:3,000
E	OIEa 1:1,000	OIEa 1:1,000	WHO1 1:10,000	WHO1 1:10,000	WHO2 1:5,000	WHO2 1:5,000	KSU 1:10,000	KSU 1:10,000	WHO2 1:5,000	WHO2 1:5,000	KSU 1:10,000	KSU 1:10,000
F	OIEa 1:1,500	OIEa 1:1,500	WHO1 1:15,000	WHO1 1:15,000	WHO2 1:7,500	WHO2 1:7,500	KSU 1:15,000	KSU 1:15,000	WHO2 1:7,500	WHO2 1:7,500	KSU 1:15,000	KSU 1:15,000
G	OIEa 1:3,000	OIEa 1:3,000	WHO1 1:30,000	WHO1 1:30,000	WHO2 1:15,000	WHO2 1:15,000	KSU 1:30,000	KSU 1:30,000	WHO2 1:15,000	WHO2 1:15,000	KSU 1:30,000	KSU 1:30,000
H	OIEa 1:10,000	OIEa 1:10,000	WHO1 1:100,000	WHO1 1:100,000	WHO2 1:50,000	WHO2 1:50,000	KSU 1:100,000	KSU 1:100,000	WHO2 1:50,000	WHO2 1:50,000	KSU 1:100,000	KSU 1:100,000

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1:10	N 1:10	0.0223 IU/ml	0.0223 IU/ml	0.0197 IU/ml	0.0197 IU/ml	0.02 IU/ml	0.02 IU/ml	0.0197 IU/ml	0.0197 IU/ml	0.02 IU/ml	0.02 IU/ml
B	P 1:10	P 1:10	15.0 IU/ml	15.0 IU/ml	2.8 IU/ml	2.8 IU/ml	0.5 IU/ml	0.5 IU/ml	0.1 IU/ml	0.1 IU/ml	0.0197 IU/ml	0.0197 IU/ml
C	6.7 IU/ml	6.7 IU/ml	5.9 IU/ml	5.9 IU/ml	6 IU/ml	6 IU/ml	5.9 IU/ml	5.9 IU/ml	6 IU/ml	6 IU/ml	5.9 IU/ml	5.9 IU/ml
D	2.23 IU/ml	2.23 IU/ml	1.97 IU/ml	1.97 IU/ml	2 IU/ml	2 IU/ml	1.97 IU/ml	1.97 IU/ml	2 IU/ml	2 IU/ml	1.97 IU/ml	1.97 IU/ml
E	0.67 IU/ml	0.67 IU/ml	0.59 IU/ml	0.59 IU/ml	0.6 IU/ml	0.6 IU/ml	0.59 IU/ml	0.59 IU/ml	0.6 IU/ml	0.6 IU/ml	0.59 IU/ml	0.59 IU/ml
F	0.447 IU/ml	0.447 IU/ml	0.39 IU/ml	0.39 IU/ml	0.4 IU/ml	0.4 IU/ml	0.39 IU/ml	0.39 IU/ml	0.4 IU/ml	0.4 IU/ml	0.39 IU/ml	0.39 IU/ml
G	0.223 IU/ml	0.223 IU/ml	0.197 IU/ml	0.197 IU/ml	0.2 IU/ml	0.2 IU/ml	0.197 IU/ml	0.197 IU/ml	0.2 IU/ml	0.2 IU/ml	0.197 IU/ml	0.197 IU/ml
H	0.067 IU/ml	0.067 IU/ml	0.059 IU/ml	0.059 IU/ml	0.06 IU/ml	0.06 IU/ml	0.059 IU/ml	0.059 IU/ml	0.06 IU/ml	0.06 IU/ml	0.059 IU/ml	0.059 IU/ml

