RABIES SEROLOGY: RELATIONSHIP BETWEEN ASSAY TYPE, INTERPRETATION, AND APPLICATION OF RESULTS

by

SUSAN M. MOORE

M.S., Kansas State University, 2005

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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Abstract

The immune status of an individual host or among a population is affected by important variables including the source and route of potential natural exposure and for vaccination consist of vaccine type, potency, and virus strain; vaccination route and schedule; and individual host factors. Although, perhaps, often overlooked, it is essential to have a basic understanding of the laboratory methods used to measure and assess the host's immune status. The precision, accuracy, sensitivity, and specificity of a method must be well defined. Moreover, an "adequate," acceptable, or diagnostic value for each method must be clearly defined so that a particular test result for a patient can be meaningfully interpreted in relation to the patient's history and clinical management. The reasons for performing rabies serology can range from diagnosis of infection to investigation of epitope specificity of an anti-rabies virus glycoprotein monoclonal antibody. Characterization of an antibody's affinity, specificity, quantity, and neutralizing function, and class/subclass are achieved by various methods. Many serological techniques developed over the past five decades differ not only in their ability to detect the function, affinity and specificity of rabies virus antibodies, but also in the ease and practicality with which they are performed. To select an appropriate method and appropriately interpret test results, it is essential to understand the specific strengths, weaknesses, and limitations of available methods. The decision to use a specific assay should start with the purpose of testing and the intended application of results. Other factors to consider are the assay complexity, degree of precision and/or accuracy, specificity and range of detection. Given the importance of RVNA levels in the prevention of human and animal rabies, guidelines for adequate vaccination should be stated in terms that are readily understood by individuals-at-risk and health care providers, both veterinary and medical, who will use the recommendations for clinical management of humans or animals. Across the

globe, the standardization of rabies serologic assays has a direct effect on the clinical use of human and animal products, including direct assessment of, and assessment of host responses to, rabies vaccines for the prevention of rabies.

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sample is inversely related to intensity of color development. The level of antibody in the test serum can be quantitated by use of a standard curve and an OD reader. **(D) Lateral Flow.** The test serum sample is mixed with inactivated virus before adding the mixture to the absorbent material of the test strip. The mixture then flows across to encounter a labeled anti-rabies virus (anti-G) antibody which will bind to any unbound inactivated rabies virus. The mixture continues to flow toward two areas (lines) of the strip bound with detection antibodies; the first detection antibody is specific for the rabies virus (anti-G), and the second detection antibody is specific for the labeled rabies virus (anti-G) antibody. If the mixture contains unbound inactivated rabies virus there will a color development at the first strip, indicating the sample did not contain enough anti-rabies virus antibody to bind the inactivated rabies virus in the mixture. If the test serum sample does contain anti-rabies virus antibody, then the labeled antibody will pass the first strip and be bound by the antibody in the second strip causing a color development at the second strip. The results will be either "presence of rabies virus antibody" (only second strip visible) or 'absence of

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I received much encouragement from my friends and family. They kept me going and listened. I especially am grateful to my children Colleen, Robert, and Chris and Shannah for being there. Lastly, but most importantly, I could not have started, persevered, or finished this academic trip without the constant encouragement of my husband Toby; my thanks to him is endless.

Dedication

To my husband, Toby, for his extreme patience and unwavering belief that I could reach my goal, I dedicate this work.

Chapter 1 - Literature Review

Introduction

> "Measurements are the basis of science.
> Therefore the methods used to assess immunological parameters and
> immunity…… need to be critically reviewed.
> Is the chosen parameter accurately measured, is it robust,
> is it a good correlate of protective immunity…..?"
> (Zinkernagel, 2002)

There are a number of variables to be considered in the assessment of immune status of an individual host or among a population. For a pathogen like rabies virus, important variables include the source and route of potential natural exposure and for vaccination consist of vaccine type, potency, and virus strain; vaccination route and schedule; and individual host factors. Although perhaps often overlooked, it is essential to have a basic understanding of the laboratory methods used to measure and assess the host's immune status. The precision, accuracy, sensitivity, and specificity of a method must be well defined. Moreover, an "adequate," acceptable, or diagnostic value for each method must be clearly defined so that a particular test result for a patient can be meaningfully interpreted in relation to the patient's history and clinical management. If these parameters are not clearly and objectively defined, conclusions based on test results from various methods may be inherently misleading. If a laboratory method such as the rapid fluorescent focus inhibition test (RFFIT) (Smith, Yager, & Baer, 1973) developed for measuring vaccine response in serum samples is applied for the analysis of biologic products such as human or equine rabies immune globulin (RIG) or rabies virus neutralizing monoclonal antibodies, the method will most likely need modifications and thus also subsequent method validation.

Serology is the study of the immunological properties of blood serum or other bodily fluids. For the most part, serology is the investigation of antibodies in serum, although assessment of immunity may be conducted on cerebrospinal fluid and other sources of fluid. Antibodies are produced by plasma cells which may be specifically activated in response to antigens, such as those from viruses and bacteria, to protect the host. The primary action of an antibody is to bind to antigen. The secondary or effector actions of antibodies include neutralization and opsonization of infectious agents, and activation of other immune mediators (see Figure 1.1). Complement activation and antibody dependent cellular cytotoxity (ADCC) are other effector functions that rely on the binding action of antibodies. Not all antibodies have effector actions. Some antibodies that bind to an antigen may not result in a biological effect because they are not effective in eliciting a secondary effect. Effector actions occur in accordance with the individual characteristics of a specific antibody structure and depend upon the class, subclass, or variable region of an antibody. In a competent host, exposure to an antigen will activate multiple immune cell clones and result in the production of a polyclonal antibody response.
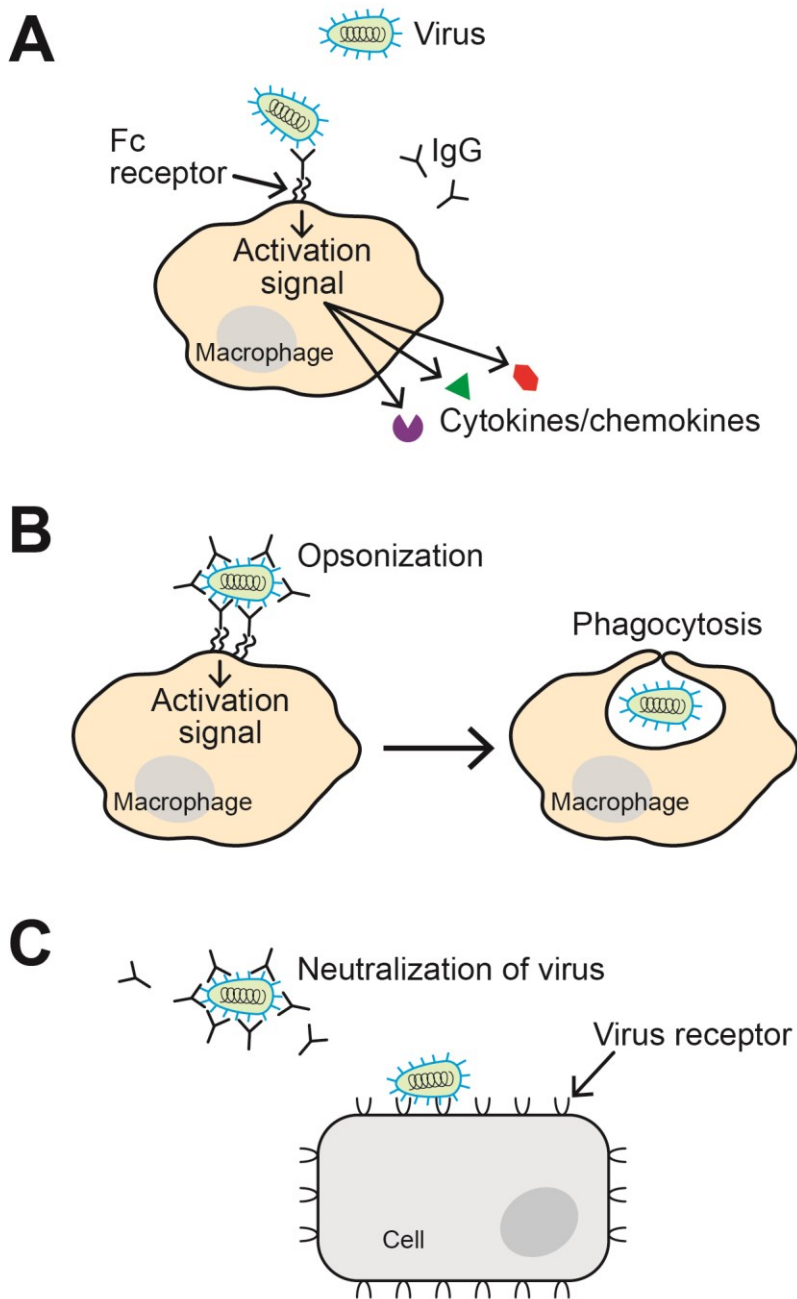
Figure 1.1 The effector functions of antibodies include: A. activation of immune cells such as macrophages to produce cytokines and chemokines through Fc receptor binding; B. Opsonization of infectious organisms induces phagocytosis of the organisms through Fc receptor binding; and C. Neutralization of virus though binding of proteins used for attachment and entry of the virus, thereby blocking infection of the cell.

Rabies virus specific antibodies are produced by the immune system in response to infection or vaccination *in vivo*, or by immune cells or molecular methods *in vitro*. The reasons for performing rabies serology can range from diagnosis of infection to investigation of epitope specificity of an anti-rabies virus glycoprotein monoclonal antibody. Characterization of an antibody's affinity, specificity, quantity, and neutralizing function, complement binding function, and class/subclass are achieved by various methods. Many serological techniques developed over the past five decades differ not only in their ability to detect the function, affinity and specificity of rabies virus antibodies, but also in the ease and practicality with which they are performed. To select an appropriate method and appropriately interpret test results, it is essential to understand the specific strengths, weaknesses, and limitations of available methods. Numerous reports indicate that protection against rabies is largely dependent upon the presence of rabies virus neutralizing antibodies (RVNA) (Hooper et al., 1998; Dietzschold, 1993). Thus, assays to detect and quantify RVNA, such as the rapid fluorescent focus inhibition test (RFFIT) (Smith et al., 1973) and the fluorescent antibody virus neutralization test (FAVN) (Cliquet, Aubert, & Sagne, 1998) are the methods recommended for quantitation purposes in rabies serology. Antigen binding assays have proven to be useful for the detection of specific isotypes of rabies virus antibodies, either using whole virions or specific viral proteins as antigen(s). The decision to use a specific assay should start with the purpose of testing and the intended application of results. Other factors to consider are the assay complexity, degree of precision and/or accuracy, specificity and range of detection. In addition, the availability of laboratory materials, instruments, and safety equipment also must be considered. It is critical to understand exactly what aspect of rabies virus specific antibodies are measured as well as the limitation of the assay in order to select the best test and also interpret and use the test results in an appropriate manner.

Investigative serology focuses on the detection and measurement of immune components in blood (usually serum) including immunoglobulins of several subclasses directed against specific epitopes. Detection of IgM and IgG classes is dependent upon the time point in the course of the humoral immune response after exposure to an antigen. In the initial or primary antibody response, IgM is produced first in relatively low levels followed by higher levels of IgG after the occurrence of class switching. If the purpose of the assay is to detect the initial response, it should be designed to detect both IgM and IgG. The specificity of the immunoglobulin produced is driven by distinct epitopes present on the rabies viral proteins used to generate the antibodies. Exposure to rabies virus, whether through vaccination with inactivated virus or through exposure, induces the formation of antibodies potentially against all viral proteins, but predominantly against the rabies virus glycoprotein (G) and nucleoprotein (N). Studies of monoclonal antibodies (Mabs) capable of neutralizing rabies virus indicate that these Mabs are directed against a number epitopes on the G of rabies virus (Tordo, 1996).

Rabies virus neutralization requires a minimum number of antibody molecules per G spike to induce steric hindrance of the virus-receptor-binding activity (Flamand, Raux, Gaudin, & Ruigrok, 1993). Another mechanism may involve conformational changes in the G protein ultimately resulting in the loss of virion receptor-binding ability (Irie & Kawai, 2002). The humoral immune response elicited by rabies virus vaccination consists of a mixture of polyclonal antibodies that influence a variety of complex neutralization mechanisms.

Specific methods to detect antibodies specific for rabies viral antigens include precipitation, agglutination, immunoelectrophoresis, radioimmunoassay, enzyme-linked immunosorbent assays (ELISA), Western blots, indirect immunofluorescence, immunoelectron microscopy and serum neutralization assays. All of these assays depend on an antibody–antigen

interaction to detect the presence of an antibody. Two basic types of assays are used: 1) assays involving primary binding activity between antibodies and antigens, and 2) functional assays to measure neutralization actions of antibodies. Although other components and products of the immune system are involved, protection from clinical rabies after infection relies heavily on the presence of RVNA. Therefore, methods to detect and quantify antibodies which can functionally neutralize rabies virus are recommended to quantify the level of immunity after rabies vaccination.

1.1 **History of Regulatory Standards**

Throughout history, various governmental entities have been responsible for regulating drug products and related methods for human health assessment. This has and continues to include measures of rabies immunity in relation to diagnosis, qualification of biologic products such as vaccines and rabies immune globulins, and response to vaccination, particularly in at-risk groups. The primary function of government regulation has been to ensure the quality and safety of drug products available for human use. Federal regulations began on a large scale in the early twentieth century when the U.S. Congress enacted the Pure Food and Drugs Act of 1906. The Food and Drug Administration (FDA) was born from this law and has oversight over products, marketing of food and drugs to consumers, and the manufacturing practices of food and drug industrial companies.

Most major regulatory standards throughout history arose from disasters. In 1976, Good Laboratory Practices (GLP) were created by FDA in response to a high percentage of studies with flawed data, falsified data, insufficient documentation, inappropriate testing facilities, and instances where experimental animals were subjected to inhumane conditions. In the 1970's, the Organization for Economic Co-operation and Development was created to ensure quality,

integrity, and reproducibility of data, a global definition of GLP. Good Manufacturing Practices (GMP) was created in 1972 in response to the Davenport Disaster in which six people died due to contaminated intravenous fluids. Good Clinical Practices (GCP) began at the end of World War II following inhumane experiments performed on humans prompting the Nuremberg Code, which outlines the proper means to conduct research. These instructions were the foundation of GCP (Milestones in US Food and Drug Law History, FDA, (2009)). Each standard has been updated continually to meet the growing demands of ensuring safety and the quality of drug research and clinical trials. Throughout the 70's and 80's, Japan, the UK and other European countries had developed their own set of GCP guidelines. Different guidelines developed and put in place in each individual country brought into question the validity of clinical trials performed in different countries. In 1996, conferences were held to unify each country's GCP codes of practice resulting in the "International Conference on Harmonization of Good Clinical Practice" ICH-GCP (Baynes, 2005). The International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use (1996) *Guideline for Good Clinical Practice E6(R1)* is available online from:

http://www.ich.org/LOB/media/MEDIA482.pdf (Accessed: 08 October 2010).

1.2 **Regulatory requirements**

Research is the cornerstone of all scientific endeavors. It is a harnessing of curiosity to solve new or existing problems, prove new ideas, or develop new theories. Research is not defined or controlled. No formal regulatory requirements exist for laboratory assay development or performance for scientific research. Primary regulations dealing with clinical laboratories like the Clinical Laboratory Improvement Amendments (CLIA) or the College of American Pathologists (CAP) specifically state that they do not have jurisdiction over research. The FDA

does not provide regulations for oversight of research testing. Even without regulatory guidance, researchers can and do produce work of the highest quality. However, any technology such as: new test methods, new vaccines, or prophylactic drugs, that may be developed in an academic setting, and eventually transferred to the industrial world must meet quality criteria in order to have any commercial potential. So can the 'assurance of quality' needed for regulatory compliance be found within basic research?

The answer to this question is based upon an understanding of what 'assurance of quality' is needed for a researcher to test at a level compliant with "Good Practices" as described for laboratory, clinical or manufacturing environments, and designated as GLP, GCP or GMP or implying any or all of the three by GxP, which GxP represents the abbreviations of these titles where x (a common symbol for a variable) represents the specific regulation. First, it must be understood what is meant by 'assurance of quality' as defined within a GxP environment. Second, it is critical to understand when to apply the different GxP regulations within the research and development applied to the prevention or treatment of a disease.

The fundamental of any requirement or standard is a complete and operational quality system that will demonstrate that there is overall control of all aspects of testing and quality. Components of a Quality System include but are not limited to the following: the study regulations or quality standards of the regulatory body, the testing and standard operational procedures (SOPs), the organization infrastructure with roles and responsibilities (i.e., study directory QA, testing personnel, management), trained and competent testing personnel, personnel safety, physical facilities, validated/calibrated equipment, validated testing procedures, certified reference materials, quality control programs (proficiency testing, conformation testing, quality control samples), an internal audit program, laboratory information systems, and

procedures for recording and archiving of data (Food and Drug Administration, 1996). These standards, if implemented within the laboratory, allow optimal laboratory operations to ensure consistent, reproducible, auditable, and reliable laboratory results of a sufficient quality for regulatory testing. Complete documentation is required to allow reconstruction of the events not only during and right after completion of the study, but also 5-10 or more years later. A quality system must be intentionally included in the study or testing process before it starts, and monitored and documented during its performance. Quality cannot be created after the work has been completed.

Drug Development Stages

Stage 1	Stage 2	Stage 3	Stage 4
Discovery	**Non-clinical**	**Clinical**	**Manufacturing**
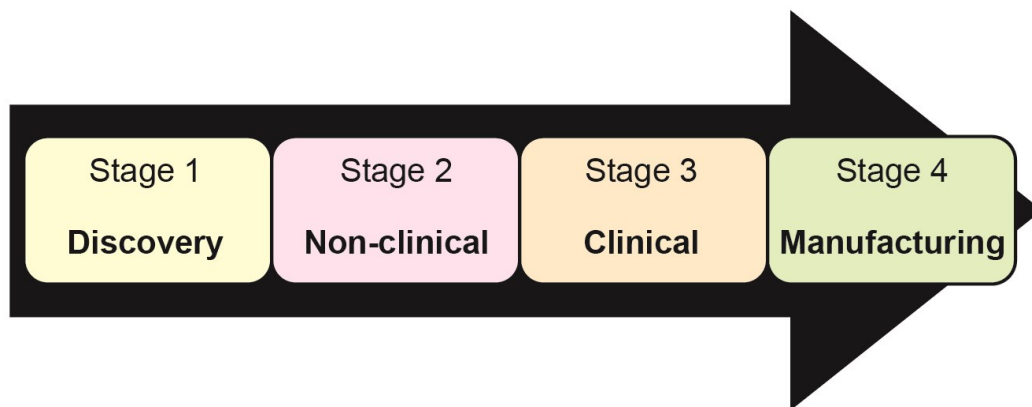
Figure 1.2 Drug development is delineated into specific stages, each with its own requirement of regulatory oversight.

With the importance of producing quality products and processes and the increase in regulatory scrutiny of all aspects of product development and the manufacturing cycle, researchers must understand what regulatory environment is applicable for each phase (see Figure 1.2) of the development of a product. Researchers need to have an organized approach to applying and/or combining the GXP functions.