Microplate #3

A	N 1:10	N 1:10	OIEa 1:30,000	OIEa 1:30,000	WHO1 1:300,000	WHO1 1:300,000	WHO2 1:150,000	WHO2 1:150,000	KSU 1:300,000	KSU 1:300,000	OIEa 1:30,000	OIEa 1:30,000
B	P 1:10	P 1:10	KSU Ref#1 1:100	KSU Ref#1 1:100	KSU Ref#2 1:100	KSU Ref#2 1:100	KSU Ref#3 1:100	KSU Ref#3 1:100	KSU Ref#4 1:100	KSU Ref#4 1:100	WHO2 1:150,000	WHO2 1:150,000
C	OIEa 1:100	OIEa 1:100	WHO1 1:1,000	WHO1 1:1,000	WHO2 1:500	WHO2 1:500	KSU 1:1,000	KSU 1:1,000	OIEa 1:100	OIEa 1:100	WHO2 1:500	WHO2 1:500
D	OIEa 1:300	OIEa 1:300	WHO1 1:3,000	WHO1 1:3,000	WHO2 1:1,500	WHO2 1:1,500	KSU 1:3,000	KSU 1:3,000	OIEa 1:300	OIEa 1:300	WHO2 1:1,500	WHO2 1:1,500
E	OIEa 1:1,000	OIEa 1:1,000	WHO1 1:10,000	WHO1 1:10,000	WHO2 1:5,000	WHO2 1:5,000	KSU 1:10,000	KSU 1:10,000	OIEa 1:1,000	OIEa 1:1,000	WHO2 1:5,000	WHO2 1:5,000
F	OIEa 1:1,500	OIEa 1:1,500	WHO1 1:15,000	WHO1 1:15,000	WHO2 1:7,500	WHO2 1:7,500	KSU 1:15,000	KSU 1:15,000	OIEa 1:1,500	OIEa 1:1,500	WHO2 1:7,500	WHO2 1:7,500
G	OIEa 1:3,000	OIEa 1:3,000	WHO1 1:30,000	WHO1 1:30,000	WHO2 1:15,000	WHO2 1:15,000	KSU 1:30,000	KSU 1:30,000	OIEa 1:3,000	OIEa 1:3,000	WHO2 1:15,000	WHO2 1:15,000
H	OIEa 1:10,000	OIEa 1:10,000	WHO1 1:100,000	WHO1 1:100,000	WHO2 1:50,000	WHO2 1:50,000	KSU 1:100,000	KSU 1:100,000	OIEa 1:10,000	OIEa 1:10,000	WHO2 1:50,000	WHO2 1:50,000

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1:10	N 1:10	0.0223 IU/ml	0.0223 IU/ml	0.0197 IU/ml	0.0197 IU/ml	0.02 IU/ml	0.02 IU/ml	0.0197 IU/ml	0.0197 IU/ml	0.0223 IU/ml	0.0223 IU/ml
B	P 1:10	P 1:10	15.0 IU/ml	15.0 IU/ml	2.8 IU/ml	2.8 IU/ml	0.5 IU/ml	0.5 IU/ml	0.1 IU/ml	0.1 IU/ml	0.02 IU/ml	0.02 IU/ml
C	6.7 IU/ml	6.7 IU/ml	5.9 IU/ml	5.9 IU/ml	6 IU/ml	6 IU/ml	5.9 IU/ml	5.9 IU/ml	6.7 IU/ml	6.7 IU/ml	6 IU/ml	6 IU/ml
D	2.23 IU/ml	2.23 IU/ml	1.97 IU/ml	1.97 IU/ml	2 IU/ml	2 IU/ml	1.97 IU/ml	1.97 IU/ml	2.23 IU/ml	2.23 IU/ml	2 IU/ml	2 IU/ml
E	0.67 IU/ml	0.67 IU/ml	0.59 IU/ml	0.59 IU/ml	0.6 IU/ml	0.6 IU/ml	0.59 IU/ml	0.59 IU/ml	0.67 IU/ml	0.67 IU/ml	0.6 IU/ml	0.6 IU/ml
F	0.447 IU/ml	0.447 IU/ml	0.39 IU/ml	0.39 IU/ml	0.4 IU/ml	0.4 IU/ml	0.39 IU/ml	0.39 IU/ml	0.447 IU/ml	0.447 IU/ml	0.4 IU/ml	0.4 IU/ml
G	0.223 IU/ml	0.223 IU/ml	0.197 IU/ml	0.197 IU/ml	0.2 IU/ml	0.2 IU/ml	0.197 IU/ml	0.197 IU/ml	0.223 IU/ml	0.223 IU/ml	0.2 IU/ml	0.2 IU/ml
H	0.067 IU/ml	0.067 IU/ml	0.059 IU/ml	0.059 IU/ml	0.06 IU/ml	0.06 IU/ml	0.059 IU/ml	0.059 IU/ml	0.067 IU/ml	0.067 IU/ml	0.06 IU/ml	0.06 IU/ml

Microplate #4

A	N 1:10	N 1:10	OIEa 1:30,000	OIEa 1:30,000	WHO1 1:300,000	WHO1 1:300,000	WHO2 1:150,000	WHO2 1:150,000	KSU 1:300,000	KSU 1:300,000	WHO1 1:300,000	WHO1 1:300,000
B	P 1:10	P 1:10	KSU Ref#1 1:100	KSU Ref#1 1:100	KSU Ref#2 1:100	KSU Ref#2 1:100	KSU Ref#3 1:100	KSU Ref#3 1:100	KSU Ref#4 1:100	KSU Ref#4 1:100	KSU 1:300,000	KSU 1:300,000
C	OIEa 1:100	OIEa 1:100	WHO1 1:1,000	WHO1 1:1,000	WHO2 1:500	WHO2 1:500	KSU 1:1,000	KSU 1:1,000	WHO1 1:1,000	WHO1 1:1,000	KSU 1:1,000	KSU 1:1,000
D	OIEa 1:300	OIEa 1:300	WHO1 1:3,000	WHO1 1:3,000	WHO2 1:1,500	WHO2 1:1,500	KSU 1:3,000	KSU 1:3,000	WHO1 1:3,000	WHO1 1:3,000	KSU 1:3,000	KSU 1:3,000
E	OIEa 1:1,000	OIEa 1:1,000	WHO1 1:10,000	WHO1 1:10,000	WHO2 1:5,000	WHO2 1:5,000	KSU 1:10,000	KSU 1:10,000	WHO1 1:10,000	WHO1 1:10,000	KSU 1:10,000	KSU 1:10,000
F	OIEa 1:1,500	OIEa 1:1,500	WHO1 1:15,000	WHO1 1:15,000	WHO2 1:7,500	WHO2 1:7,500	KSU 1:15,000	KSU 1:15,000	WHO1 1:15,000	WHO1 1:15,000	KSU 1:15,000	KSU 1:15,000
G	OIEa 1:3,000	OIEa 1:3,000	WHO1 1:30,000	WHO1 1:30,000	WHO2 1:15,000	WHO2 1:15,000	KSU 1:30,000	KSU 1:30,000	WHO1 1:30,000	WHO1 1:30,000	KSU 1:30,000	KSU 1:30,000
H	OIEa 1:10,000	OIEa 1:10,000	WHO1 1:100,000	WHO1 1:100,000	WHO2 1:50,000	WHO2 1:50,000	KSU 1:100,000	KSU 1:100,000	WHO1 1:100,000	WHO1 1:100,000	KSU 1:100,000	KSU 1:100,000

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1:10	N 1:10	0.0223 IU/ml	0.0223 IU/ml	0.0197 IU/ml	0.0197 IU/ml	0.02 IU/ml	0.02 IU/ml	0.0197 IU/ml	0.0197 IU/ml	0.0197 IU/ml	0.0197 IU/ml
B	P 1:10	P 1:10	15.0 IU/ml	15.0 IU/ml	2.8 IU/ml	2.8 IU/ml	0.5 IU/ml	0.5 IU/ml	0.1 IU/ml	0.1 IU/ml	0.0197 IU/ml	0.0197 IU/ml
C	6.7 IU/ml	6.7 IU/ml	5.9 IU/ml	5.9 IU/ml	6 IU/ml	6 IU/ml	5.9 IU/ml	5.9 IU/ml	5.9 IU/ml	5.9 IU/ml	5.9 IU/ml	5.9 IU/ml
D	2.23 IU/ml	2.23 IU/ml	1.97 IU/ml	1.97 IU/ml	2 IU/ml	2 IU/ml	1.97 IU/ml	1.97 IU/ml	1.97 IU/ml	1.97 IU/ml	1.97 IU/ml	1.97 IU/ml
E	0.67 IU/ml	0.67 IU/ml	0.59 IU/ml	0.59 IU/ml	0.6 IU/ml	0.6 IU/ml	0.59 IU/ml	0.59 IU/ml	0.59 IU/ml	0.59 IU/ml	0.59 IU/ml	0.59 IU/ml
F	0.447 IU/ml	0.447 IU/ml	0.39 IU/ml	0.39 IU/ml	0.4 IU/ml	0.4 IU/ml	0.39 IU/ml	0.39 IU/ml	0.39 IU/ml	0.39 IU/ml	0.39 IU/ml	0.39 IU/ml
G	0.223 IU/ml	0.223 IU/ml	0.197 IU/ml	0.197 IU/ml	0.2 IU/ml	0.2 IU/ml	0.197 IU/ml	0.197 IU/ml	0.197 IU/ml	0.197 IU/ml	0.197 IU/ml	0.197 IU/ml
H	0.067 IU/ml	0.067 IU/ml	0.059 IU/ml	0.059 IU/ml	0.06 IU/ml	0.06 IU/ml	0.059 IU/ml	0.059 IU/ml	0.059 IU/ml	0.059 IU/ml	0.059 IU/ml	0.059 IU/ml