In a rabies research environment, all information and data collected during the basic research stage may be inadmissible in the regulatory findings if not obtained through a quality system in compliance with GxP regulations. The quality system in place while the research is being performed will dictate whether the information and data will be suitable for regulatory evaluation. Table 1.1 details the regulatory requirements that must be complied with for particular products under development.

The intended use of the data to be generated will define the recommended regulatory level of testing for a product or drug. During the first few stages of drug or treatment studies, including basic research to drug discovery, oversight is not provided by any regulatory agency as shown in Figure 1.3. During the preclinical development stage, compliance with GLP is required (2011). GLP is only relevant to non-clinical (human) testing and deals with the organization, processes and conditions under which these studies are planned, performed, monitored, recorded and reported. The primary purpose of these (non-human evaluations) studies is generally safety testing for the drug and/or product. Clinical evaluation in humans would follow successful safety studies. During the human testing phase, GCP is the basis for quality standards and regulatory compliance. GCP addresses source data for clinical trials and applies to human research studies where the rights, integrity and confidentiality of trial subjects are protected. Within the US, GCP used to support human diagnostics and health-care are established by Centers for Medicare & Medicaid Services (CMS) regulations and accrediting organizations such as Clinical laboratory Improvement Amendments (CLIA). GCP must be instituted in all laboratory testing during the clinical trials from Phase I (to demonstrate tolerance of the test drug and to define human pharmacokinetics), through Phase II (where the dose–effect relationship is confirmed), to Phase III (full-scale, often multicenter, clinical efficacy trials in hundreds and thousands of patients).

The final phase is manufacturing. Any laboratory testing preformed on the manufactured product and the actual manufacturing of the product will be completed in compliance with GMP standards. These regulations and instructions are covered in FDA, Code of Regulations Title 21.

Table 1.1 Regulatory Requirements – Laboratory Testing

Type of Testing	Applicable Regulatory Standard
Drug Product	GMP
Drug Product in Animals	GLP
Human Specimens/Trials	GCP/CLIA
Non clinical safety studies development of drugs	GLP
Basic research	Non-Regulated Testing
Studies to develop new analytical methods	Non-Regulated Testing
Discovery of drug product	Non-Regulated Testing
Discovery of disease	Non-Regulated Testing

GxP Regulations from Research to Manufacturing

Basic Research

Disease Discovery

Drug Discovery

Preclinical Development

Clinical Trials I, II, III

Manufacturing

GLP GCP GMP

← No GLP/GMP →

GLP is study based   GLP is process based
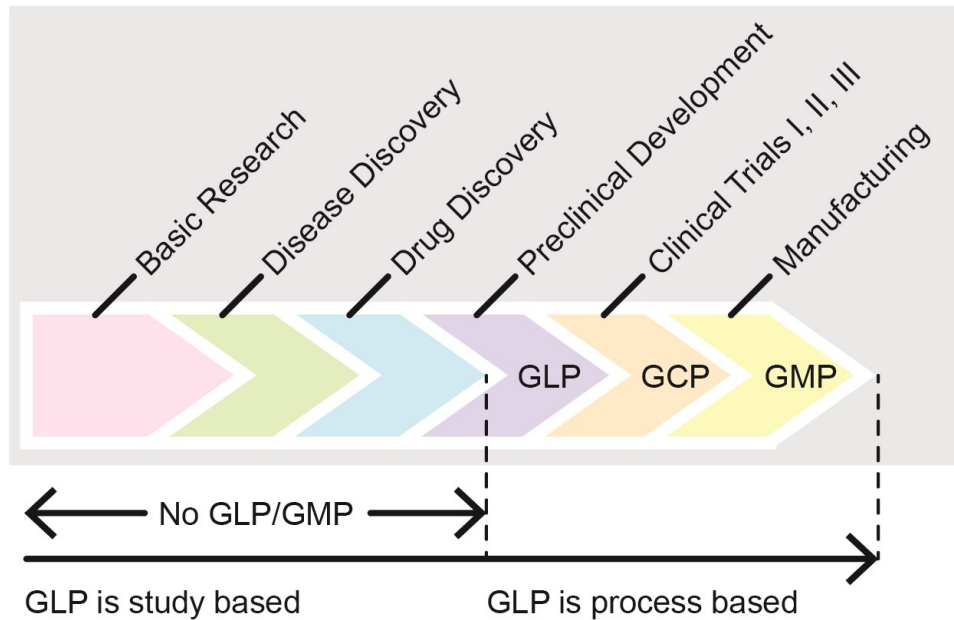
Figure 1.3 The intended use of the data to be generated will define the recommended regulatory level of testing for a product or drug.

Test method development is essential to ensure reproducible and defensible data, and thus, product quality. A test method must be developed and validated for use to analyze samples during the early development of a drug produced. As the drug development progresses from nonclinical study through clinical trials to commercialization, the test method will need to follow a similar progression. Throughout the process of drug research and test development, method validation and revalidation must be considered. Method validation is defined by all regulatory standards as to which components must be tested, the acceptance criteria for each component and the test method. GXP requirements do not specifically state what components or to what degree method validation must be completed during the phases of drug production. The reason is due to the fact that drug products and test methods will necessarily evolve through the course of product development. The purpose of the assay is linked to the type of trial and the drugs intended purpose. As an example (GLP and GMP), the FDA regulation 21 CFR 211.194(a)(2) specifically

states that users of analytical methods in the U.S. Pharmacopeia/National Formulary (USP/NF) are not required to validate the accuracy and reliability of these methods, but merely verify their suitability under actual conditions of use. However, if testing within a clinical trial setting, CLIA or ICH regulations would be utilized depending upon the requests or needs of the company sponsoring the testing. CLIA specifically states how an assay is to be validated for use. FDA regulation CFR Section 493.1253 Standard: Establishment and Verification of Performance Specifications states the performance characteristics: accuracy, precision, analytical specificity, analytical sensitivity to include interfering substances, reportable range of test results for the test system, verify the reference intervals (normal values), determine calibration and control procedures and documentation of all activities specified above (1992). In ICH Q7A: "Changes are expected during development, and every change in product, specifications, or test procedures should be recorded adequately" (ICH, 2001). Above all else, method validation must prove the test method is fit for the purpose of the testing to which it is applied. The performance characteristics such as sensitivity, specificity, accuracy, precision, lower limit of detection, linearity, and reproducibility for each testing method must be analyzed. It is the laboratory's responsibility to prove suitability or competency of the test method 'in house' before and during testing. It is possible that a method that functions satisfactorily in one laboratory, fails to operate in the same manner in another. It is considered unacceptable for the researcher/laboratorian to use a published 'validated method' without demonstrating their capability in the use of the method (AOAC International, 2002).

The extent of and expectations from early phase method validation are lower than the requirements in the later stages of development. The validation exercise becomes larger and more detailed and collects a larger body of data to ensure that the method is robust and

appropriate for use at the commercial site. The final method must be validated for the intended use whether it is to test the actual product or test the product in use. During the research and testing process, partial or full method validation may be required. Logically any assay whether it is fully or partially validated must be scientifically sound, suited for its intended purpose and stage of product or drug development, and capable of generating reliable results. Any method that is newly developed must always be fully validated first prior to being used within a laboratory.

To adapt a quality system within the research stage, the technician performing the testing must have the scientific and technical understanding, knowledge of the product (drug, vaccine, etc.), and the ability to execute the quality functions of analytical method validation. All technicians performing the testing must have appropriate training to promote an understanding of the testing principles involved with the method validation, proper documentation of the data as *well as understanding of how to interpret the data, and an understanding of the cross-functional relationships of the testing, product, companies, and patients. This means that fundamental quality system components must be applied at the bench level.*

1.3 **Assuring Quality Results**

The only way to know if a method has the performance characteristics that "fit the purpose" for which it will be used is to define the test method through validation. Just as there are consequences for selecting an unsuitable assay for a given purpose, erroneous conclusions can be made if the capabilities and limitations of the assay are not considered when interpreting results. A method with acceptable accuracy and precision levels for measurement of antibodies in a potency range of 0.1 to 10.0 IU/mL in a serum matrix cannot claim the same accuracy and precision levels for higher potency samples or samples in a different matrix or body fluid without

validation experiments to evaluate these adaptations of the method. The method parameters important for a qualitative assay are sensitivity, specificity, and predictive value. In addition to sensitivity and specificity, a quantitative assay requires definition of accuracy (closeness to the true value), precision (repeatability of the measure), linearity, and reportable range. Recent publications are examples of RFFIT or modified RFFIT validations performed for specific purposes—evaluation of clinical trial samples for a human monoclonal antibody combination for the post-exposure treatment of rabies and vaccine potency evaluation (Kostense et al., 2012; Kramer, Bruckner, Daas, & Milne, 2010). Robustness evaluation describes the ability of the method to perform to set criteria during normal laboratory conditions, including normal variations of equipment performance, reagent lots, or between different personnel. Biologic variation must be considered separately from analytical variation. For example, two test results from the same sample may vary solely on the basis of the receptivity of cells to virus infection; cells used last week may have different virus infectivity characteristics than the cells used in subsequent testing. The variation from these types of factors is separate from other sources of variation. For repeat measures of the same sample, there are statistical tools to set the expected variance and for determining what variance is evidence of a significant difference, such as minimum significance ratio (MSR) (Khan & Findlay, 2009). Measurement or detection of rabies virus antibodies can be influenced by interference. Interference can be caused by cross reacting antibodies, non-specific binding, and matrix effect (hemolysis, lipemia, or "dirty" samples, etc.). Interference can occur not only with the antibody of interest in the sample but also in the interaction of the detected or competing antibodies in the assay. Naturally occurring proteins in samples, such as albumins, fibrinogen, and complement factors, can result in assay interference (Selby, 1999). Results from samples with interfering factors can be misleading if the effects of

these interfering factors are not considered. In most cases, interference will occur at low levels and will not cause measurement problems at higher dilutions or in samples with high potency, since specific binding is stronger than the weaker interference reaction. When interference is suspected or needs to be ruled out, samples may be evaluated by an alternative method in which the effect of interfering factors is minimized so that specific activity may be detected and measured.

The lower limit of detection (LOD) is affected by interference and the assay parameters. If the purpose of testing is determination of the presence or absence of rabies virus antibodies, defining the lowest level of antibodies that an assay can reliably detect is critical. However if the ability to accurately and precisely measure low levels of rabies virus antibodies is important, as in evaluation of passive rabies virus immunoglobulin levels in post-exposure treatment, then defining an assay's lower limit of quantitation (LLOQ) is required. Cut-off values assigned to an assay depend both on the LOD or LLOQ and the purpose of testing. If the application of the rabies serology testing is to identify low levels of rabies virus antibodies and exclude false negative test results, the cut-off level should be low, but this may yield some false positive test results. Conversely, a higher cut-off value (i.e., above a level which might allow some false positive test results) would identify only true positive test results which would be acceptable if the purpose of the method is to reliably identify only those individuals. The trade-off is that a high cut-off level would increase the number of false negatives (i.e., exclude some true positives that are low). The probability of false positive and false negatives is related to the precision of the assay. Assays with a high variability particularly at the cut-off level would exclude some true positive samples with potency values close to the cut-off level and conversely identify some true

negatives as positive. Upon repeat testing, these samples could measure either positive or negative.

The matrix of the sample can affect the LOD and LLOQ for a specific method, therefore whenever the sample matrix is altered; re-evaluation of this parameter is required. Any change that impacts the sensitivity of an assay will also change the LOD. Indeed, any change in the procedure or sample may require re-validation to determine the effect on the established performance characteristics. Method variations listed in Table 1.2 (for either binding assays or neutralization assays) can be used to customize a method for certain purposes, such as measurement of antibodies from a particular species, or within a range of potency values, but the changes implemented to customize an assay may also result in changes in the performance characteristics of a method.

Table 1.1.2 'Fit for Purpose' method variations that can be applied to neutralization or antigen binding assays

Neutralization Assays	Antigen Binding Assays
Strain of challenge virus	Antigen – virus strain
Dose of challenge virus	Antigen – virus protein(s)
Cell type	Whole virus
Serial dilution scheme	Purified protein
Detection system	Detection system
Fluorescent-labeled antibody	Species specific or non-species specific
Enzyme-labeled antibody	Immunoglobulin specific for class or subclass
Modified challenge virus	Platform – slides, plates, or beads
(ex. Green Fluorescent Protein insert)	

Immunity can be measured by different methods. It is natural to compare the results from different methods and it is important to consider how the comparison is made. Although it is very common to evaluate agreement between methods by a correlation coefficient, conclusions based on this value are improper. According to at least one well-known medical statistician, the best way to conduct a method comparison is to calculate the "mean and standard deviation of the between-method-differences" (Altman, 1991). It is not enough to just generate and examine the data, it is essential to apply appropriate statistical tools. A functional understanding of statistics or collaboration with a statistician is often essential for these exercises. The application of statistics to evaluations of immunoassay performance is a specialized area of competence, and is of particular importance when the assay will be used to determine acceptance of biologics (Findlay et al., 2000).

As previously mentioned, there are some critical components that are essential to consider, identify, and control to ensure precise and accurate measurements for serum neutralization assays, such as strain and dose of challenge virus, cell type, and reference standards. For results to be comparable over time from the same laboratory and possibly between laboratories, these components must be standardized. Whenever any critical steps or components are changed as may be necessary for a specific purpose, the modified method will require re-validation. A standard reference rabies immune globulin serum (SRIG) provides a defined potency standard in international units per mL (IU/mL). By comparison of the SRIG result to the test sample result the value of the test sample is standardized and comparable. But if the SRIG used is not the same or not calibrated against a known standard, discrepant results can occur (Yu et al., 2012). The value of the test sample result is standardized through comparison with the SRIG result in that assay at that time. It is essential for the SRIG to be precisely described for

each batch of test results. If the standards are not identical or not calibrated against a known standard, results cannot be directly compared between assay runs and between laboratories. Standard reference serum of equine source may perform differently than human SRIG such that batches of test results will yield different values depending upon the control serum (Haase, Seinsche, & Schneider, 1985). The potency assigned to a SRIG by one method may not be the same in a different method and cannot be automatically assumed. For example, a control serum at 0.5 IU/mL in the RFFIT method may perform at 0.7 Equivalent units per mL (EU/mL) in an ELISA-based assay. If this standard with a known performance by RFFIT was directly applied as the standard for an ELISA and assumed to perform at 0.5 EU/mL, the ELISA results would bias toward the exclusion of some samples which might meet a RFFIT 0.5 IU/mL value. Control serum such as an SRIG needs to be fully characterized by a new method and its potency needs to be assigned in units applicable to that particular method (Moore & Hanlon, 2010). A comparison of two international SRIG products in current use, WHO 1st international rabies immune globulin and WHO 2nd international rabies immune globulin, over several years show that reference serum can lose potency over time, with the 1st RIG lower in potency by 2.5% in 1997 to 19% lower in 2012 by RFFIT, yet higher in potency by ELISA (see Table 1.3) illustrates the importance of calibration and monitoring of the RIG in use in a particular laboratory and for a particular assay. If the challenge virus of an assay is substantially different than the virus source for a vaccine, the serologic results from clinical trials may underestimate responses to the vaccine (Moore, Ricke, Davis, & Briggs, 2005; Brookes & Fooks, 2006). The same is true for antigen binding assays where the virus strain and type (whole or protein) used in the detection system should ideally be the same, to obtain the most informative results.

Table 1.3 Comparison of the WHO international standard anti-rabies immunoglobulin, human – 1st and 2nd

Laboratory/Year	Difference in potency (WHO 1st/WHO 2nd)	Method of Testing:
FDA/CDC/KSU		
1997	2.5% lower	RFFIT
KSU		
2006	12% lower	RFFIT
2006	36% lower	FAVN
2006	22% higher	Direct ELISA
2012	19% lower	RFFIT
2012	34% lower	FAVN
2012	15% higher	Direct ELISA

Despite the potential negative effect of a change in how a method is performed, there are good reasons to introduce variations to a procedure. These may include the need to measure rabies virus antibodies from a specific species which may require a change in the detection system or the need to measure potency of samples which are beyond the normal linear range of testing and hence may require a pre-dilution to achieve a different range of sample dilutions to be tested. Method validation reveals the robustness and limitations of assay and its performance characteristics. In addition to method validation, conducting continual monitoring of method performance increases the chances that potential problems will be quickly identified. Regular participation in proficiency programs is one way to monitor performance of the method and also assists in the identification of drifts and trends. If good quality control practices are in place, results may be comparable between laboratories even when there are differences in procedure. For example, nine laboratories performing the RFFIT for different purposes (from +/- screening to regulated quantitative measurements) and executing different RFFIT procedures (including

SRIG source, virus strain, cell type used) recently participated in a voluntary exchange of an informal proficiency panel of samples. All nine laboratories identified the RVNA negative sample as below their assigned cut-off and had reported values within two-fold of the average of all measurements for the remaining five RVNA positive samples. Even if laboratories are following the same protocol and using the same components, the agreement in results for the same sample can vary based on method variables related to environment, personnel training and equipment performance. In other words, methods can be standardized, but unless the laboratories are adhering to the same quality assurance standards, the results may still demonstrate greater variability than is ideal. Acceptance criteria for precision and accuracy are different depending upon on the type of assay. Cell-based assays, such as serum neutralization, are inherently more variable and thus are allowed greater variability than binding assays. The precision of binding assays is generally expected to be in the range of 5-20%, while cell-based assays may be allowed a precision variability of 30%, and up to 50% (2001; Chaloner-Larsson, Anderson, & Egan, 1997). In general, for serological titration assays a two-fold difference in replicate measurements is commonly recognized as the upper level of reproducibility (Wood & Durham, 1980). The precision of an assay should be taken into account when reviewing rabies serology results in relation to survival of experimental challenge, inter-laboratory comparisons, and proficiency testing, as well as when establishing acceptable levels for proof of sero-conversion or an adequate response to rabies vaccination.

1.4 **Assay Selection**

Knowing the specific component of immunity that is the focus of a particular investigation is paramount in selection of the suitable assay. Similarly, it is essential to understand the performance characteristics of a particular method and its limitations, to

determine whether the method can generate appropriate results that can answer a particular question. Rabies virus antibodies are measured for several different reasons. The reasons for these measurements will influence the requirements for method sensitivity, specificity, precision, accuracy, linear range, limit of detection and robustness of the method. The requirements for specific reagents, instrumentation or facilities may vary according to the particular methods. The consequence of selecting an improper method can be as simple as getting a result that does not answer an academic question, thus leading the research down a wrong path; or, as complex as providing incomplete or misleading information that will be used to make essential health care decisions, whether veterinary or medical, for the prevention of clinical rabies. For example, if there is an encephalitis suggestive of clinical rabies, evaluating a sample from a human or animal with an assay which can only detect IgG antibodies could be insensitive or misleading because IgM antibodies, which are produced before IgG, may remain undetected. Thus, a negative test result would be misleading. Besides the consequence of using an unsuitable method for individual diagnosis, ambiguous results add potentially incorrect information to the body of data compiled for typical antibody responses in rabies patients.

Laboratory tests for rabies virus antibodies are used for research, human vaccination decisions, pet travel permits, wildlife vaccination program evaluations, and pharmaceutical product licensure. No one method will be the ideal fit for all purposes. The method that will "fit" must be defined by the characteristics of rabies virus antibodies that are most important or by the parameter of interest. For example, to research the difference between monoclonal antibodies produced against the glycoprotein of the ERA rabies virus strain, a serum neutralization assay is essential if the ultimate purpose of the monoclonal is therapeutic. The challenge virus used in the serum neutralization assay should be considered. If the purpose of the monoclonal antibody is

use as a therapeutic agent, then the challenge virus used should be one that is most closely related to the rabies virus variants that are enzootic in the regions where the biologic is intended for use. Moreover, if the monoclonal antibody is intended for eventual licensure, the laboratory method selected must be an approved, validated method that is recognized by the licensing authority. Conversely, if the purpose of the monoclonal antibody is use in diagnostic testing to differentiate ERA infected brains from brains infected with other strains, then the method best used to illustrate the difference in monoclonal antibodies would be an IFA using ERA infected cells. Below are specific assay requirements that apply to some common reasons for measuring rabies virus antibodies:

- Standardized for comparable results between laboratories and over time (clinical trial testing, human testing for vaccine response either for post-exposure or pre-exposure, oral-bait program evaluation, pet travel)

- Detection of low levels in an initial response to infection or vaccination/ability to measure low levels of IgM and IgG (clinical diagnosis, evaluation of post-exposure treatment, some research purposes)

- Cost effective (to obtain screening results from large numbers of samples)

- Adaptable for detecting difference immunoglobulin subclasses (research and clinical)

- Adaptable for detecting specificities or antibodies from difference species (research and surveillance)

- Approved by regulatory authorities (biologic product testing, pet travel)

- Low technology or low level bio-containment facilities (field research or in areas of developing countries)

Consideration of the sample is also a factor in choosing the proper assay. Attempting to measure a F(ab')$_2$ product with an ELISA whose secondary detection system relies on binding to the Fc portion of the immunoglobulin would be futile. A blocking or competitive ELISA or a serum neutralization assay would be a better 'fit' for this purpose because these assays do not rely on the complete structure of the immunoglobulin, only the antigen binding portion, F(ab')$_2$ for detection. In human laboratory medicine, it is not uncommon to screen samples using a sensitive assay to identify positive samples from negative samples and then follow the sensitive screening tool with confirmatory testing with a more specific assay to identify the true positive samples and exclude the false positives. Several methods can be used effectively for screening purposes. Depending on the screening goal, assays such as ELISA using whole virus antigen, lateral flow with a positive or negative readout, and IFA can identify samples that potentially contain rabies virus specific antibodies. Testing with a western blot technique can confirm the specificity of the antibodies detected in the screening assay or testing with a serum neutralization (SN) method can confirm the neutralizing function of the antibodies. A screening method with lower accuracy (result may not be the true value), but higher precision (repeat measurements are clustered closely although they may not be near the true value) may be more useful for oral baiting surveillance, if it is quick, standardized and simple, than a more accurate method that is cumbersome, time-consuming, and more variable. For the purpose of evaluation of oral baiting campaigns, determination of individual "protection" is less important than herd immunity levels and the ability to confidently compare results between laboratories and over time.

1.5 Serum Neutralization Assays

Rabies serum neutralization assays are distinguished by the ability to detect the neutralization activity of specific antibodies *in vitro* and therefore attempts to measure the

potential protective action of these antibodies *in vivo*. Technical performance of rabies virus

neutralization assays requires the use of infectious virus and can be labor intensive and time

consuming. There are two rabies serum neutralizing assays recognized by the World Health

Organization (WHO) and the World Organisation for Animals Health (OIE) to measure RVNA:

the rapid fluorescent focus inhibition test (RFFIT), described in 1973 by Smith et al. (Smith et

al., 1973); and the fluorescent antibody virus neutralization test (FAVN), developed in 1997 by

Cliquet et al. (Cliquet et al., 1998). Measurement of RVNA by serum neutralization assays is

based on the same principle as the mouse neutralization test (MNT), extensively employed in

early rabies serology work. The MNT involves the injection of test serum dilutions in mice

followed by a challenge with a standard dose of rabies virus, with the read-out being mortality

among the mice (Atanasiu, 1973). While this is truly a "real" measurement of the protective

function RVNA in the serum, the biological variation of individual mouse immunity as well as

possible interference of other immune effectors made it difficult to standardize the test. With the

development of *in vitro* methods such as the RFFIT, improvements in sensitivity and

standardization were achieved.

Both the RFFIT and the FAVN tests consist of incubation of dilutions of heat-inactivated

serum with a fixed amount of live rabies virus for 60–90 minutes at 37°C. Measurement of

residual virus infectivity is accomplished by detection of virus in cell culture via a labeled anti-

rabies virus antibody and subsequent calculation of the quantitative titer by the number

microscopic fields containing virus infected cells. The RFFIT method is conducted in multi-

chamber slides (see Figure 1.4 A). Serum is serially diluted fivefold and tested in each well.

Variations of the RFFIT include the use of microtiter plates in place of the slides and the use of

twofold or threefold dilutions. The rabies challenge virus should contain 30–100 50% tissue

culture infective dose ($TCID_{50}$). After the virus is added to the diluted serum, the slides are incubated at 37°C for 90 minutes, after which baby hamster kidney (BHK) or mouse neuroblastoma (MNA) cells are added to each of the wells. Diethylaminoethyl-Dextran (DEAE-Dextran) has been used, typically at a 0.01 µg/mL concentration, in some variations of the RFFIT to enhance susceptibility of the cells to rabies virus infection (Kaplan, Wiktor, Maes, Campbell, & Koprowski, 1967). The slides are generally incubated at 37°C in a 2-5% CO_2 incubator for 20–24 hours, although the incubation period is extended to 48 hours in some variations of the method conducted in microtiter plates. The wells containing an adherent monolayer of cells are washed and the cells are fixed with 80% cold acetone. FITC-conjugated anti-rabies virus antibody directed against the rabies virus nucleoprotein (N) is added in order to detect virus-infected cells. In 8-well chamber slides, 20 fields of each well are examined using a fluorescent microscope for the presence of fluorescence in the cells, an absence of which indicates antibodies in the sample neutralized virus and the presence of which indicates a lack of antibodies. The titer of RVNA in the serum sample being analyzed is defined as the dilution at which 50% of the observed microscopic fields contain one or more infected cells. Mathematical calculation using the Reed and Muench formula, Spearman-Karber formula or Probit method will determine the exact quantitative titer of RVNA in the serum sample. Alternatively, the quantitative titer of RVNA can be more simply defined, but with less precision, as the highest serum dilution where 100% viral inhibition occurred, thus indicating that there were no infected cells at that dilution and all subsequent higher dilutions exhibit infected cells (Habel, 1996; Aubert, 1996). Transcribing a serum dilution value into a standardized and more globally recognized measure of IU/mL is achieved by a simple calculation wherein the value from a serum sample being tested is compared to the serum dilution value of a reference serum standard

containing a specific amount of RVNA previously tested and verified to be accurate (Velleca &

Forrester, 1981). The quality of the test components as well as the skill and expertise of the

technician conducting the test, including the analysis of the microscopic readout, can

substantially affect the precision of RFFIT test results.

To simplify and reduce the subjectivity of the microscopic counting step, the FAVN

method uses four replicates of serum using threefold dilutions in microtiter wells and scores each

well as either positive or negative for the presence of rabies virus infected cells after a 48-hour

incubation. A direct comparison of the two methods demonstrated no statistically significant

differences in results when conducted in a laboratory adhering to good quality assurance

standards (Briggs et al., 1998). Precision and repeatability of virus neutralization test results can

be controlled by strict adherence to the dose and strain of the challenge virus used and the source

of the standard reference serum. Early published reports that compared different laboratory

RFFIT results reported that the use of a high infective dose of challenge virus resulted in reduced

sensitivity for testing low titered sera, whereas a low viral dose of challenge virus could result in

lower precision when testing high titered sera such as rabies virus immunoglobulin (RIG)

preparations (Fitzgerald, Baer, Cabasso, & Vallancourt, 1975). In addition, the use of an equine

RIG as the reference standard to determine IU/mL values resulted in significantly different titer

results than when a human RIG reference standard was used (Lyng, Bentzon, & Fitzgerald,

1989). Measuring RVNA from people vaccinated with a vaccine prepared with a parent virus

strain heterologous to the challenge virus strain in the RFFIT (usually CVS-11) can result in

lower titers than if a homologous challenge strain is used (Moore et al., 2005). Rabies virus

neutralization tests identify the presence of all classes of immunoglobulin in a sample (both IgM

and IgG) and therefore will be able to detect the early production of rabies virus antibody after exposure or vaccination, but may not be as sensitive as the IFA (Smith, 1991).

Because the virus neutralization testing method depends on the measurement of residual, or 'non-neutralized' rabies virus infecting the cells, the presence of interference factors in the sera or culture media that adversely affects cell (and ultimately viral) growth will mimic virus neutralization by non-specifically inhibiting viral growth. Any inhibition of viral growth not directly due to neutralizing antibody will give a false positive result. As with ELISA techniques, some steps of the virus neutralization test can be automated, especially when performed using micro-titer plates, (e.g., the addition of media to the plates, serial dilution of the serum samples, addition of virus and anti-rabies virus conjugate) as well as some of the more tedious steps such as plate washing, allowing the application of these methods to high-throughput testing. Automated reading of FAVN and RFFIT reduces the work-time required for the microscopic analysis readout and aids in the minimization of errors (Peharpre et al., 1999). No significant difference was noted when comparison was made between the rabies virus antibody levels reported by automated and non-automated reading, but the automated reading resulted in lower sensitivity. The expense of the equipment required to conduct automated reading of the RFFIT or FAVN, the requirement for a consistent cell monolayer and need for a good quality FITC conjugate limits the practicality of this enhancement, especially for laboratories that do not conduct large numbers of tests. As an alternative to microscopic fluorescence measurement, a microneutralization test (RAMIN), and the indirect immunoperoxidase virus neutralization (IPVN) technique and the modified FAVN, employ a mouse anti-rabies virus antibody and a peroxidase anti-mouse conjugate enabling automated reading by a spectrophotometer (Mannen et al., 1987; Cardoso, Silva, Albas, Ferreira, & Perri, 2004; Hostnik, 2000). In each of these studies,

a good correlation was confirmed between traditional rabies virus neutralization methods and the

modifications that were made to each test. Other modifications take advantage of molecular

techniques to prepare recombinant viruses to use in place of the standard challenge virus, CVS-

11, for standardization of fluorescence or for adaptability to detect different specificities of

antibodies. Modified CVS-11 expressing green fluorescent protein (GFP), eliminates the need

for FITC-conjugated anti-rabies virus antibody (Khawplod et al., 2005). Using this modified

CVS-11 in combination with flow cytometry to detect residual virus present after incubation

with serum, reportedly increases the sensitivity because each cell is individually assessed for

viral infectivity, creating a more precise percentage of viral inhibition (Bordignon et al., 2002). A

new method developed by Wright et. al. (2008) utilizes pseudotype viruses—lentivirus vectors

expressing rabies virus glycoprotein and a reporter (e.g., *lacZ*, GFP) (Wright et al., 2008). By

expressing the glycoprotein from different rabies virus strains, a panel of pseudotypes can be

used in cross-species comparison studies. Because the pseudoviruses are replication incompetent

particles, this method is applicable in areas where high level bio-containment facilities are not

available.
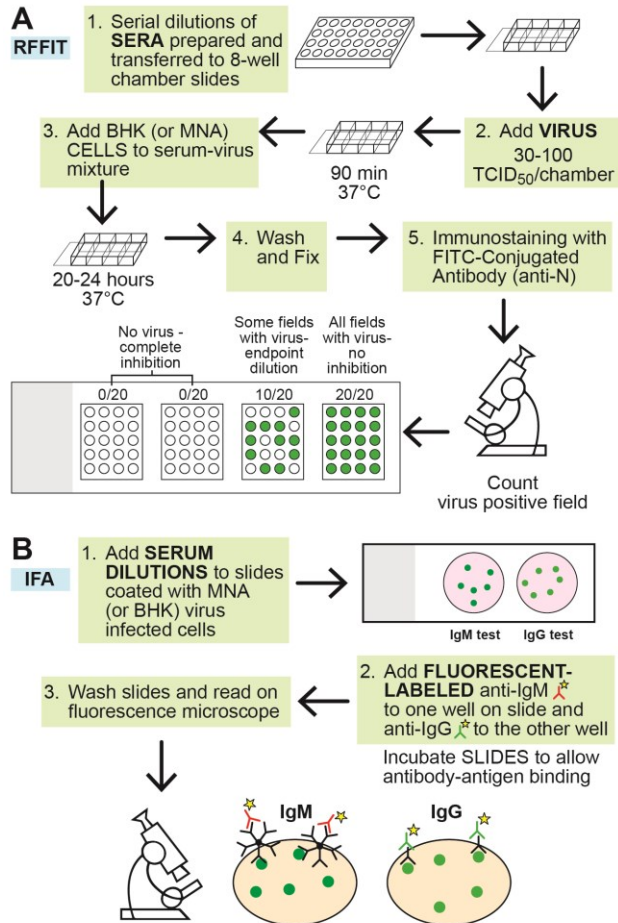
Figure 1.4 (A). RFFIT procedure. Serum is serially diluted in a 96 well plate and transferred into 8-well chamber slides. The rabies challenge virus is added to the diluted serum, the slides are incubated at 37°C for 90 minutes, after which baby hamster kidney (BHK) or mouse neuroblastoma (MNA) cells are added to each of the wells. The slides are incubated at 37°C in a 2-5% CO_2 incubator for 20–24 hours. The wells containing an adherent monolayer of cells are washed and the cells are fixed with 80% cold acetone. FITC-conjugated anti-rabies virus antibody directed against the rabies virus N is added in order to detect virus-infected cells. In 8-well chamber slides, 20 fields of each well are examined, using a fluorescent microscope, for the presence of fluorescence in the cells indicating the presence of non-neutralized rabies virus. The titer of RVNA in the serum sample being analyzed is defined as the dilution at which 50% of the observed microscopic fields contain one or more infected cells. **(B) IFA technique.** Test serum in added to slides fixed with rabies virus-infected cells. Rabies virus antibodies in the serum bind to antigens on rabies virus proteins present in the infected cells and are subsequently detected by FITC-labeled anti-IgG or anti-IgM. Slides are read on a fluorescence microscope to evaluate the slides for the presence of labeled antibodies.
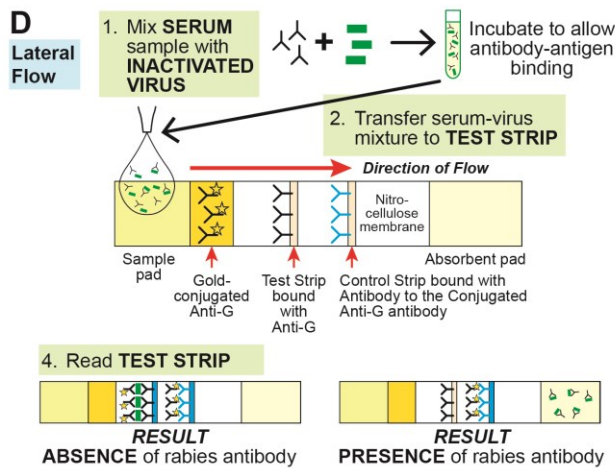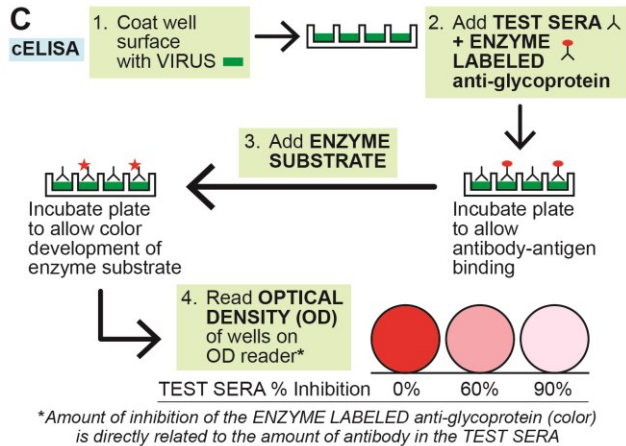
Figure 1.4 (C). Competitive ELISA. A labeled rabies virus antibody competes with the rabies virus antibodies in the test sample. The test serum sample and an enzyme-labeled anti-rabies virus antibody is incubated with the inactivated rabies virus antigen on the well surface. The amount of enzyme-labeled antibody is detected by adding a conjugate for color development; amount of rabies virus antibody in the serum sample is inversely related to intensity of color development. The level of antibody in the test serum can be quantitated by use of a standard curve and an OD reader. **(D) Lateral Flow.** The test serum sample is mixed with inactivated virus before adding the mixture to the absorbent material of the test strip. The mixture then flows across to encounter a labeled anti-rabies virus (anti-G) antibody which will bind to any unbound inactivated rabies virus. The mixture continues to flow toward two areas (lines) of the strip bound with detection antibodies; the first detection antibody is specific for the rabies virus (anti-G), and the second detection antibody is specific for the labeled rabies virus (anti-G) antibody. If the mixture contains unbound inactivated rabies virus there will a color development at the first strip, indicating the sample did not contain enough anti-rabies virus antibody to bind the inactivated rabies virus in the mixture. If the test serum sample does contain anti-rabies virus antibody, then the labeled antibody will pass the first strip and be bound by the antibody in the second strip causing a color development at the second strip. The results will be either "presence of rabies virus antibody" (only second strip visible) or 'absence of rabies virus antibody" (first and second strip visible).

1.6 **Binding Assays**

Binding assays are methods that detect or measure immunoglobulin molecules by their ability to bind specifically to their target antigen. This binding can be detected by use of secondary detection systems usually bound to a color development system for visualization or quantitation by optical density (OD) or fluorescent measurement. ELISA assays are the most commonly used binding assay. ELISA assays may be based on indirect, competitive, and blocking approaches. Other binding assays are lateral flow and indirect fluorescent antibody (IFA). Western blots are used to identify the fine specificities of antibodies. Antigen binding assays such as ELISAs and IFAs are rapid, simple and often do not require manipulation of infectious rabies virus during the assay, although preparation of antigen may involve live virus. These assays rely on the interaction of the antibody and antigen, regardless of the ability of the antibody to neutralize rabies virus, and are useful for the detection of rabies virus binding antibodies. An assay with whole virus as the target antigen may be useful to identify the presence of rabies virus antibodies specific for the different antigens on the rabies virus. Conversely, purified viral proteins can be used to distinguish the specific composition of antibodies which may be present. Binding assays are able to identify the subclass of rabies virus antibodies (for example, IgM and IgG) by using a conjugated anti-subclass Ig antibody as the secondary antibody.

The IFA technique involves adding test serum to slides fixed with rabies virus-infected cells (see Figure 1.4 B.). Rabies virus antibodies in the serum bind to antigens on rabies virus proteins present in the infected cells and are subsequently detected by FITC-labeled anti-IgG or anti-IgM. A fluorescence microscope is required to evaluate the slides for the presence of labeled antibodies. Quantification of the antibodies can be accomplished by serial dilution of the serum

to determine the antibody titer. Because infected cells are used as the source of rabies virus antigen, antibodies with specificities to different rabies virus proteins can detected and also antibodies to cellular antigens. The possibility of detecting non-rabies of antibody binding that is not specific to rabies antigen (i.e., autoantibodies, antibodies to cellular antigens, etc.) is important to consider when evaluating the results.