Microplate #5

A	N 1:10	N 1:10	WHO1 1:300,000	WHO1 1:300,000	S4 1:100	S4 1:100	S12 1:100	S12 1:100	S20 1:100	S20 1:100	S28 1:100	S28 1:100
B	P 1:10	P 1:10	KSU Ref#1 1:100	KSU Ref#1 1:100	S5 1:100	S5 1:100	S13 1:100	S13 1:100	S21 1:100	S21 1:100	S29 1:100	S29 1:100
C	WHO1 1:1,000	WHO1 1:1,000	KSU Ref#2 1:100	KSU Ref#2 1:100	S6 1:100	S6 1:100	S14 1:100	S14 1:100	S22 1:100	S22 1:100	S30 1:100	S30 1:100
D	WHO1 1:3,000	WHO1 1:3,000	KSU Ref#3 1:100	KSU Ref#3 1:100	S7 1:100	S7 1:100	S15 1:100	S15 1:100	S23 1:100	S23 1:100	S31 1:100	S31 1:100
E	WHO1 1:10,000	WHO1 1:10,000	KSU Ref#4 1:100	KSU Ref#4 1:100	S8 1:100	S8 1:100	S16 1:100	S16 1:100	S24 1:100	S24 1:100	S32 1:100	S32 1:100
F	WHO1 1:15,000	WHO1 1:15,000	S1 1:100	S1 1:100	S9 1:100	S9 1:100	S17 1:100	S17 1:100	S25 1:100	S25 1:100	S33 1:100	S33 1:100
G	WHO1 1:30,000	WHO1 1:30,000	S2 1:100	S2 1:100	S10 1:100	S10 1:100	S18 1:100	S18 1:100	S26 1:100	S26 1:100	S34 1:100	S34 1:100
H	WHO1 1:100,000	WHO1 1:100,000	S3 1:100	S3 1:100	S11 1:100	S11 1:100	S19 1:100	S19 1:100	S27 1:100	S27 1:100	S35 1:100	S35 1:100

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1:10	N 1:10	0.0197 IU/ml	0.0197 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
B	P 1:10	P 1:10	15.0 IU/ml	15.0 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
C	5.9 IU/ml	5.9 IU/ml	2.8 IU/ml	2.8 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
D	1.97 IU/ml	1.97 IU/ml	0.5 IU/ml	0.5 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
E	0.59 IU/ml	0.59 IU/ml	0.1 IU/ml	0.1 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
F	0.39 IU/ml	0.39 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
G	0.197 IU/ml	0.197 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
H	0.059 IU/ml	0.059 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown

Microplate #6

A	N 1:10	N 1:10	WHO1 1:300,000	WHO1 1:300,000	S39 1:100	S39 1:100	S47 1:100	S47 1:100	S55 1:100	S55 1:100	S63 1:100	S63 1:100
B	P 1:10	P 1:10	KSU Ref#1 1:100	KSU Ref#1 1:100	S40 1:100	S40 1:100	S48 1:100	S48 1:100	S56 1:100	S56 1:100	S64 1:100	S64 1:100
C	WHO1 1:1,000	WHO1 1:1,000	KSU Ref#2 1:100	KSU Ref#2 1:100	S41 1:100	S41 1:100	S49 1:100	S49 1:100	S57 1:100	S57 1:100	S65 1:100	S65 1:100
D	WHO1 1:3,000	WHO1 1:3,000	KSU Ref#3 1:100	KSU Ref#3 1:100	S42 1:100	S42 1:100	S50 1:100	S50 1:100	S58 1:100	S58 1:100	S66 1:100	S66 1:100
E	WHO1 1:10,000	WHO1 1:10,000	KSU Ref#4 1:100	KSU Ref#4 1:100	S43 1:100	S43 1:100	S51 1:100	S51 1:100	S59 1:100	S59 1:100	S67 1:100	S67 1:100
F	WHO1 1:15,000	WHO1 1:15,000	S36 1:100	S36 1:100	S44 1:100	S44 1:100	S52 1:100	S52 1:100	S60 1:100	S60 1:100	S68 1:100	S68 1:100
G	WHO1 1:30,000	WHO1 1:30,000	S37 1:100	S37 1:100	S45 1:100	S45 1:100	S53 1:100	S53 1:100	S61 1:100	S61 1:100	S69 1:100	S69 1:100
H	WHO1 1:100,000	WHO1 1:100,000	S38 1:100	S38 1:100	S46 1:100	S46 1:100	S54 1:100	S54 1:100	S62 1:100	S62 1:100	S70 1:100	S70 1:100

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1:10	N 1:10	0.0197 IU/ml	0.0197 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
B	P 1:10	P 1:10	15.0 IU/ml	15.0 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
C	5.9 IU/ml	5.9 IU/ml	2.8 IU/ml	2.8 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
D	1.97 IU/ml	1.97 IU/ml	0.5 IU/ml	0.5 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
E	0.59 IU/ml	0.59 IU/ml	0.1 IU/ml	0.1 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
F	0.39 IU/ml	0.39 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
G	0.197 IU/ml	0.197 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
H	0.059 IU/ml	0.059 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown

Microplate #7

A	N 1:10	N 1:10	WHO1 1:300,000	WHO1 1:300,000	S74 1:100	S74 1:100	S82 1:100	S82 1:100	S90 1:100	S90 1:100	S98 1:100	S98 1:100
B	P 1:10	P 1:10	KSU Ref#1 1:100	KSU Ref#1 1:100	S75 1:100	S75 1:100	S83 1:100	S83 1:100	S91 1:100	S91 1:100	S99 1:100	S99 1:100
C	WHO1 1:1,000	WHO1 1:1,000	KSU Ref#2 1:100	KSU Ref#2 1:100	S76 1:100	S76 1:100	S84 1:100	S84 1:100	S92 1:100	S92 1:100	S100 1:100	S100 1:100
D	WHO1 1:3,000	WHO1 1:3,000	KSU Ref#3 1:100	KSU Ref#3 1:100	S77 1:100	S77 1:100	S85 1:100	S85 1:100	S93 1:100	S93 1:100	S101 1:100	S101 1:100
E	WHO1 1:10,000	WHO1 1:10,000	KSU Ref#4 1:100	KSU Ref#4 1:100	S78 1:100	S78 1:100	S86 1:100	S86 1:100	S94 1:100	S94 1:100	S102 1:100	S102 1:100
F	WHO1 1:15,000	WHO1 1:15,000	S71 1:100	S71 1:100	S79 1:100	S79 1:100	S87 1:100	S87 1:100	S95 1:100	S95 1:100	S103 1:100	S103 1:100
G	WHO1 1:30,000	WHO1 1:30,000	S72 1:100	S72 1:100	S80 1:100	S80 1:100	S88 1:100	S88 1:100	S96 1:100	S96 1:100	S104 1:100	S104 1:100
H	WHO1 1:100,000	WHO1 1:100,000	S73 1:100	S73 1:100	S81 1:100	S81 1:100	S89 1:100	S89 1:100	S97 1:100	S97 1:100	S105 1:100	S105 1:100