Early ELISA methods, such as the one described by Nicholson and Prestage in 1982 used inactivated whole virus as the antigen and anti-IgG as the secondary antibody (Nicholson & Prestage, 1982). This technique offered greater specificity over the IFA since the only source for binding is whole virus coated to the surface of the well and not in a cell where there are other antigens present as possible targets for interfering antibodies. Modification of the ELISA by Grassi in 1989 improved the assay specificity further by using purified rabies virus glycoprotein (G) antigen allowing detection of anti-rabies G antibodies (Grassi, Wandeler, & Peterhans, 1989). In contrast to early ELISA assays, there is a higher degree of correlation between RFFIT and G-protein ELISA due to the fact that most neutralizing antibodies are directed against the G protein. The secondary antibody employed by ELISA methods may be species specific but if staphylococcus Protein A is employed, the method can be applied to samples from a number of species because Protein A binds to the Fc portion of the IgG of many species.

Other types of ELISAs include competitive (cELISA) and blocking ELISA. Both methods involve the use of a labeled rabies virus antibody to either compete with (cELISA) or to detect antigen not blocked by (blocking ELISA) the rabies virus antibodies in the test sample. In the blocking ELISA, incubation of the serum sample with the inactivated rabies virus antigen on the well surface is followed by addition of an enzyme-labeled anti-rabies virus antibody. Any unbound (unblocked) inactivated rabies virus is bound by the labeled antibody. The amount of

enzyme-labeled antibody is detected by adding a conjugate for color development; the amount of rabies virus antibody in the serum sample is inversely related to intensity of color development. The level of antibody in the test serum can be quantitated by use of a standard curve and an optical density (OD) reader. Competition for rabies virus binding between the anti-rabies virus antibodies in a serum sample with a labeled-anti-rabies virus monoclonal antibody is the basis of the cELISA (see Figure 1.4 C). Similar to the blocking ELISA, the labeled antibody is measured and used to determine the level of anti-rabies virus antibody in the sample. Both of these methods can reduce the effect of non-specific binding because the antibody that is measured is a purified reagent antibody. Use of different reagent monoclonal antibodies in these assays allows detection of various specificities of rabies virus antibodies that may be present in the sample (i.e., use of a labeled anti-rabies virus N will detect anti-N in the sample and use of a labeled anti-rabies virus G will detect anti-G in the sample).

An electrochemiluminescent (ECL) adaption of the blocking ELISA method employs microtiter plates fitted with a series of electrodes at the bottom of the wells. Applying an electrical current across the electrodes causes the generation of a luminescent signal by the chemical energy ligand-binding reactions. Quantitation of the signal converts the measurement to antibody concentration. This method has been applied to measurement of proteins and has the potential for greater sensitivity and faster results compared to the traditional ELISA method (Guglielmo-Viret, Attree, Blanco-Gros, & Thullier, 2005; Ma, Niezgoda, Blanton, Recuenco, & Rupprecht, 2012).

Lateral flow immunoassays to detect and measure antibodies are useful for field work and in areas where a low tech screening method is required. By adapting the concepts of ELISA to an absorbent test strip, the testing process is simplified to progress across a straight line by

having the sample interacting with the assay reagents sequentially (see Figure 1.4 D.). A version

of this method for detecting rabies virus antibody requires an initial step of mixing the serum

sample with inactivated virus before adding the mixture to the absorbent material of the test strip.

The mixture then flows across to encounter a labeled anti-rabies virus antibody which will bind

to any unbound inactivated rabies virus. The mixture continues to flow toward two areas (lines)

of the strip bound with detection antibodies; the first detection antibody is specific for the rabies

virus, and the second detection antibody is specific for the labeled anti-rabies virus antibody. By

using this design, if the mixture contains inactivated rabies virus bound with labeled anti-rabies

virus antibody, there will a color development at the first strip, indicating the sample did not

contain enough anti-rabies virus antibody to bind the inactivated rabies virus in the mixture. If

the sample contains anti-rabies virus antibody, then the labeled antibody will pass the first strip

and be bound by the labeled anti-rabies virus antibody causing a color development at the second

strip. The results will be either positive (only second strip visible) or negative (first and second

strip visible) for the presence of anti-rabies virus antibody in the test serum sample. The level of

antibody to define positive or negative is set and defined by the design of the assay and can only

be altered by concentration or dilution of the serum sample. Its simplicity and portability allows

use by operators with a minimal amount of education and training. The lateral flow assay is

useful for point-of-care situations where an initial rapid screening result would determine

whether or not further action, such as additional testing or vaccination, was necessary.

ELISA methods have several advantages including the fact that they are rapid, require

little expertise, do not need high-level biohazard facilities to be performed and several steps of

the procedure can be automated (i.e., serial dilution of the sera, addition of reagents and optical

density reading). Additionally, software packages are available to calculate endpoint titers or

antibody concentration, thus allowing for objective reading and interpretation. The disadvantages of ELISA methods include the restrictive nature of the conjugated antibody or protein A that limits the isotype of immunoglobulin detected. The use of species-specific anti-IgG confines the utility of the assay to a certain species. Additionally, although the use of protein A increases test application to several species, it does not react with all forms of IgG3 and therefore will lead to an underestimation of the level of rabies virus-specific antibodies in serum containing higher proportions of IgG3 antibodies (Carpenter, 1997). The degree of non-specific binding detected by an ELISA will depend on the purity of the antigen preparation and the efficiency of the coating step because immunoglobulins will non-specifically adhere to glass, plastic, and also to contaminating material (i.e., mycoplasma) or cell culture components. Quantification of IgG antibodies that bind to rabies virus will <u>not</u> precisely demonstrate the level of <u>protective</u> virus neutralizing antibodies present in the sera. Therefore, the ELISA method is not appropriate for attempting to measure the amount of RVNA. Reporting of ELISA results in IU/ml is not reflective of this unit of measurement as defined by the WHO, where 1 International Unit of neutralizing activity is present per mg of protein. Therefore, the use of IU/ml to describe an antigen-binding assay for a rabies virus titer result is misleading. Since not all binding antibodies neutralize virus, whether for rabies virus or other pathogens, titers obtained from antigen-binding assays are not biologically identical to RVNA titers and therefore, ELISA-based test results should only be ordered, used and interpreted by informed health care providers, whether veterinary or human, or researchers, for the optimal prevention of rabies.

1.7 **Defining "adequate" or "minimum" response to rabies vaccination**

It is well known that vaccination resulting in production of rabies virus neutralizing antibodies (RVNA) prevents rabies in persons who have been in contact with a rabid animal.

There is no specific RVNA level equivalent to protect against rabies virus infection and progression to the disease has ever been or is likely to be established due to the unethical nature of conducting efficacy studies in humans who have various levels of RVNA and to determine survival rates. Humans who have an increased risk of rabies exposure are vaccinated pre-exposure to provide protection for unnoticed exposures and to reduce the vaccination schedule upon known exposure. This population should have periodic RVNA titer checks to evaluate the need for booster vaccinations. There are two major sources of guidelines in regard to an adequate response to rabies vaccination: the World Health Organization (WHO) Expert Committee on Rabies and the Advisory Committee on Immunization Practices (ACIP). Because the acceptable level given by these two guidelines are different and there is lack of understanding of how these levels were obtained and what they mean, there is confusion in the medical and veterinary fields about how to interpret rabies serology results in regard to booster vaccination decisions. Health professionals need a clear guideline to reference in making life-saving decisions about rabies treatment, for both pre- and post-exposure situations. The guideline instructions should clearly define the acceptable RVNA level in terms applicable to the recommended laboratory methods and clarify if there are situations where a different level may apply.

Because rabies is preventable by vaccination which produces rabies virus neutralizing antibodies (other immune mediators may be at play but are not readily measured), use of methods to quantitate RVNA are preferred when testing for vaccine response in humans. Currently the most utilized method for this purpose is the RFFIT (and modified RFFIT methods). Not only should the method for this purpose be confirmed to measure neutralizing antibodies, but it should also be standardized to allow comparison to other laboratories and to established guidelines for human vaccination and vaccine manufacturer's instructions. The method needs to

provide results that can be related to the guidelines in regards to units of measure and ability to detect the level stated. While no specific RVNA level has been identified as representing absolute protection under all circumstances and in all hosts against all rabies virus variant infections, RVNA levels attained by the majority of subjects in vaccine clinical trials formed the basis for the levels currently recognized as the minimal adequate response in vaccinated humans.

Although the ACIP defines a method, it does not define the adequate level of RVNA in standardized terms. "Complete neutralization at a serum dilution of 1:5 in the RFFIT" can represent different titers and IU/mL values in laboratories which perform "modified" RFFIT assays. Besides standardizing the value reported for rabies serology, defining the specific parameters and standard reagents that comprise acceptable methods for RVNA measurement would aid in interpreting results for the determination of the need for booster vaccination.

The ACIP states rabies serology should be performed by the RFFIT and both the OIE and WHO recommended methods are serum neutralization assays (FAVN and RFFIT), but other rabies serology methods are available, particularly ELISA methods, and may be inadvertently ordered. This may be especially true if samples from humans are collected and sent for "rabies" titer through a commercial human medical laboratory. Understanding the method and then interpretation of the result is essential for the optimal management of humans and animals. Indirect ELISA methods detect and measure the presence of rabies virus specific antibodies based on their binding ability; they do not measure the neutralizing ability of the antibodies (Irie & Kawai, 2002). The relationship between the level of binding antibodies and neutralizing antibodies cannot be predicted and is not linear. The validity of a method is unique to each laboratory and the parameters of a validation should be carefully considered. Method validation documents performance standards and includes identification and verification of the lower limit

of quantitation (LOQ). This is the lowest level that will produce accurate and precise results. Performance of a method within each laboratory that generates a rabies serology result and consideration of the rationale behind the two different definitions of adequate rabies vaccine response will need to be considered toward the development of clear language in regard to clinical management of persons or animals at-risk for exposure or following an exposure.

1.8 **Conclusions**

Rabies serological laboratories serve a critical function in rabies prevention programs. They are vital for providing reliable information required for human diagnoses, vaccine evaluations, animal surveillance and epidemiological studies and for routine testing of professionals working in the field of rabies. As outlined in the chapter, accurate rabies serological testing can be highly complex. The choice of the correct method for testing depends directly on what the intended purpose of the test results will be. Understanding the principle and limitations of the assay chosen and strict adherence to key components of testing will ensure appropriate results for decision-making.

Guidelines for adequate vaccination should be readily understood by individuals-at-risk (whether human or animal) and health care providers, both veterinary and medical, who will use the recommendations for clinical management of individuals. The guidelines should clearly define the adequate response by test method; result format; and in consideration of method validation and method performance, especially the ability to differentiate a specific antibody response from a non-specific (or "false positive") result.

Across the globe, there are different official regulatory standards and guidelines, both national and international for drugs and related product approvals. Each has a common goal to ensure the integrity of the laboratory data; protect human welfare; and provide safe and effective

products. These goals can only be met if all aspects of the testing and phase developments are held to the appropriate regulatory standard and monitored throughout the development process. The implementation of these standards must be from the beginning of the work at bench level to the end of the process which often continues during clinical use of products intended for the improvement or protection of human and animal health, including direct assessment of, and assessment of host responses to, rabies vaccines and sources of polyclonal and monoclonal products for the prevention of rabies.

1.9 **Acknowledgement**

1.10 **References**

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Chapter 2 - Factors influencing the serological test results

in rabies virus neutralizing antibody titers:

The use of homologous vs. heterologous challenge virus strain

2.1 **Abstract**

The effect that the relatedness of the viral seed strain used to produce rabies vaccines and the strain of challenge virus used to measure rabies virus neutralizing antibodies after vaccination was evaluated. Serum samples from 173 subjects vaccinated with either purified Vero cell rabies vaccine (PVRV) produced from the Pitman-Moore (PM) seed strain of rabies virus or purified chick embryo cell rabies vaccine (PCECV) produced from the Flury low egg passage (Flury-LEP) seed strain of rabies virus were tested in parallel assays using a homologous and a heterologous testing system. In the homologous testing system, CVS-11 was used as the challenge virus in the assay to evaluate the humoral immune response in subjects vaccinated with PVRV and Flury-LEP was used for subjects vaccinated with PCECV. In the heterologous testing system, CVS-11 was used as the challenge virus in the assay to evaluate subjects vaccinated with PCECV and Flury-LEP was used for subjects vaccinated with PVRV. Although the difference in Glycoprotein (G) protein homology between the CVS-11 and Flury-LEP rabies virus strains has been reported to be only 5.8%, the use of a homologous testing system resulted in approximately 30% higher titers for nearly two-thirds of the samples from both vaccine groups compared to a heterologous testing system. The evaluation of equivalence of the immune response after vaccination with the two different vaccines was dependent upon the type of testing system, homologous or heterologous, used to evaluate the level of rabies virus neutralizing antibodies. Equivalence between the vaccines was achieved when a homologous testing system was used but

not when a heterologous testing system was used. The results of this study indicate that the strain of virus used in the biological assays to measure the level of rabies virus neutralizing antibodies after vaccination could profoundly influence the evaluation of rabies vaccines.

2.2 Introduction

The immune response to rabies vaccination involves activation of rabies virus-specific B cells which differentiate into plasma cells producing antibody and memory B cells. Although antibodies specific for the rabies virus glycoprotein (G) and nucleoprotein (N) proteins (as well as other rabies viral proteins) are produced after vaccination, published reports indicate that it is the antibodies specifically directed against antigenic components of the G protein that neutralize the rabies virus (Lafon, Edelman, Bouvet, Lafage, & Montchatre, 1990). Rabies virus-specific CD4+ T cells, primarily induced by the rabies virus N protein, assist in B cell immunoglobulin class switching and immunoglobulin production. The effector CD4+ T cells are also differentiated into central and effector memory T cells. Due to the lack of a well-established, practical method to measure the cellular immune response against rabies virus and because rabies virus neutralizing antibodies (RVNA) are critical for protection against rabies infection, the standard method for determining the immune response that has occurred after vaccination is to measure the level of RVNA in sera. The World Health Organization (WHO) recognizes two RVNA tests for the measurement of humoral immunity after rabies vaccination: the Rapid Fluorescent Focus Inhibition Test (RFFIT) and the Fluorescent Antibody Virus Neutralization Test (FAVN). Both assays utilize the Challenge Virus Standard (CVS-11) strain of rabies virus as the challenge virus to quantitate the neutralizing activity of RVNA produced in response to rabies vaccine (Smith, Yager, & Baer, 1973; Cliquet, Aubert, & Sagne, 1998). Previous studies have demonstrated the significant influence that the strain of challenge virus used in testing has

on the measurement of vaccine potency (Blancou et al., 1989; Ferguson, Wachmann, Needy, & Fitzgerald, 1987). Indeed, published studies indicate that higher vaccine potency values are achieved when a homologous challenge virus is used for potency testing as compared to when a heterologous challenge virus strain is used. A similar effect has been demonstrated in the serological test results from serum samples assayed for the presence of specific antibody against different genotypes of lyssaviruses including rabies virus. For example, higher RVNA titers were obtained against rabies virus (lyssavirus genotype 1) as opposed to lyssavirus genotypes 2-7 when the source of the antibody is pooled sera from person vaccinated against rabies virus (lyssavirus genotype 1) (Smith, 2002). Additionally, another study reported variations in RVNA titer values when two different CVS strains were used as the challenge virus (Smith, 1991).

There are several cell culture rabies vaccines licensed for use throughout the world. Many of these vaccines are produced from different rabies virus seed strains including: Pitman-Moore (PM) rabies virus strain used to produce human diploid cell rabies vaccine (HDCV), purified Vero cell rabies vaccine (PVRV) and purified duck embryo cell rabies vaccine (PDEV); Flury high egg passage (Flury-HEP) or Flury low egg passage (Flury-LEP) rabies virus strain used to produce two different types of purified chick embryo cell rabies vaccine (PCECV); and Kissling rabies virus strain of Challenge Virus Strain used to produce rabies vaccine adsorbed (RVA). The PM and Kissling rabies virus strains originated from the brain of a rabid cow in France in 1882 and the Flury LEP strain originated from a human patient in the USA who died of rabies in 1939. Investigation of the phylogenic trees of the G and N rabies virus proteins originating from different vaccine seed strains indicate a closer relationship exists between the PM and CVS strains of rabies virus than between the Flury LEP and the CVS strains (Figure 2.1). Published reports also indicate areas of differences exist between the amino acid sequence of the G protein

of CVS and PM and the G protein of CVS and Flury LEP rabies virus strains (Figure 2.2). It is important to note that there are no amino acid differences in the known, mapped antigenic sites (Tordo, 1996). However, six of the eight known antigenic sites (epitopes) of the G protein are conformational and any amino acid changes in close proximity to these epitopes could potentially affect the folding of the protein (Tordo, 1996). Additionally, the transmembrane region has been reported to affect folding of the ectodomain resulting in subtle conformational changes of the antigenic sites (Maillard & Gaudin, 2002). The production of RVNA involves a process of fine tuning of specificity resulting in the selection of B cell clones with the highest avidity to a specific antigen. The potential differences in the G protein antigenic sites of the original seed virus strains used in the production of rabies virus could result in the preferential production of antibodies with the highest affinity for antigenic sites resembling the vaccine seed virus strain. Thus, the strain of challenge virus used in an RVNA assay and the type of vaccine that a person was vaccinated with could profoundly influence the serological test results after vaccination. If this proved to be correct, RVNA assays using homologous testing systems (wherein the strain of challenge virus used in the testing assay is closely related to the seed virus used to produce the vaccine that a subject received) would report higher titer values than heterologous testing systems (wherein the strain of challenge virus used in the testing system is less closely related to the seed virus used to produce the vaccine that a subject received). The following study was conducted to determine the influence that the strain of rabies virus used as the challenge virus in a serological assay (homologous versus heterologous) and the strain of seed virus used in the production of the rabies vaccine that a subject received has on the quantitative evaluation of RVNA.
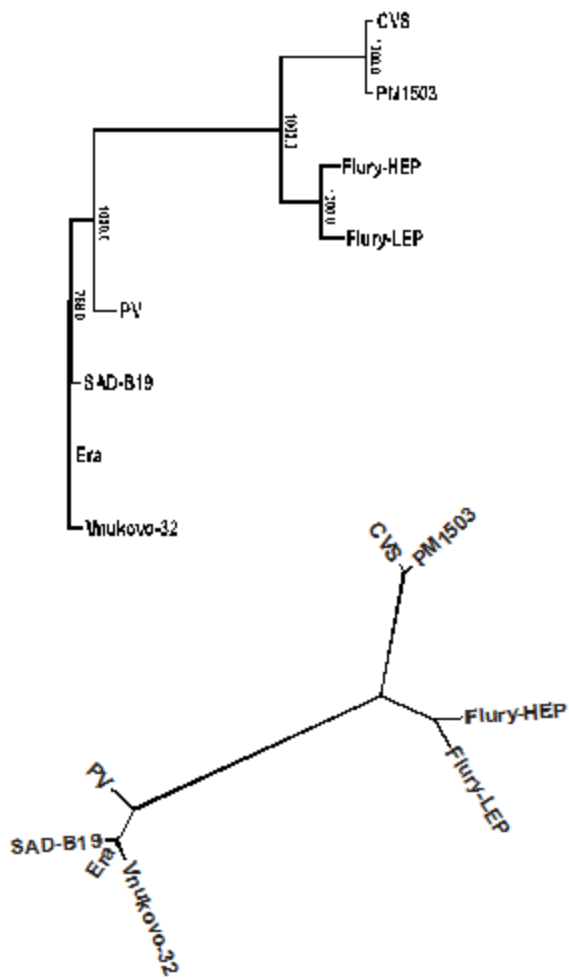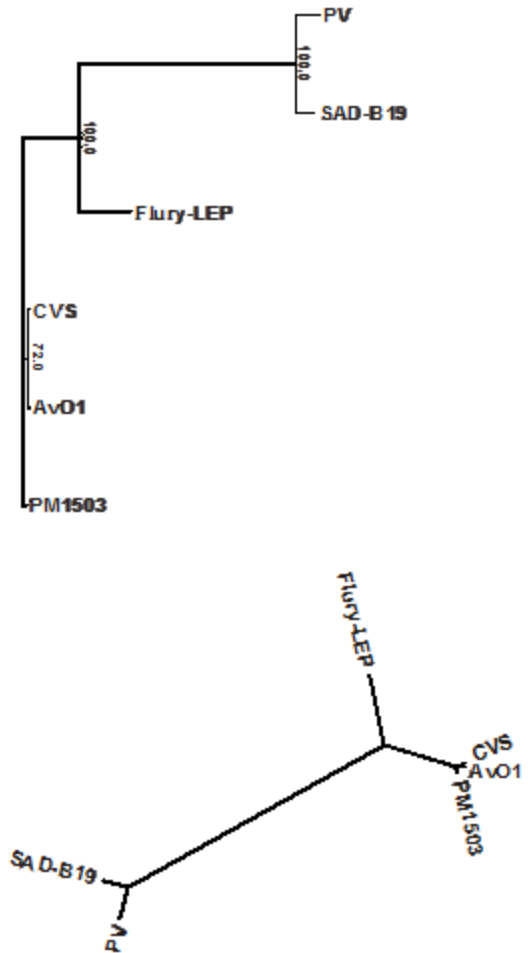
Figure 2.1 Phylogenic relationship of rabies virus strains (courtesy of Iris Stalkamp, Institut für Virologie, Giessen, Germany).

Figure 2.2 Amino acid alignment of the rabies glycoprotein from Flury, CVS and PM strains (courtesy of Iris Stalkamp, Institut für Virologie, Giessen, Germany). There are fewer amino acid sequence changes from CVS to PM (filled arrows) than CVS to Flury (open arrows). The changes are not in areas of the mapped antigenic sites of the rabies glycoprotein (shaded triangles). The transmembrane sequence is indicated by the boxed area.

2.3 Materials & Methods

2.3.1 *Challenge Virus*

Two strains of rabies virus were evaluated as the challenge virus fin the RFFIT assays used to quantitate the amount of RVNA present in serum samples. The CVS-11 strain was obtained from the Centers for Disease Control and Prevention (Atlanta, Georgia). Seed virus of the CVS-11 was grown on BHK cells to produce stock virus. The Flury LEP strain was obtained from Chiron Corporation (Marburg, Germany), stock virus was grown in primary chicken fibroblasts. Stock virus preparations were titered to obtain a working dilution of 50 $TCID_{50}$.

2.3.2 *Serum samples*

Serum samples used in the analyses were obtained from subjects that had received the same simulated post-exposure vaccination regimen with either PCECV (n=86) or PVRV (n=87). Subjects did not receive rabies immunoglobulin (RIG). Serum samples that were collected on day 14 and day 90 after initial vaccination were included in the study. Serum samples were randomly placed into five testing groups (Groups 1 through 5). Each group contained from 60 to 120 serum samples including samples from subjects vaccinated with PCECV as well as subjects vaccinated with PVRV. All serum samples were coded to ensure that testing was conducted blindly and unsorted by vaccine group.

2.3.3 *Equilibration of working dilution of challenge virus*

The working dilution of the challenge virus was equilibrated to 50 $TCID_{50}$ for both the CVS-11 and the Flury LEP rabies virus strains. The titer of the challenge virus was calculated for each test set of serological samples in order to assure equivalence in testing criteria. For all test runs, the titer of the challenge virus was maintained within one standard deviation of the average calculation (41.1 $TCID_{50}$) for virus titer throughout the entire evaluation.

2.3.4 *Serological testing*

The RFFIT, using CVS-11 and Flury LEP as the challenge virus strains in parallel, was used to assay all serum samples, as previously described (Smith, 1996). Briefly, 100 µL of each serum sample, in duplicate, was diluted in serial five-fold dilutions and loaded into 8-well lab-tek chamber slides after which 100 µL of the challenge virus, at a concentration of 50 TCID50, was added. Slides were incubated at 37°C for 90 minutes after which 200µL of a suspension of 5 X 10^5 BHK cells was added to each well. Slides were placed in a 5% CO2 incubator at 370C for 24 hours. After incubation, the slides were washed and fixed in 80% cold acetone, dried and stained with FITC conjugated anti-rabies antibody (Chemicon, Temecula, CA). Twenty fields/well were examined under 160X magnification using a fluorescence microscope for the presence of rabies virus and RVNA titers were calculated using the Reed and Muench method. Reciprocal titers were used in the evaluation in order to eliminate the need to calculate international units using titer results obtained form an international rabies reference serum that originated form subjects only vaccinated with a rabies vaccine produced from a PM seed strain of rabies virus.

2.3.5 *Statistical analyses:*

After all serum samples were tested separately with both the CVS-11 and the Flury LEP rabies challenge virus strains, the identification of the two vaccination groups (PVRV and PCECV) was unblinded and the RVNA titers were statistically analyzed to determine the effect of serological testing by means of a homologous vs. heterologous test. To determine whether any strain-dependent difference in neutralizing antibody was magnified at higher titers, the titer results (both day 14 and day 90) were sorted into response groups, the geometric mean titer (GMT) of the groups was calculated, and the GMT by challenge virus was compared. Additionally, to determine whether maturation of the antibody response amplified the differences

in GMT, the titer responses by day of serum drawn were sorted and the GMT of the groups was

calculated and compared by challenge virus.

Table 2.1 Titer of Challenge Virus Standard (CVS-11) and Flury low egg passage (Flury-LEP), the two rabies virus strains used as the challenge viruses for each serological testing group

Serological testing group	Titer of CVS-11	Titer of Flury-LEP
1	42.1	41.1
2	40	41.2
3	41	40
4	41.4	42.3
5	41.1	40.9
Geometric mean	41.1	41.1

Virus titer is expressed in TCID50.

Table 2.2 Rabies virus neutralization antibody (RVNA) values for Rapid Fluorescent Focus Inhibition Test (RFFIT) using a homologous challenge virus testing system and a heterologous challenge virus testing system.

Vaccine administered	RFFIT testing system Challenge virus	GMT (Range)	
		Day 14	Day 90
PCECV	Homologous	1855	265
	Flury-LEP	(320 to 6300)	(45 to 1500)
	Heterologous	1275	192
	CVS-11	(145 to 5400)	(45 to 1100)
PVRV	Homologous	2364	284
	CVS-11	(360 to 8500)	(45 to 9500)
	Heterologous	1448	196
	Flury-LEP	(70 to 8500)	(45 to 19700)

Serum samples were obtained from subjects vaccinated with purified chick embryo cell rabies vaccine (PCECV) or purified Vero cell rabies vaccine (PVRV) and were assayed using CVS-11 and Flury-LEP as the challenge viruses in the RFFIT.

2.4 **Results**

The virus titer of CVS-11 and Flury LEP, used as the challenge virus in each of the five

serological testing groups, remained consistently equivalent throughout the testing period (Table

2.1). There was a similar wide range of RVNA titers obtained for each vaccination group, independent of whether CVS-11 or Flury-LEP was used as the challenge virus strain (Table 2.2). There were two outlier reciprocal titer values in the PVRV vaccination group, 9,500 and 19,700, exhibited by the same subject on days 14 and 90, respectively. The GMT for each group indicates higher RVNA titers are reported when a homologous challenge virus strain was used in the serological assay. The RVNA test results of individual serum samples indicated that there was a clear trend to report higher titers when a homologous testing system (CVS-11 used as the challenge virus in the RFFIT for testing sera from subject vaccinated with PVRV and Flury-LEP used as the challenge virus in the RFFIT for testing sera from subjects vaccinated with PCECV) rather than a heterologous testing system (Flury-LEP used as the challenge virus in the RFFIT for testing sera from subjects vaccinated with PVRV and CVS-22 used as the challenge virus in the RFFIT for testing sera from subjects vaccinated with PCECV) was used (Figure 2.3).
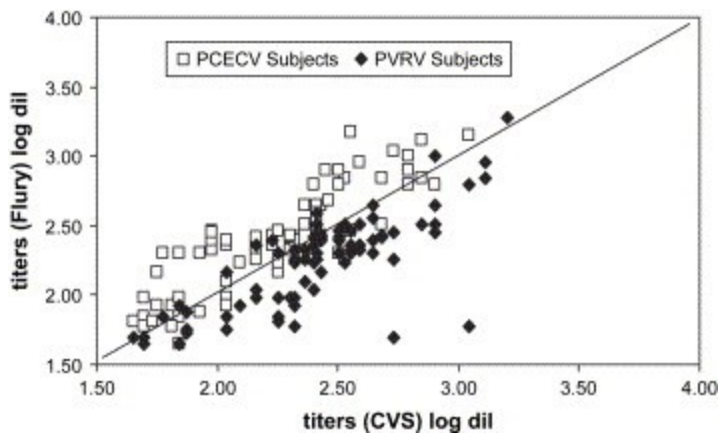
Figure 2.3 Serum samples from day 90 after administration of purified chick embryo cell rabies vaccine (PCECV) or purified Vero cell rabies vaccine (PVRV) given in a post-exposure prophylaxis regimen were analyzed twice by RFFIT. In one assay CVS-11 was utilized as the challenge virus in the RFFIT and in the second RFFIT, Flury-LEP was utilized as the challenge virus. The rabies virus neutralization titer (RVNA) result obtained for each serum sample was plotted according to the challenge virus used in the RFFIT. The line of unity represents expected RFFIT values that would be equivalent regardless of whether CVS-11 or Flury-LEP rabies virus was used as the challenge virus for patients vaccinated with PCECV or PVRV. Similar results were seen with the day 14 results (data not shown).

The RVNA values in both vaccination groups included titers in the low, medium and high range, regardless of which challenge virus used in the assay. Low, medium, and high ranges were designated for this set of results to determine possible trends associated with the strength of the antibody response. Nearly two-thirds of the samples from each vaccine group reported higher titers when a homologous challenge virus strain was used for the RFFIT assay, 63% for PCECV and 65% for PVRV. Approximately 30% of the serum samples tested in each vaccine group reported titers that were the same value or similar (within one standard deviation) regardless of whether they were assayed using a homologous or a heterologous challenge virus strain.

The percent reduction of reported RVNA titers, when switching from a homologous testing system to a heterologous testing system was 23%, 47%, and 33% respectively for the low, medium, and high response groups in the PVRV vaccination group and 27%, 25%, and 40% in the PCECV vaccination group (Figure 2.4). Thus, there was no clear trend of higher or lower RVNA titers related to the type of testing system, and whether the serum tested belonged to the low, medium, or high response group. The overall reduction in RVNA titer values when switching from a homologous challenge virus assay to a heterologous challenge virus assay was 33% in reported RVNA titer values for the PVRV vaccination group, and a 31% reduction in reported RVNA titer values for the PCECV vaccination group.

On both day 14 (data not shown) and day 90 the GMTs were higher when a homologous challenge virus system was used for the PVRV and PCECV vaccination groups (Figure 2.5).
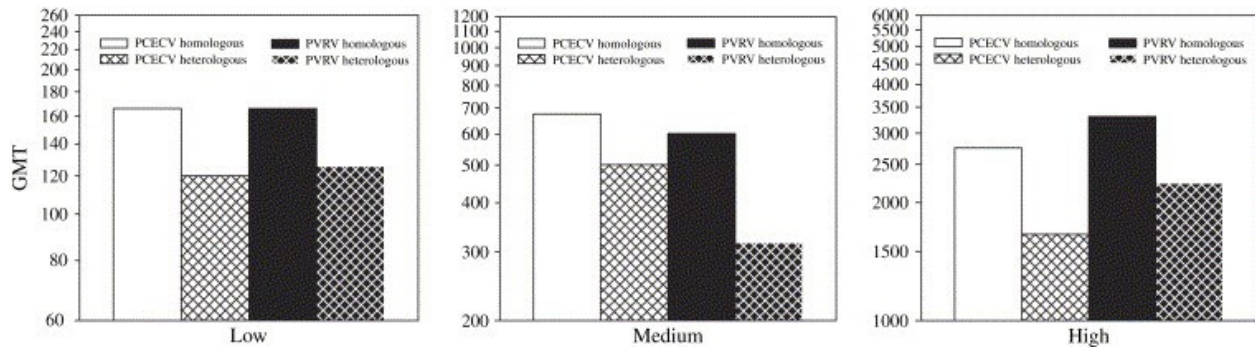
Figure 2.4 Depicted are the geometric mean titers (GMT) of serum samples analyzed by the Rapid Fluorescent Focus Inhibition Test (RFFIT) and separated into high, medium and low titer results. Serum samples were collected from subjects vaccinated with purified Vero cell rabies vaccine (PVRV) or purified chick embryo cell rabies vaccine (PCECV), and tested with RFFIT in either a homologous or heterologous testing system. A homologous testing system included sera from subjects vaccinated with PCECV and analyzed by RFFIT using the Flury-LEP as the challenge and sera from subjects vaccinated with PVRV and analyzed by RFFIT using the CVS-11 as the challenge virus. A heterologous testing system included sera from subjects vaccinated with PCECV and analyzed by RFFIT using the CVS-11 challenge virus and sera from subjects vaccinated with PVRV and analyzed by RFFIT using the Flury-LEP as the challenge virus.
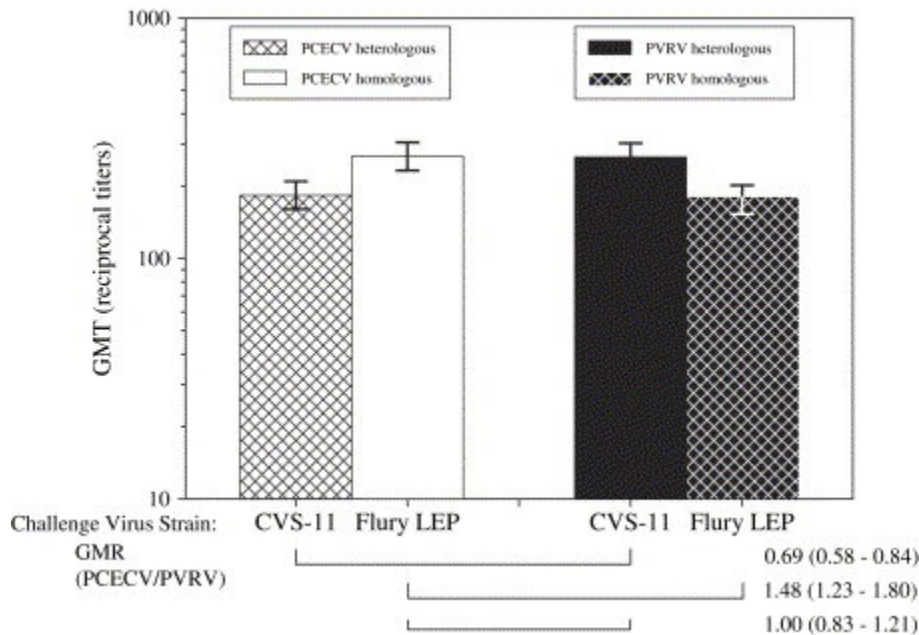
Figure 2.5 Serum samples from day 90 after administration of purified chick embryo cell rabies vaccine (PCECV) or purified Vero cell rabies vaccine (PVRV) given in a post-exposure prophylaxis regimen were analyzed by the Rapid Fluorescent Focus Inhibition Test (RFFIT) for rabies virus neutralizing antibodies, using different challenge virus strains. Depicted are geometric means of reciprocal titers (GMT), error bars represent 90% confidence intervals. Geometric mean ratios (PCECV/PVRV) of the different challenge strain comparison groups were calculated (90% confidence intervals in parentheses), resulting in equivalent titers when using the homologous challenge strain Flury-LEP for PCECV and CVS-11 for PVRV.

2.5 **Discussion**

Neutralizing antibodies play a critical role in immune protection against rabies infection. Therefore, it is appropriate to utilize RVNA assays to measure the immune response after rabies vaccination rather than relying on antibody-binding assays, which do not measure the function of the antibodies produced. Indeed, currently the most accepted approach for measuring the immune response to rabies antigen is to measure the amount of RVNA in serum. In the United States, the Advisory Committee on Immunization Practices (ACIP) recommends that RVNA testing should be performed by virus neutralization assays; those persons at risk of contracting rabies should have their RVNA levels measured periodically; and a booster should be administered to persons at risk of contracting rabies when their RVNA titer falls below complete neutralization of a specific quantity of rabies at a 1:5 serum dilutions by the RFFIT (the World Health Organization recognizes this level to be 0.5 IU/mL) (Centers for Disease Control and Prevention, 1999; World Health Organization, 1992). The evaluation of serological levels of RVNA is also appropriate for patients who may be immunosuppressed, or when a patient may have had a severe adverse reaction to the vaccine. Finally, new rabies vaccines are evaluated, licensed and approved for use partly by assessing the level of RVNA produced after vaccination in human subjects enrolled in clinical trials.

As mentioned earlier, the CVS-11 strain of rabies virus, generally used as the challenge virus in RFFIT assays that are used to measure RVNA, differs in how closely it is related to the PM and Flury strains of rabies viruses that are used in the production of human rabies vaccines (Figures 2.1 and 2.2). These differences are in some instances located in the areas that are in close enough proximity to the antigenic sites (and also in the transmembrane region) to potentially affect the conformation of the antigenic sites. It is possible that the differences

between the strains of seed virus used in the production of rabies vaccines are enough to cause slight conformational changes in the antigen-binding site of the antibodies that are induced after vaccination. These slight differences in the antigen-binding site could cause the antibody to have a higher affinity for a challenge virus used in an in vitro assay that more closely resembles the antigen that caused its production in the first place. The results of our study indicate that the degree of homology between the strain of challenge virus used in the RFFIT to measure the immune response after vaccination and the strain of seed virus used to produce the vaccine that subjects received profoundly affects the reported RVNA values. The use of challenge virus strains with equivalent titers in RFFIT assays resulted in approximately 30% higher RNVA titers in two-thirds of the serum samples we analyzed when a homologous testing system was used. In addition, the level of the RVNA titer (high, medium, or low) had no obvious or consistent effect on the percentage of titer difference reported between the testing systems.