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1:10	N 1:10	0.0197 IU/ml	0.0197 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
B	P 1:10	P 1:10	15.0 IU/ml	15.0 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
C	5.9 IU/ml	5.9 IU/ml	2.8 IU/ml	2.8 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
D	1.97 IU/ml	1.97 IU/ml	0.5 IU/ml	0.5 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
E	0.59 IU/ml	0.59 IU/ml	0.1 IU/ml	0.1 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
F	0.39 IU/ml	0.39 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
G	0.197 IU/ml	0.197 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
H	0.059 IU/ml	0.059 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown

Microplate #8

A	N 1:10	N 1:10	WHO1 1:300,000	WHO1 1:300,000	S109 1:100	S109 1:100	S117 1:100	S117 1:100	S125 1:100	S125 1:100	S133 1:100	S133 1:100
B	P 1:10	P 1:10	KSU Ref#1 1:100	KSU Ref#1 1:100	S110 1:100	S110 1:100	S118 1:100	S118 1:100	S126 1:100	S126 1:100	S134 1:100	S134 1:100
C	WHO1 1:1,000	WHO1 1:1,000	KSU Ref#2 1:100	KSU Ref#2 1:100	S111 1:100	S111 1:100	S119 1:100	S119 1:100	S127 1:100	S127 1:100	S135 1:100	S135 1:100
D	WHO1 1:3,000	WHO1 1:3,000	KSU Ref#3 1:100	KSU Ref#3 1:100	S112 1:100	S112 1:100	S120 1:100	S120 1:100	S128 1:100	S128 1:100	S136 1:100	S136 1:100
E	WHO1 1:10,000	WHO1 1:10,000	KSU Ref#4 1:100	KSU Ref#4 1:100	S113 1:100	S113 1:100	S121 1:100	S121 1:100	S129 1:100	S129 1:100	S137 1:100	S137 1:100
F	WHO1 1:15,000	WHO1 1:15,000	S106 1:100	S106 1:100	S114 1:100	S114 1:100	S122 1:100	S122 1:100	S130 1:100	S130 1:100	S138 1:100	S138 1:100
G	WHO1 1:30,000	WHO1 1:30,000	S107 1:100	S107 1:100	S115 1:100	S115 1:100	S123 1:100	S123 1:100	S131 1:100	S131 1:100	S139 1:100	S139 1:100
H	WHO1 1:100,000	WHO1 1:100,000	S108 1:100	S108 1:100	S116 1:100	S116 1:100	S124 1:100	S124 1:100	S132 1:100	S132 1:100	S140 1:100	S140 1:100

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1:10	N 1:10	0.0197 IU/ml	0.0197 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
B	P 1:10	P 1:10	15.0 IU/ml	15.0 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
C	5.9 IU/ml	5.9 IU/ml	2.8 IU/ml	2.8 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
D	1.97 IU/ml	1.97 IU/ml	0.5 IU/ml	0.5 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
E	0.59 IU/ml	0.59 IU/ml	0.1 IU/ml	0.1 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
F	0.39 IU/ml	0.39 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
G	0.197 IU/ml	0.197 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
H	0.059 IU/ml	0.059 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown

Microplate #9

A	N 1:10	N 1:10	WHO1 1:300,000	WHO1 1:300,000	S144 1:100	S144 1:100	S152 1:100	S152 1:100	S160 1:100	S160 1:100	S168 1:100	S168 1:100
B	P 1:10	P 1:10	KSU Ref#1 1:100	KSU Ref#1 1:100	S145 1:100	S145 1:100	S153 1:100	S153 1:100	S161 1:100	S161 1:100	S169 1:100	S169 1:100
C	WHO1 1:1,000	WHO1 1:1,000	KSU Ref#2 1:100	KSU Ref#2 1:100	S146 1:100	S146 1:100	S154 1:100	S154 1:100	S162 1:100	S162 1:100	S170 1:100	S171 1:100
D	WHO1 1:3,000	WHO1 1:3,000	KSU Ref#3 1:100	KSU Ref#3 1:100	S147 1:100	S147 1:100	S155 1:100	S155 1:100	S163 1:100	S163 1:100	S172 1:100	S172 1:100
E	WHO1 1:10,000	WHO1 1:10,000	KSU Ref#4 1:100	KSU Ref#4 1:100	S148 1:100	S148 1:100	S156 1:100	S156 1:100	S164 1:100	S164 1:100	S173 1:100	S173 1:100
F	WHO1 1:15,000	WHO1 1:15,000	S141 1:100	S141 1:100	S149 1:100	S149 1:100	S157 1:100	S157 1:100	S165 1:100	S165 1:100	S174 1:100	S174 1:100
G	WHO1 1:30,000	WHO1 1:30,000	S142 1:100	S142 1:100	S150 1:100	S150 1:100	S158 1:100	S158 1:100	S166 1:100	S166 1:100	S175 1:100	S175 1:100
H	WHO1 1:100,000	WHO1 1:100,000	S143 1:100	S143 1:100	S151 1:100	S151 1:100	S159 1:100	S159 1:100	S167 1:100	S167 1:100	S176 1:100	S177 1:100