In most cases the choice of challenge virus strain used in a rabies virus neutralization assay would not play a critical role in the evaluation of RVNA titers; for example, periodic titer evaluations and the determination of an immune response after post-exposure prophylaxis where the exact titer level is less important than the actual detection of neutralizing antibody. In addition, the strain of rabies virus used in a rabies virus neutralization assay is unlikely to be a determining factor in the measurement of RVNA titers in persons whose pre- exposure series may be from one vaccine source and subsequent booster(s) from another source. Similarly, persons who have had a rabies exposure will have an immune response to the rabies antigens in the exposure strain and to the vaccine strain confounding the mix of antibodies produced. For all of the above mentioned reasons it would provide no benefit to measure the RVNA response by a separate rabies virus strains. In contrast, the measurement of the humoral immune response after

rabies vaccination for the specific purpose of evaluating a new vaccine makes the choice of the challenge virus used in a rabies virus neutralizing assay extremely important. When the RVNA levels against vaccines made from two different parent strains are compared using an assay that employs a particular challenge virus strain in the testing system, the combined effect of the quantity, functionality, and specificity of the respective antibody response is measured. As demonstrated by this study, if the challenge virus used in the assay is more closely related to one parent virus strain than to the other, the titer results obtained will be biased toward the homologous vaccine. Most importantly, the evaluation methods used to confirm an absence of significant difference between the immune response produced by two vaccines involve statistical comparisons of the GMT by the geometric mean ratio (GMR). The Food and Drug Administration (FDA) defines bioequivalence as "pharmaceutical equivalents whose rate and extent of absorption are not statistically different when administered to patients or subjects at the same molar dose under similar experimental conditions."(Food and Drug Administration & Health and Human Services, 2004). In comparing statistical evaluation of each vaccine, the confidence intervals (CI) of the GMR are examined. When the lower limit of the 95% CI is greater than 50% and the interval includes 100%, "non-inferiority" is achieved. To determine the stricter standard of "bio-equivalence", 90% CI of the GMR must lie within 80%-125%. If this bioequivalence test is applied for the day 90 results in our study, the GMTs obtained for PCECV are inferior to PVRV when serum samples from subjects vaccinated with PCECV are tested in a heterologous testing system using the CVS-11 strain of challenge virus. Conversely, the GMTs obtained for PVRV are inferior to PCECV when serum samples from subjects vaccinated with PVRV are tested in a heterologous testing system using the Flury LEP strain of challenge virus (Figure 2.5). However, when a homologous testing system is used to test the

serum samples for subjects in each vaccination group, not only are the two vaccines non-inferior, they are equivalent.

2.6 **Conclusions**

This report ascertains that the choice of challenge virus strain used in rabies virus neutralization assays to evaluate the production of RVNA titers after vaccination should be taken into consideration when the titer values will be used for the evaluation of new or existing vaccines. Clearly, if quantifying the immune response to vaccine is objective, then using a homologous rabies virus strain in the testing would most appropriately reflect this goal. Finally, it is important to remember that modern cell culture vaccines are highly effective and cross-protection between strains has been demonstrated (Briggs, 2002; Lodmell, Smith, Esposito, & Ewalt, 1995). The use a heterologous or homologous testing system to evaluate the level of RVNA as a measure of complete 'protection' against rabies infection is incorrect. To date, the level of RVNA required to be 'protective' against infection in humans is not known for several reasons, the most important of which is that it is unethical to conduct challenge experiments in humans to determine the level of RVNA required for protection. On the other hand, the use of rabies virus neutralizing antibody testing systems to measure the immune response to specific rabies antigens and the response to rabies vaccines should not only be accurate and precise, but also meaningful.

2.7 **Acknowledgments**

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Chapter 3 - Rabies vaccine booster decision and rabies serology: how to interpret results

3.1 Abstract

For people at risk of rabies exposure, the protection afforded by pre-exposure rabies vaccination is a significant health assurance measure. Though rabies vaccines are among the safest and most successful vaccines made, the level of immunity induced can wane over time while the level of risk may not. Periodic rabies titer checks are recommended by the two main sources of rabies prevention guidelines, the Advisory Committee on Immunization Practices (ACIP) in the United States and the World Health Organization (WHO). While both guidelines provide very similar instructions, there are some differences, such as the level of RVNA, that represent evidence of seroconversion or adequate response. These different levels--complete neutralization at a 1:5 serum dilution and 0.5 IU/mL--in combination with different rabies serology methods in practice can lead to misinterpretation when decisions about rabies booster vaccinations are made. Given the importance of rabies vaccination in the prevention of rabies in at-risk individuals and in people undergoing post-exposure vaccinations, the language in the ACIP guidelines in regards to rabies serology results and testing should be updated to provide applicable booster vaccination guidance and understandable definition of adequate response as related to rabies serology.

3.2 Introduction

Immunization against rabies with the vaccine produced by Pasteur in 1885, and which remained unchanged until the advent of tissue culture vaccines, involved multiple vaccinations and was not without serious consequences (Steele, 1975). In contrast, modern tissue culture vaccines are safe and effective (Wunner & Briggs, 2010). The safety of the vaccine combined

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with the knowledge of the protective effects of RVNA upon rabies exposure led to the practice of pre-exposure vaccination for people at frequent or continuous risk of exposure. Initially, it was suggested that this population receive a booster vaccination every two years to ensure ongoing 'protection' by RVNA (Centers for Disease Control, 1976). RVNA was measured in the early days of rabies vaccine development by the mouse neutralization test (MNT), a cumbersome *in vivo* test. Over-vaccination concerns and the implementation of a rapid test (RFFIT) for RVNA influenced a change from periodic booster vaccination to boostering only when the RVNA level falls below a level representing adequate protection (Centers for Disease Control and Prevention, 1980).

Pre-exposure vaccination serves two functions: one, to protect the person in case of unnoticed rabies exposure; two, to eliminate the need for passive immunization and reduce the number of vaccinations in the event of a rabies exposure (Centers for Disease Control and Prevention, 2008; World Health Organization, 2012). The main protective effect of rabies vaccination is the production of rabies virus neutralizing antibodies (RVNA) (Hooper et al., 1998). Because the longevity of RVNA varies per person, guidelines recommend periodic checks to determine RVNA level and to administer a booster vaccination if the level is too low (Centers for Disease Control and Prevention, 2008; World Health Organization, 2012). However, the two main guidelines, the Advisory Committee on Immunization Practices (ACIP) and World Health Organization (WHO), while providing very similar instructions, differ when it comes to defining the adequate vaccination response which, along with other variables related to testing methods, can cause difficulty in deciding when a booster vaccination is needed.

Rabies is universally feared due to the mortality rate--nearly 100%--and the ensuing horrific death. Though canine rabies, the primary source of human rabies cases globally, has

been eliminated in the United States in recent years, some areas remain rabies endemic due to wildlife rabies. Spillover from wildlife into domestic species and humans can occur (Leslie et al., 2006). Those whose occupation and location put them at risk of rabies exposure know that rabies pre-exposure vaccination is a protective measure. Most know that measurement of rabies antibodies is required to show continued protection. What may not be clear is that different laboratory methods measure different aspects of the antibody response and that defining the seroconversion involves both the method and the specific guidelines followed. The Kansas State University Rabies Laboratory routinely tests serum samples from humans and animals for the level of RVNA. People at risk of rabies exposure undergo rabies pre-exposure vaccination and monitor their RVNA levels per the ACIP and WHO guidelines to determine if a booster vaccination is required based on the RVNA result obtained upon rabies titer check.

The document globally referenced for defining adequate protection from rabies is the 1992 Report WHO Expert Committee on Rabies (World Health Organization, 1992). The applicable section concerns persons who have undergone pre-exposure rabies vaccination. It simply states if a person's RVNA titer is below 0.5 IU/mL, a booster vaccination is recommended. The ACIP first issued recommendations for RVNA titer testing in 1980 (Centers for Disease Control and Prevention, 1980); mentioning "a titer < 1:16" is considered proof of RVNA presence. In subsequent ACIP updates, this level has been described in various ways (see Table 3.1). The current ACIP version (2008) states the level as "complete neutralization at a 1:5 dilution of serum in the RFFIT." Both the 0.5 IU/mL and the complete neutralization at a 1:5 serum dilution levels are referenced in the rabies vaccine instructions of the two vaccines in use in the United States--RabAvert and IMOVAX. Numerous publications have mentioned the levels together and if not clearly stated, it can easily be interpreted or inferred that these levels

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are the same--one in IU/mL format and one in titer format (Dreesen, 1999). This is not the case.

To understand the two different levels, a description of the origins of these levels is helpful.

Table 3.1 Guidelines for humans pre-exposure vaccinated and at risk of rabies exposure

Agency/Year		Booster vaccination recommended if level is below:	Method of Testing:
WHO			
	1992	0.5 IU/mL	MNT or RFFIT; ELISA only with caution
	2005	0.5 IU/mL	RFFIT or FAVN; ELISA if RFFIT not available
	2013	0.5 IU/mL	RFFIT or FAVN; ELISA
ACIP			
	1976	None, boosters recommended every 2 years	None stated
	1980	1:16 titer or booster every 2 years	RFFIT
	1984	1:5 titer per CDC; 0.5 IU/mL per WHO	RFFIT
	1991	1:5 titer [a]	RFFIT
	1999	Complete neutralization at a 1:5 serum dilution [b]	RFFIT
	2008	Complete neutralization at a 1:5 serum dilution [c]	RFFIT

[a] Recommended response 2-4 weeks after either pre- or post-exposure vaccination is complete neutralization at a 1:25 serum dilution which is equivalent to the WHO level of 0.5 IU/mL

[b] Recommended response 1-2 weeks after post-exposure vaccination is complete neutralization at a 1:5 serum dilution

[c] RVNA titer most properly reported according to a standard as IU/mL

The level of 0.5 IU/mL was recommended at the Joint WHO/IABS Symposium on the

Standardization of Rabies Vaccines for Human Use Produced in Tissue Culture held in Marburg,

West Germany in November 1977 (Bogel, 1978). After the results of several international

human rabies vaccine trials were presented, recommendations were given by specific Working

Groups. According to Dr. Alexander Wandeler, a member of the 1992 Expert Committee on

Rabies and a participant in the 1977 Joint WHO/IABS Symposium on Rabies Vaccines, the

suggestion that low levels of virus inhibiting activity in sera may not be due to the presence of

specific antibody came from the observation that virus inhibition was found in a fair proportion

of sera of patients before they received any vaccination. Most of the nonspecific inhibition was

reported in sera at dilutions of 1:5 or lower; a few results were higher (Kuwert et al., 1978;

Wandeler, 2014). The resulting assumption was that a serum should show virus inhibiting activity at dilutions of 1:10 and higher in order to give some confidence of the presence of specific antibody. To be on the safe side, virus neutralization at a 1:20 dilution as specificity threshold was suggested. The representative of IABS (F.T.Perkins) insisted that international units (IUs), not titers or serum dilutions be used to define this specificity threshold. Based on a variety of opinions Symposiums attendees agreed that a titer of 1:20 approximated 0.5 IU/mL (Wandeler, 2014). The Working Group for vaccine potency requirements of reduced immunization schedules and pre-exposure vaccination stated:

> "The group suggests 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."((Bogel, 1978), p.270)

Based on the subsequent reports of rabies antibody levels attained after pre- or post-exposure vaccination series, the level of 0.5 IU/mL was globally accepted as proof of seroconversion (Smith, 2000). Similarly, the designated level in the ACIP was also based on RFFIT results from human vaccine trials in the 1970's and the observation that non-specific inhibition (false positive) reactions were never seen at serum dilutions 1:25 or greater in the RFFIT (Smith, 2000). This led to the conclusion that if a specific RVNA titer result was detected (above complete neutralization at a 1:5 serum dilution), then seroconversion had been achieved. The level described in the ACIP is approximately 0.1 IU/mL in the RFFIT as originally described (Moore & Hanlon, 2010). Both levels are referenced in the manufacturer's inserts for both current available rabies vaccines in the United States (IMOVAX and RabAvert) as an adequate or protective level of vaccine response (package insert, 2014; package insert, 2006).

It should be noted that in none of these documents is it stated that a vaccinated human with an antibody titer of 0.5 IU/mL is protected against rabies virus infection. Therefore, both

levels, 0.5 IU/mL and 0.1 IU/mL, though five-fold different, are based on the same rationale--

specific detection of RVNA. The difference is the degree of confidence that the designated level

can be assured to be a true measurement and not a "false positive." The WHO does not define

the assay to be used for antibody measurement, but in rationale recognizes the differences in

testing methods and laboratory capabilities. The variance in rabies serology results was described

in early publications on methods; wide differences in RVNA levels were obtained by MNT and

RFFIT and by different laboratories (Bogel, 1978; Fitzgerald, Gallagher, Hunter, Spivey, &

Seligmann, Jr., 1978). The ACIP states that rabies serology should be performed by the RFFIT,

thereby designating a single approved method. Neither the WHO or ACIP addresses the issue of

whether the assay used has been validated to human clinical standards (CLIA, ICH, etc.) in the

laboratory producing the results. Though the ACIP defines a method, it does not define the

adequate level of RVNA in standardized terms. "Complete neutralization at a serum dilution of

1:5 in the RFFIT" can represent different titers and IU/ml values in laboratories which perform

"modified" RFFIT assays. The ACIP states rabies serology should be performed by the RFFIT,

but other rabies serology methods are available to the public, particularly ELISA methods.

Indirect ELISA methods detect and measure the presence of rabies specific antibodies based on

their binding ability; they do not measure the neutralizing ability of the antibodies (Irie & Kawai,

2002). The immune response to rabies vaccination involves antibody production that is

polyclonal. Each individual production includes clonal antibodies that vary in affinity, avidity,

and ability to neutralize the rabies virus. Therefore, the relationship between the level of binding

antibodies and neutralizing antibodies is not constant; in one person, the ELISA result will be

higher than the RFFIT result and in another person, the opposite can be true. Applying the

"adequate" RVNA levels as described by WHO and ACIP to ELISA results may not be

advisable. A laboratory's method validation should also be considered. Validation of a method verifies performance standards and includes identification and verification of the lower limit of quantitation (LLOQ) (FDA, 2001). This is the lowest level that will produce accurate and precise results. The LLOQ is the level that, by implication based on the history stated above, both WHO and ACIP recognize as showing adequate response to rabies vaccination. Examination of the method/laboratory used to produce a rabies serology result and the rationale behind the two definitions of adequate rabies vaccine response can aid in developing clear language for guidelines in rabies vaccination.

In May of 2010, the Rapid Fluorescent Focus Inhibition Test (RFFIT) results reported by the KSU Rabies Laboratory underwent a formatting change (from titer to IU/mL) in response to the ACIP recommendations. At the same time, instead of only referencing ACIP guidelines, the WHO recommendations were also included in the rabies serology report. By including the WHO recommendations, the interpretation of results more closely aligned with the vaccine manufacturers' package inserts. In addition, areas outside the U.S. follow the WHO recommendations exclusively. These changes highlighted the difference between the two guidelines. This difference is not new, but the existence of different levels may not have been commonly known. The ACIP recommends the use of the Rapid Fluorescent Focus Inhibition Test (RFFIT) because other methods [i.e., Enzyme-linked Immunosorbent Assay (ELISA), Lateral Flow] do not measure RVNA specifically and therefore cannot be correlated to the RFFIT. Because applying the WHO level will result in more vaccinated individuals falling within the "need for booster" group and ELISA methods may produce results not correlated with the guidelines levels, these important components relied on for vaccination decisions require clarification. Possibly related to the decision to reference both the ACIP and WHO rabies

vaccination guidelines on the laboratory reports, several veterinary schools reported an increase in numbers of students who require rabies booster vaccination.

The KSU Rabies Laboratory performs the RFFIT test as recommended by both the WHO and ACIP for measuring human RVNA levels for booster vaccination decisions. Because there were concerns about the number of veterinary students requiring booster vaccination in recent years subsequent to the referencing both ACIP and WHO guidelines on the rabies serology reports, a retrospective study of rabies serology result from a subset of Veterinary Schools who have submitted samples for rabies titer check in the years 2005 to 2014 was performed. The objective was to determine whether a trend or change in the percentage of veterinary students requiring rabies vaccine booster exists over the years examined and to identify any differences between the schools.

3.3 **Material & Methods**

3.3.1 *Rabies serology*

The study was performed under the Kansas State University IRB protocol 7062. The RFFIT, using CVS-11 as the challenge virus strain, was used to assay all serum samples, as previously described (Velleca & Forrester, 1981). The RFFIT assay has been validated for the purpose of measuring antibody response to rabies vaccination in the KSU Rabies Laboratory. Using the laboratory information management system (LIMS), RFFIT results were collated from the veterinary student serum samples submitted from six Veterinary School between the years 2005 to 2014. Four of the schools had student sample submissions for each of those years; one school had sample submissions for all years except 2005 and 2008; and the remaining school had sample submissions from 2005 to 2011. The average number of samples submitted per school per year ranged from 114 to 201 for the years 2005 to 2014. Results were sorted into groups per

RVNA level as reported (< 0.1 IU/mL, >/= 0.1 IU/mL, or >/= 0.5 IU/mL) for each school per

year. Percent of samples in each group was calculated per school and per year. Additionally, the

results were grouped by titer decision level (using ACIP level of < 0.1 IU/mL and WHO level of

<0.5 IU/mL as the levels to determine need for booster) and percentages calculated per school

and per year.

3.3.2 *Statistical analysis*

Using the statistical software Analyze-it (Leeds, United Kingdom), percentages in each

group were analyzed by ANOVA for difference between years and between schools; if

differences were found, Bonferroni pairwise comparison was performed.
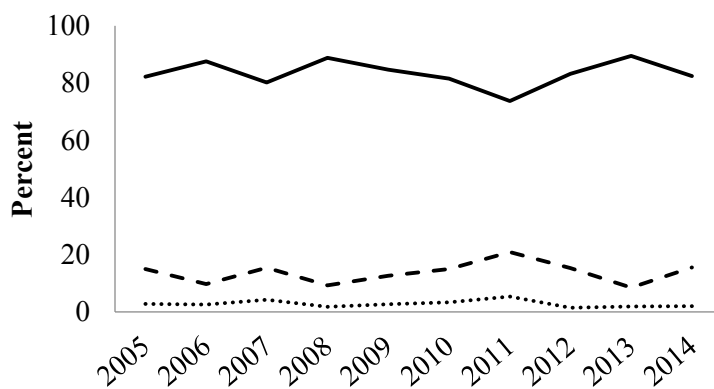
Figure 3.1 Percentages of student rabies titers >/= 0.5 IU/mL (solid line), >/= 0.1 and < 0.5
IU/mL (dashed line) and < 0.1 IU/mL (dotted line) for all veterinary schools combined for the
years 2005 to 2014.

3.4 **Results**

There has not been a significant change in the percent of individuals who fall below

published levels of minimum response to vaccination per this retrospective study of rabies

serology results from six veterinary schools who annually submit veterinary student serum

samples to the KSU Rabies Laboratory for rabies titer checks for the years 2005 to 2014 (see

Figure 3.1). Leaving out the outlier values for one school (D) from 2007, the percentages (0-8%

for the ACIP level and 6-31% for the WHO level) are similar to previously reported for pre-exposure vaccinated individuals; 2-7% of vaccinated patients fail to continue to have antibody levels at complete neutralization at a 1:5 serum dilution after 2 years (Centers for Disease Control and Prevention, 2008), and 10-30% of persons having RVNA levels below 0.5 IU/mL one year after pre-exposure vaccination (Rodrigues et al., 1987; Strady et al., 1998; Banga, Guss, Banga, & Rosenman, 2014). No obvious trend upwards or downwards in the percentage of students falling into each response group was noted. To illustrate the difference in percentages of students who require a booster vaccination based on their RFFIT results compared against the ACIP versus the WHO level, the percentages were graphed per year (see Figure 3.2). Table 3.2 lists the mean percentages and range of students requiring vaccine booster, per year. No statistically significant difference between schools for percentages by ACIP or WHO level; or between years by ACIP level, but there was a statistically significant difference between years by WHO level ($p < 0.0005$). Bonferroni pairwise comparison determined significant differences between the percentages of student requiring vaccine booster per the WHO level in the comparisons: 2011-2006, 2011-2008, 2011-2009, and 2011-2013. An evaluation of RFFIT results from student and employee groups from school A demonstrated that the percentages of persons requiring vaccine booster by either ACIP or WHO level did not peak or dip in the same years, data not shown.

Table 3.2 Average percent (and percent range) of students requiring rabies vaccine booster per the WHO or ACIP RVNA level guideline for the years 2005 to 2014

Year	ACIP	WHO [a]
2005	3	16
	(0 to 6)	(15 to 23)
2006	3	12
	(0 to 8)	(9 to 17)
2007	6	22
	(1 to 13)	(10 to 44)
2008	2	12
	(1 to 3)	(6 to 15)
2009	3	14
	(1 to 4)	(10 to 19)
2010	4	20
	(1 to 6)	(10 to 25)
2011	5	26 [b]
	(2 to 8)	(21 to 31)
2012	2	18
	(0 to 4)	(11 to 22)
2013	2	11
	(1 to 3)	(7 to 14)
2014	2	19
	(1 to 3)	(13 to 24)

[a] A statistically significant difference in the percent of students requiring rabies booster vaccine per the WHO level was determined between the years evaluated by ANOVA (p=0.0005).
[b] A Bonferroni pairwise comparison determined a significant difference between the years 2011 vs. 2006, 2011 vs. 2008, 2011 vs. 2009, and 2011 vs. 2013.

A

B
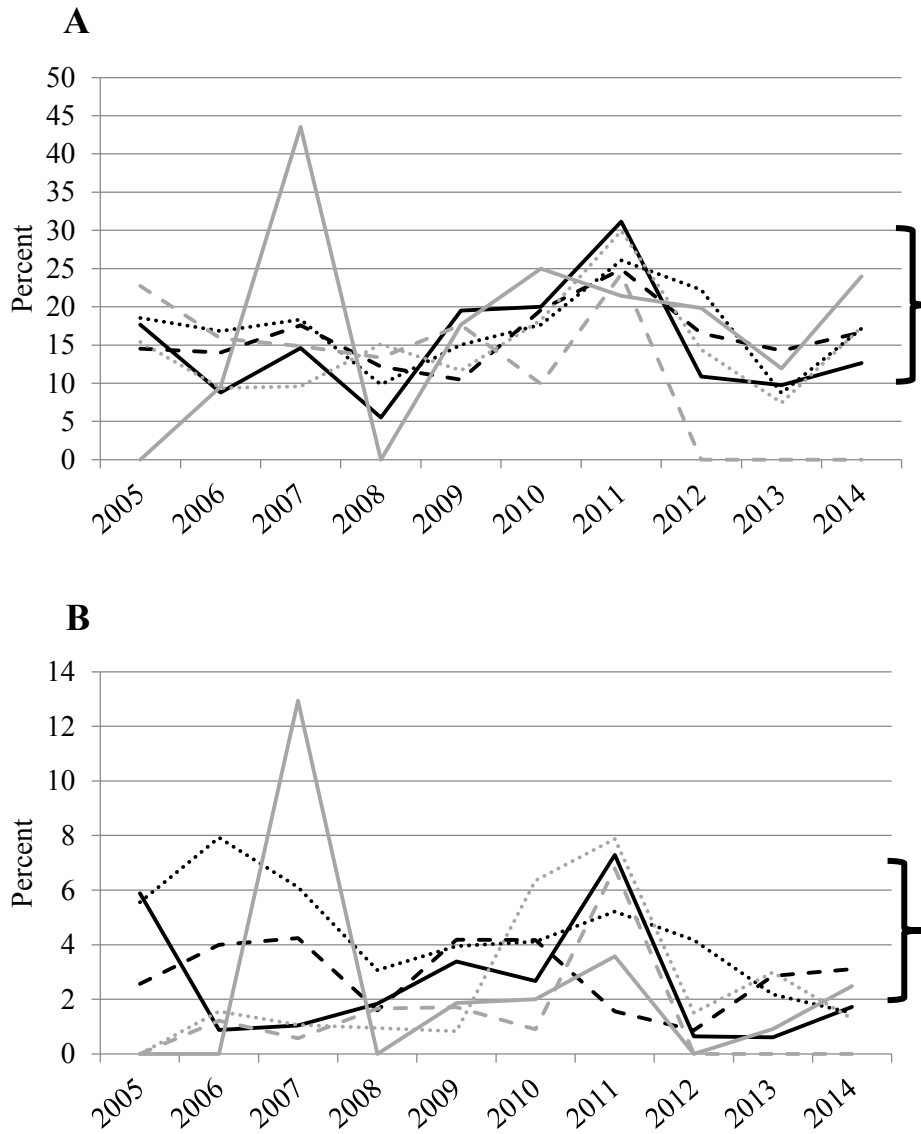
Figure 3.2 Percentages of students requiring rabies booster vaccination per the WHO guideline (RVNA <0.5 IU/mL) in panel A or per the ACIP guideline (RVNA <0.1 IU/mL) in panel B for the yars 2005 to 2014 by school: A (solid black line), B (dotted black line), C (dashed black line), D (solid grey line), E (dotted grey line) and F (dashed grey line). The expected percentages per previous studies is marked with a bracket on the right of the graph in both panel A and panel B.

3.5 **Discussion**

For obvious reasons, the RVNA levels assigned as adequate cannot be scientifically verified; therefore animal challenge studies provide information about the relationship between rabies serology results and survival from challenge. In a review article summarizing rabies challenge studies, valid cut-off values for RFFIT results in cats and dogs, of 0.1 and 0.2 IU/mL respectively, were identified (Aubert, 1992). The level determined by a prior study in dogs was similar, dogs with titers below 1:20 succumbing to rabies challenge and the statistically predicted risk of rabies death decreasing with rising titers up to 1:50 (Bunn & Ridpath, 1984). There have been very few rabies cases in humans who have had pre-exposure vaccination. Of the three published accounts, one patient was vaccinated with an experimental vaccine and did not have a detectable titer one year post-vaccination (Winkler, Fashinell, Leffingwell, Howard, & Conomy, 1973), the second had a titer of 1:32 five months prior to the exposure (MMWR, 1977), and the third did not have a titer test after vaccination, but did not have a detectable titer at time of clinical onset (Bernard et al., 1985). In an investigation of the third case, 9 of 11 other persons vaccinated at the same time, with the same vaccine lot and same schedule as the patient were found to have no detectable rabies titer.

The level of protection as defined by rabies antibody levels cannot be ascertained for humans. As with most vaccines, high and low responders as well as range of longevity of response are expected due to many factors, including individual polygenetic major histocompatibility complex (MHC) genetic make-up (Kuwert, Barsenbach, Werner, & Mardus, 1981; Haralambieva et al., 2013). There has been no change in the recommendations of either WHO since 1992 or ACIP since 1999. There has been no change in the percentage of individuals maintaining an adequate response to rabies vaccination based on the result of this study in

comparison to historical data, but there continues to be a misunderstanding of the relationship between the two guidelines and between results of different rabies serology methods.

Vaccine titer checks are used to assure the vaccinated person has continued humoral immune response to the vaccine, with recommendation to boost if there is low to no response. A detectable level should be sufficient for most persons at frequent risk of rabies exposure in the United States where rabies awareness and availability of rabies vaccination post-exposure is good and canine rabies is not endemic. A detectable level is the level validated (to human clinical standards) for the method in use and in the laboratory performing the testing. This level should be robust enough to account for the variability of human serum samples and of the method. The percentage of unvaccinated persons whose serum samples demonstrate non-specific inhibition of virus in the RFFIT at the 0.1 IU/mL is nearly double the percentage at the 0.2 IU/mL level, indicating the lower the level set for proof of adequate response to vaccination the larger the number of "false positives" reported. The primary purpose of rabies titer checks is to determine a detectable response to vaccination, and the prevention of rabies is thought to rely on RVNA; it follows that a low probability of "false positives" should be the primary criteria for an assay used for rabies titer checks. Per RFFIT method validation and historical data at KSU Rabies Laboratory, 0.2 IU/mL is the level where there is high confidence of a specific, detectable rabies antibody response (Kostense et al., 2012). A factor to keep in mind for consideration of assigning a level for rabies booster decisions are the timing of the blood draw. The majority of the antibody response shortly after the vaccination series has begun will be IgM which may not provide much protection in interstitial areas of the body, therefore a high level detected in the early days of response may not be as "protective" as a lower level of IgG several weeks, months, and years after vaccination. The level of 0.5 IU/mL was determined to be the level expected 4

weeks post-vaccination using serum neutralization assays (MNT and RFFIT). For post-exposure vaccination response evaluation, considerations such as the rabies virus variant, severity and location of the bite exposure, and the immune system condition of the patient can influence clinical decisions of the required level. The patient's physician(s) should make these decisions and consult with epidemiologists and public health professionals.

3.6 Conclusions

The extent of rabies antibody response to vaccination varies per individual, but within an expected range for both the immediate (early-high and late-low) and the longevity response. Besides defining rabies exposure, risk groups, and vaccination schedules and regimens; national and international guidelines and vaccine instructions need to unambiguously state the RVNA level that is considered to be an adequate response to vaccination, both for pre-and post-exposure situations. This level should be termed in a standard format (IU/mL) and with consideration of methods that are recommended and validated for this purpose. Given the importance of rabies vaccination in the prevention of rabies in at-risk individuals and in people undergoing post-exposure vaccinations, the language in the guidelines in regards to rabies serology results and testing should be updated to provide applicable booster vaccination guidance and an understandable definition of adequate response.

3.7 Acknowledgements

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Chapter 4 - Differences between rabies serology methods affect cut-off values for determination of adequate vaccine response

4.1 **Abstract**

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

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

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

4.2 **Introduction**

There are several different laboratory methods for detection and measurement of the (Bogel, 1978) rabies antibodies, yet the 0.5 IU/mL level is the one globally recognized marker of adequate response to vaccination in humans (World Health Organization, 2012). Often forgotten are the circumstances surrounding the origin of this determination. The World Health Organization (WHO) Expert Committee on Rabies (from the 3rd/1957 through the 6th/1973 reports) states that the vaccination response should be verified in the serum one month after vaccination by detection of RVNA, but no specific antibody level is specified. A Working Group convened during the 1978 Joint WHO/International Association of Biological Standardization (IABS) symposium defined a cut-off value after review of RVNA levels obtained from several clinical trial studies for the newly developed cell culture rabies vaccines. The conclusion was presented "that the serum be tested four weeks after the last inoculation and at that time a minimum value of 0.5 IU per ml be attained to demonstrate seroconversion"(Bogel, 1978). In the WHO Expert Committee on Rabies Report from 1984, the 0.5 IU/mL cut-off value is described as the level expected one month after vaccination; and that booster vaccinations are required until that level is reached (World Health Organization, 1984). The key points are that the 0.5 IU/mL antibody level was determined based on results from serum neutralization methods

(Mouse Neutralization Test or MNT and Rapid Fluorescent Focus Inhibition Test or RFFIT), was for specific time point (one month after vaccination), and was for a specified purpose (demonstrate seroconversion). To extrapolate this value to other methods, time points, and purposes is not supported without further investigation. In addition, referring to the level as protective is a misinterpretation of the original intent.

SN and ELISA rabies serology methods have been compared many times and the findings are similar: correlation is fair to good (Nicholson & Prestage, 1982; Welch, Anderson, & Litwin, 2009; Elmgren & Wandeler, 1996) (Grassi, Wandeler, & Peterhans, 1989; Esterhuysen, Prehaud, & Thomson, 1995; Cliquet et al., 2004; Feyssaguet et al., 2007). This is not surprising as both methods are quantitating, in different ways, the specific rabies antibody response to rabies vaccination. The reasons SN and ELISA results cannot be considered statistically comparable for all samples are: the methods measure different characteristics of rabies antibodies (neutralizing function for SN and binding function for ELISA); and the normal antibody response to rabies vaccination is polyclonal, producing immunoglobulins with various epitope specificities, affinities, Ig subclasses, and neutralizing abilities--a unique polyclonal response per individual (Moore & Hanlon, 2010). This means the relationship of binding antibody measurement to neutralizing function will be variable between individuals. Similar issues have been described for discordant estimates of seroprevalence to mumps and measles using SN and ELISA methods (Mancuso, Krauss, Audet, & Beeler, 2008; Latner et al., 2014).

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

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

4.3 Materials & Methods

4.3.1 *Serum Samples*

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

4.3.2 *Serological testing*

All serum samples were tested with both a SN and ELISA method.

The SN method, the RFFIT, was performed as previously described (Velleca & Forrester, 1981) and validated for use with human sera at the KSU Rabies Laboratory. The challenge virus strain used was CVS-11. RVNA titer values were standardized to IU/mL values by comparison

with the Standard Rabies Immune Globulin (SRIG) (WHO 1st international RIG/Lot R-3 FDA/CBER).

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

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

4.3.3 *Statistical analysis*

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

4.4 **Results**

The anti-rabies glycoprotein levels (EU/mL) as measured by ELISA peaked at day 30 on average though there was a lesser peak at day 14 as compared to day 30 while the peak IU/mL values as measured by RFFIT was at day 14 (see Figure 4.1). The anti-rabies glycoprotein levels were consistently lower than the RVNA levels on day 14 and day 30 for all groups (to greater extent in groups B and C) and nearly equal to the RVNA levels at day 90. The range of IU/mL (RFFIT) and EU/mL (ELISA) were similarly wide in each group and at each day with the widest ranges occurring at days 14 and 30 (see Table 4.1). Subjects with the highest levels of response were identified by both methods; two subjects in Group B both had RFFIT and ELISA results of $\geq$200 IU/mL or EU/mL at day 14.
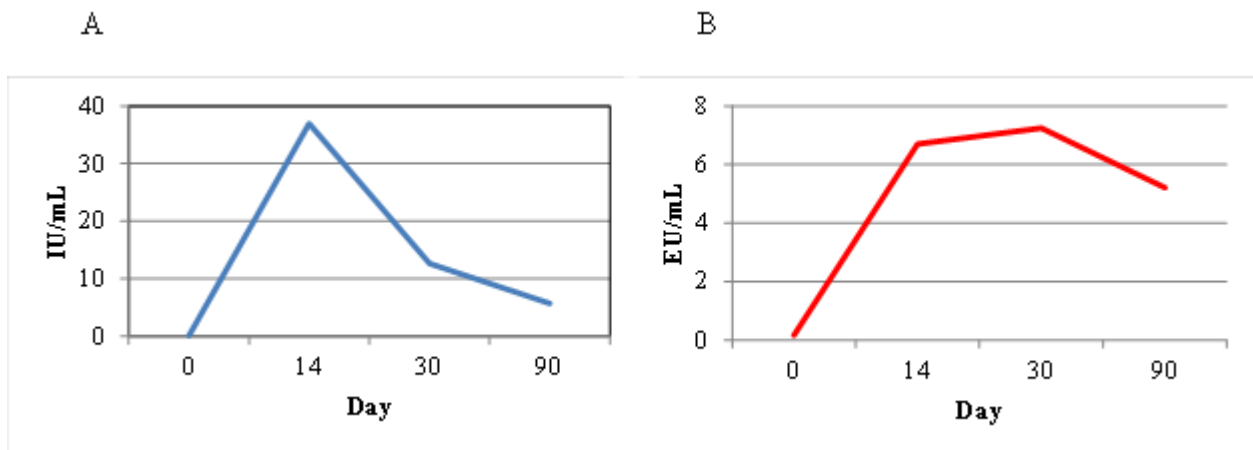
Figure 4.1 Kinetics of rabies vaccine response as measured by RFFIT (RVNA IU/mL) in panel A and as measured by indirect ELISA (anti-rabies glycoprotein EU/mL) in panel B.

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

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

The relationship between RFFIT and ELISA results per individual as examined in all groups/subjects was variable and particularly affected by day of blood collection (see Figure 4.2 A). At 14 days post-vaccination, the majority of subjects had RFFIT results more than 50% higher than their ELISA result. On day 30 the results were of a mixed relationship with some subjects having higher RFFIT than ELISA results, others with results within 50%, and a smaller portion of subjects with higher ELISA than RFFIT results. By day 90, the majority of subjects had ELISA and RFFIT results that were comparable within 50%; the next largest group having ELISA results than their RFFIT results; and the smallest group had higher RFFIT results than their ELISA results. When each vaccine group was examined separately the same trend was observed but differing proportions in each category (see Figure 4.2 B). For example, in Group A there are approximately equal numbers of subjects with RFFIT and ELISA results within 50% as there are subjects with higher RFFIT/lower ELISA result whereas for Group C, nearly all the

subjects have higher RFFIT than ELISA results. The higher the RVNA level reached by a group the more discrepant the results between the two methods were at day 14 and day 30.
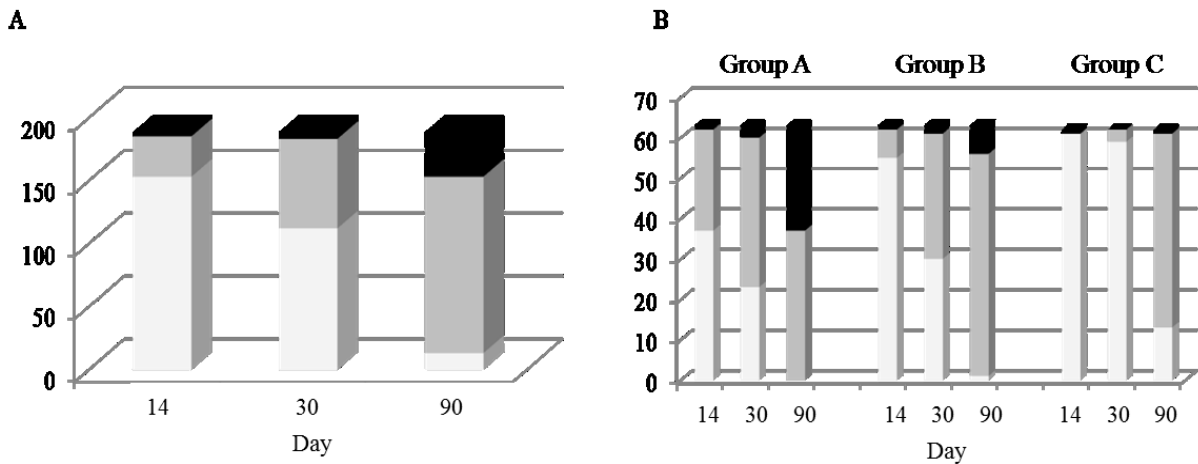
Figure 4.2 The relationship of RFFIT IU/mL to ELISA EU/mL at different time points from vaccination overall (panel A) and by group (panel B). The number of subjects with RFFIT IU/mL greater than their ELISA EU/mL is in light gray, the number of subjects with RFFIT IU/mL with 50% of their ELISA EU/mL values is in gray, and the number of subjects with RFFIT IU/mL less than their ELISA EU/mL is in black.