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1:10	N 1:10	0.0197 IU/ml	0.0197 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
B	P 1:10	P 1:10	15.0 IU/ml	15.0 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
C	5.9 IU/ml	5.9 IU/ml	2.8 IU/ml	2.8 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
D	1.97 IU/ml	1.97 IU/ml	0.5 IU/ml	0.5 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
E	0.59 IU/ml	0.59 IU/ml	0.1 IU/ml	0.1 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
F	0.39 IU/ml	0.39 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
G	0.197 IU/ml	0.197 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown
H	0.059 IU/ml	0.059 IU/ml	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown

Microplate #10

A	N 1:10	N 1:10	WHO1 1:300,000	WHO1 1:300,000	S179 1:100	S179 1:100						
B	P 1:10	P 1:10	KSU Ref#1 1:100	KSU Ref#1 1:100	S180 1:100	S180 1:100						
C	WHO1 1:1,000	WHO1 1:1,000	KSU Ref#2 1:100	KSU Ref#2 1:100								
D	WHO1 1:3,000	WHO1 1:3,000	KSU Ref#3 1:100	KSU Ref#3 1:100								
E	WHO1 1:10,000	WHO1 1:10,000	KSU Ref#4 1:100	KSU Ref#4 1:100								
F	WHO1 1:15,000	WHO1 1:15,000	S176 1:100	S176 1:100								
G	WHO1 1:30,000	WHO1 1:30,000	S177 1:100	S177 1:100								
H	WHO1 1:100,000	WHO1 1:100,000	S178 1:100	S178 1:100								

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1:10	N 1:10	0.0197 IU/ml	0.0197 IU/ml	unknown	unknown						
B	P 1:10	P 1:10	15.0 IU/ml	15.0 IU/ml	unknown	unknown						
C	5.9 IU/ml	5.9 IU/ml	2.8 IU/ml	2.8 IU/ml								
D	1.97 IU/ml	1.97 IU/ml	0.5 IU/ml	0.5 IU/ml								
E	0.59 IU/ml	0.59 IU/ml	0.1 IU/ml	0.1 IU/ml								
F	0.39 IU/ml	0.39 IU/ml	unknown	unknown								
G	0.197 IU/ml	0.197 IU/ml	unknown	unknown								
H	0.059 IU/ml	0.059 IU/ml	unknown	unknown								

6.2 Test Procedure

A. Controls, References and Serum Distribution

1. Control Distribution:

Controls are not ready-to-use and should be diluted at 1:10. Dispense 90µl of sample diluent, and add 10µl of the kit negative control into wells A1 and A2, and 10µl of the positive control to wells B1 and B2.

2. Distribution of References and Serum:

Dispense 90µl of sample diluent, and add 10µl of either reference or serum sample into the test wells and mix gently. Strips should always be placed on the frame so that both washer and reader can be used. Cover the wells with adhesive film, cut to the necessary length by the number of strips used. Mix plate manually by gentle shaking or by using plate agitator.

3. Incubation of the plate: 1 hour ± 5 minutes at +37 ± 3°C

4. Plate Washing: Wash buffer: dilute the concentrated washing solution (W)

1:10 in distilled or demineralized water. Carefully remove the adhesive film and wash 4 times.

B. Addition of the Conjugate

1. Preparation of the conjugate: Dilute the concentrate (CJ) 1:10 in the conjugate diluent (CD). 2mL are needed for one strip, meaning 200µl of CJ in 1.8 mL of CD.

2. Distribution of the Conjugate: Add 100µl of diluted conjugate to all the wells and cover with a new piece of adhesive film.

3. Incubation of Conjugate: 1 hour ± 5 minutes at +37 ± 3°C

4. Plate Washing: Carefully remove the adhesive film and wash 4 times.

C. Revelation

1. Addition of the Substrate: Add 100µl of buffered peroxidase substrate (PS)

per well. Do not cover with adhesive film at this stage. Mix by gentle shaking the plate manually or use a plate agitator to ensure correct homogenization.

2. Incubation of substrate: Incubate for 30 ± 5 minutes at laboratory temperature (+23 ± 5°C)

shielded from light.

3. Addition of the Stop Solution: Add 50µl of stop solution (S) per well. Mix by gentle shaking the plate manually or by using a plate agitator. Make certain that no bubbles occur in the wells.

4. Measure of the Optical Density: Measure the optical density (OD) bichromatically at 450 and 630 nm or monochromatically at 450 nm (in the yellow band). Reading bichromatically is strongly recommended. Should a monochromatic reader be used, ensure the cleanliness of the bottom of the wells prior to reading.

D. Test Validation

The results of each test run (or for each plate) are validate:

A. When the OD obtained with the positive control is ≥ 0.3000, and

B. When the OD obtained with the negative control is < 0.50 x OD P, and

C. When the correlation coefficient between the Neperian logarithm (ln) ODs and in Rabies Ab concentrations for the *WHO* standard serum is > 0.95.

E. Expression and Interpretation of the Results

The method of titer calculation will be completed using the Regression Curve. The calculations will be accomplished by utilizing an Excel spread-sheet obtained by Zoetis specifically designed for this ELISA. Calculations will include:

1. Calculate the average OD value for each sample tested and each WHO serum dilution. Also calculate OIE and KSU reference serum from the WHO standard curve. Also compare results against each other.

2. Calculate the Neperian Logarithm (ln) value for each average OD and the ln Value of the Rabies Ab concentration for each WHO, KSU and OIE dilution.

3. Plot the ln (OD) (Y axis) as a function of the ln (Rabies Ab concentration) (X axis) in order to draw the reference curve for the WHO, KSU and OIE standard serum.

4. Use all individual results obtained for the WHO, KSU and OIE standard serum dilutions, perform a linear regression curve between ln Rabies Ab concentrations (expressed in EU/mL) (equivalent units per mL) and ln (OD), to establish the corresponding mathematics

model:

$$\ln[\text{Rabies Ab concentration (EU/mL)}] = a + b * \ln OD$$

For each tested sample, calculate the average OD value and then the Rabies antibody concentration of the sample expressed as:
«equivalent units per ml» (EU/mL), from the established model: Sample Rabies Ab concentration (EU/mL) = $e^{(a + \hat{b} \ln OD)}$

5. If the calculated titer is > 0.6, the sample is considered as protected.

6. If the calculated titer is < 0.6, the sample is considered as not protected (a confirmation utilizing FAVN testing methodology can be performed).

6.3 Abbreviations

ELISA – Enzyme-linked Immunosorbent Assay
KSU – Kansas State University
OIE – World Organization For Animal Health
MNT – Mouse Neutralizing Test
PPE – Proper Protective Equipment
RFFIT – Rapid Fluorescent Focus Inhibition Test
RVNA – Rabies Virus Neutralizing Antibody
WHO – World Health Organization

Reference List

(1) World Health Organization (2013). *WHO Guide for Rabies Pre and Post Exposure Prophylaxis in Humans.* 11:15, Mar 12, 2014 Retrieved from http://www.who.int/rabies/WHO_Guide_Rabies_Pre_Post_Exposure_Prophylaxis_Humans_2013.pdf.

(2) Moore, SM, Hanlon, CA (2010). *Rabies-Specific Antibodies: Measuring Surrogates of Protection against a Fatal Disease.* Retrieved 12:00, Mar 11, 2014 from http://www.plosntds.org/article/info%3Adoi%2F10.1371%2Fjournal.pntd.0000595.

(3) Moore, SM, Gordon, CR, Briggs, DJ (2007). Rabies Serology. Jackson, AC, Wunner, WH (Ed.), *Rabies Second Edition* (pp. 471). United Kingdom: Elsevier