Using 0.5 IU/mL for RFFIT and 0.5 EU/mL for ELISA as the cut-off values, the number of subjects achieving an adequate response (percentage of subjects with results equal to or above cut-off value at day 14) between groups leads to different conclusions by method (see Table 4.2). Using ELISA results, Group A's response is inferior to Group B's at (34% versus 100% achieving adequate response level, respectively); using RFFIT results the two groups are more similar in response with 76% Group A and 100% Group B subjects reaching the cut-off levels. However in the comparison of Group C to Group B, the groups appear to produce nearly the same percentages above the cut-off values by both test methods, 92% versus 100% by ELISA, and 100% versus 100% by RFFIT. The groups by day were analyzed using the student's t-test to determine whether use of method would affect the assessment of group differences. The vaccine responses as measured by ELISA for Group A compared to Group B and for Group C compared to Group B are determined to be significantly different ($p = 0.05$) at both day 14 and day 30, while

the response as measured by RFFIT determined only Group A compared to Group B at day 14 were significantly different.

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

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

Head to head RFFIT/ELISA comparison of individual results demonstrated a wider discrepancy at day 14 than at day 90 (see Figure 4.3). For example, graphing the Group C individual responses clearly shows the varying kinetics of RVNA and anti-rabies glycoprotein response by both time (days from vaccination) and magnitude (level) (see Figure 4.4) due in large part to the inability of the ELISA method to detect IgM (only IgG anti-rabies glycoprotein binding measured) while RFFIT detect neutralizing antibody of both IgG and IgM class. The variation of response by individual is demonstrated in Table 4.3 by selected subjects, some of the largest and smallest discrepant ELISA versus RFFIT results are noted. The best agreement between RFFIT and ELISA result values for individuals was found at day 90 (with the majority of values within 50%) and yet large variation in results (both in value and in which method produced higher values) from the two methods were present at all the time points.
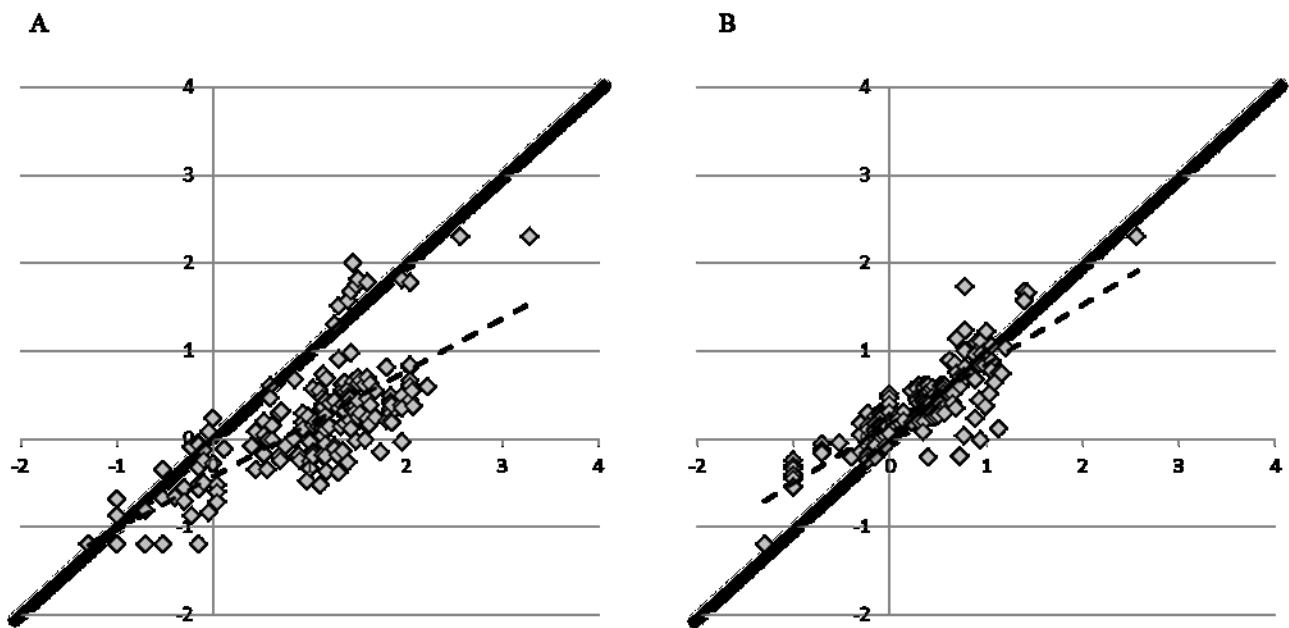
Figure 4.3 Correlation of RFFIT IU/mL to ELISA EU/mL results at day 14 (panel A) and at day 90 (panel B). Result values were log transformed and are displayed with the RFFIT results on the x-axis and the ELISA results on the y-axis. The regression line is the black dashed and the line of identity is the solid black.
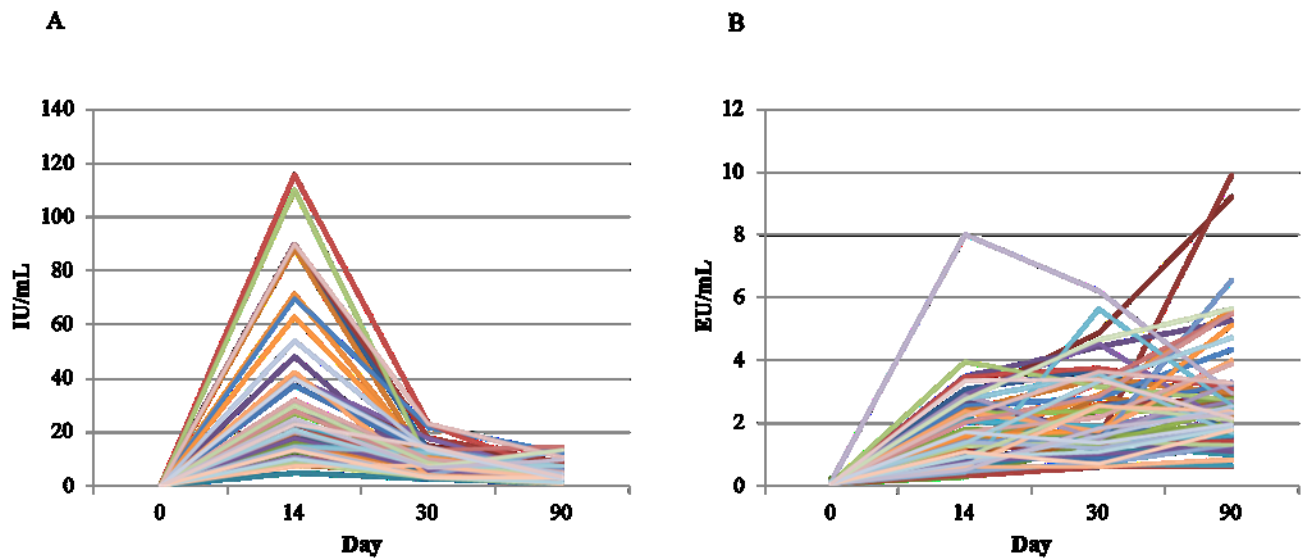
Figure 4.4 Individual results, RFFIT IU/mL in panel A and ELISA EU/mL in panel B, for subjects in group C. The results of one subject with very high ELISA (63.96 EU/mL) and moderately high RFFIT (32.0 IU/mL) results at day 14 were not plotted to allow illustration of the peak of the majority of subjects results.

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

Group C - Day 14				Group C - Day 90		
Subject #	IU/mL	EU/mL		*Subject #*	IU/mL	EU/mL
9	13	0.297		*75*	**11.3**	**11.295**
11	4.9	0.625		*88*	**2.4**	**2.623**
17	13.5	2.16		*105*	6	53.18
23	10	0.554		*165*	**2.5**	**2.559**
39	110	3.981		*171*	7.7	1.678
42	42.4	2.374		*180*	11	3.256

4.5 **Discussion**

In a broad view, both SN and ELISA methods for rabies serology measure the presence of antibody through a specific antibody-antigen interaction. Although other components of the immune system are involved, protection from clinical rabies after infection relies heavily on the presence of RVNA (Hooper, 1998). Because SN rabies serological methods detect the neutralization activity of RVNAs *in vitro*, mimicking the protective action of these antibodies *in vivo*, they are the best methods to quantify the level of immunity after rabies vaccination and subsequently the need for booster administration. The technical performance of SN methods, such as the RFFIT, requiring high levels of biosafety facilities and expertise makes the use of these methods a difficult proposition in some areas of the world. Antigen binding assays such as indirect and competitive ELISA methods are rapid, simple, and do not require manipulation of live rabies virus which makes them a practical alternative to SN methods. Simply replacing the method used for rabies antibody measurement and using the same cut-off value for "positive" and "negative" for purposes of determination of adequate response to rabies vaccination is problematic due to the inherent differences in how and what the different type of methods

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

The results of this study indicate there is no consistent relationship between the two measures of rabies antibody response to vaccination (SN/RFFIT and indirect ELISA/Bio-Rad kit) either in degree of agreement or in direction of response (one always higher than the other) for individual subjects. Even though there is a larger numerical difference between the values produced by the two assays at day 14 and day 30, there are still individuals with significantly

different responses at day 90 where the primary class of antibody is expected to be IgG, demonstrating that antibody class alone is not the sole source of difference in the measures.

The agreement of the two methods results from all subjects at all time points tested was determined by the kappa test. Using the 0.5 value for both methods gives an agreement value of 94.0%, kappa statistic 0.56 for determination of adequate vaccine response (see Table 4.4). To attempt the establishment of a cut-off level that ensures all "positive" ELISA results include only those subjects with a RVNA level of 0.5 IU/mL or above for measures on day 14, 30, and day 90, a level of 1.0 EU/mL was identified as meeting this criteria. Nonetheless using the 1.0 EU/mL for the ELISA cut-off level and 0.5 IU/mL for RFFIT reduces the overall agreement of results to 81.2%, kappa statistic 0.32 and as a consequence 106 discrepant results, all of which are >/=0.5 IU/mL by RFFIT and <1.0 EU/mL by ELISA. Of these 106, 76% are from day 14 and 30 and 40% are below 1.0 IU/mL by RFFIT. This conservative cut-off level is designed for no overestimation of the neutralizing antibody level by ELISA testing in this set of subjects, for both early and late measures of antibody response and allows the use of the ELISA method as a screening test. Used in this manner, the ELISA would identify adequately vaccinated individuals (individuals that would be expected to have RVNA equal to or above 0.5 IU/mL); and individuals with ELISA results below 1.0 EU/mL would require testing by RFFIT to identify those with levels at or above 0.5 IU/mL. Based on this study, an estimated retest rate would be 18.8% for samples drawn between 14 and 90 days after vaccination. Alternatively, if a less conservative level of 0.25 EU/mL for ELISA is used for comparison of with the 0.5 IU/mL RFFIT level, an overall agreement of results is 96.1%, but it allows 16 discrepant ELISA "positive"/RFFIT "negative" findings, hence labeling those subjects with antibody responses as adequate based solely on antibody binding levels. Use of this cut-off value scheme allows an

improved agreement of the comparison of "percentage above cut-off level" between Groups A and B for the ELISA results, with Group A at 70% and Group B at 100% subjects above cut-off at day 14. If the lower limit of quantitation (LLOQ) for both methods are used as cut-off levels (0.2 IU/mL for RFFIT at KSU and 0.125 EU/mL for the Bio-Rad ELISA), the agreement of methods becomes 97.2% for this set of subjects at time points of days 14, 30, and 90.

If each time point is considered independently for assignment of ELISA result cut-off for best agreement and kappa statistic with the 0.5 IU/mL cut-off for RFFIT results, at day 14 it was 0.25 EU/mL, and at day 90 it was 0.5 EU/mL; whereas on day 30 the use of the LLOQ for both methods as the cut-off value resulted in the highest agreement and kappa statistic values (see Table 4.4). Comparing individual results by the t-test pairwise indicates that only at day 90 was there no significant difference between the measures by RFFIT and ELISA (see Figure 4.5).

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

Cut-off value scheme – RFFIT/ELISA		Day 14	Day 30	Day 90	Overall
0.5 IU/mL / 0.5 EU/mL					
	Agreement %	89.9	95.7	96.3	94.0
	kappa	0.56	0.32	0.68	0.560
0.5 IU/mL / 1.0 EU/mL					
	Agreement %	70.7	81.4	91.5	81.2
	kappa	0.26	0.12	0.61	0.320
0.5 IU/mL / 0.25 EU/mL					
	Agreement %	96.3	99.5	92.6	96.1
	kappa	0.78	0.80	0.12	0.590
0.2 IU/mL / 0.125 EU/mL					
	Agreement %	95.7	100.0	95.7	97.2
	kappa	0.48	1	0.19	0.37
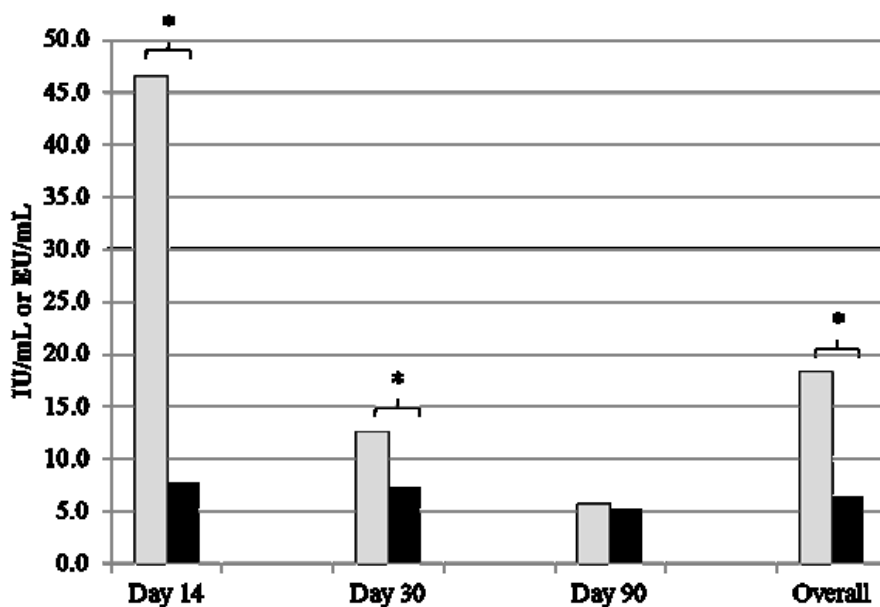
Figure 4.5 Average result values, IU/mL for RFFIT (gray bars) and EU/mL for ELISA (black bars). Significant difference (p<0.05) between method results were noted at days 14 and 30 as well as overall (*).

4.6 **Conclusions**

Rabies vaccine response can be measured by both antigen-binding and serum neutralization methods, but these measures are not the same due to differences in what and how each test measures. It is not expected that RFFIT and ELISA results have a consistent relationship since RFFIT measures neutralizing function of the rabies specific antibodies (IgG and IgM), which is not proportional in a defined degree to the binding ability of the rabies specific IgG antibodies as detected by the Bio-Rad ELISA kit. Assigning the same cut-off level for both test methods will never result in agreement for all individuals. The relationship of RFFIT and ELISA results over various time points post vaccination, as illustrated in this study, demonstrates further that one cut-off is not appropriate, though good agreement can be achieved by assigning logical cut-offs considering time point of blood draw post-vaccination for groups of subjects if not on an individual basis. Evaluating the kinetics of the RVNA/anti-glycoprotein

response, combined with laboratory validation of the specific test method and consideration of the use of the results (vaccine efficacy, determination of booster, detection of vaccine bait-uptake) is a logical approach for setting a useful cut-off for both RFFIT and ELISA methods. The importance of choosing the method 'fit for purpose' AND ensuring that the testing procedure is conducted appropriately with adequate quality assurance procedures in place cannot be overstated for a measure as important as the RVNA level—the most important immune component for the prevention of clinical rabies.

4.7 **Acknowledgements**

4.8 References

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Chapter 5 - The Detection of Antibodies to Vaccine and Field Strains of Rabies Virus in Horses by a Multiplex Microsphere-Based Assay

5.1 Abstract

Rabies is a fatal neurological disease caused by a RNA virus in the family Rhabdoviridae. Prevalence among horses in North America is steadily increasing. The majority of rabies viruses isolated from positive horses in Kansas are skunk strains; however several bat strains have also been identified. The hypothesis that horses vaccinated with laboratory adapted rabies strains have weak antibody titers to virus variants occurring in nature was investigated. A multiplex-bead-based indirect immunoassay was developed to screen sera obtained from vaccinated horses against rabies antigens isolated from seven rabies virus isolates. This multi-analyte technology (xMap) was designed to quantify equine IgG binding to viral antigens derived from the seven different rabies virus strains simultaneously. Characterization of the dominant viral proteins in the antigen preparations was performed by silver stain of SDS-PAGE. Coupling of viral proteins G and N derived from three laboratory rabies strains to three sets of xMag beads was confirmed. The 7-plex set of rabies antigen coated xMap beads was tested against serum samples obtained from18 horses before and after rabies booster vaccination. The results indicate that all horses were able to increase their vaccine response after booster, but a variety of responses were recorded to rabies strains that differ from the vaccine strain.

5.2 Introduction

Rabies is a fatal neurological disease caused by a negative single-stranded RNA virus in the Rhabdoviridae family that is transmissible to all mammals. Although the incidence in horses is low, the disease has public health significance because of the potential for human exposure to the saliva and infected tissues from wildlife and domestic animals. The numbers of exposed

horses are increasing steadily, 44 cases in horses and mules were reported nationally in a surveillance study in 2011, an 18.9% increase compared to 2010 (Blanton, Dyer, McBrayer, & Rupprecht, 2012). Six rabies virus strains have been isolated from wildlife in Kansas, 2 skunk strains, one raccoon, and three bat strains. In Kansas and Nebraska, of the eleven positive horse cases sequenced in 2012, 9 were typed as south central skunk and 2 as north central skunk. In 2011 of the 6 positive horse cases sequenced, 4 were south central skunk, 1 north central skunk and 1 raccoon. Six of the seven cases in 2010 were south central skunk and the remaining case was raccoon.   Although bat strains have been isolated in positive horse cases nationally, it occurs rarely and they were not isolated in horses from 2010 to 2012 in Kansas and Nebraska where three bat virus strains are present including *Eptesicus fuscus* (big brown bat), *Lasiurus cinereus* (hoary bat) and *L. borealis* (eastern red bat). South central skunk is the main rabies strain from North-Central Nebraska to Texas (KSVDL, 2015; Centers for Disease Control and Prevention, 2011). Horses are commonly housed outdoors and are at an increased risk for exposure to rabid wildlife, especially with urbanization of wildlife habitats.

Several lyssavirus genotypes exist and include the classical rabies virus (RABV) with worldwide distribution and others with more restricted distribution and reservoirs such as Australian Bat Lyssavirus (ABLV), European Bat Lyssavirus 1 and 2 (EBLV1, EBLV2), Lagos Bat, Mokola, and Duvenhage. The American Association of Equine Practitioners (AAEP) currently includes rabies as a core vaccine in all equids (http://www.aaep.org/rabies.htm). There are three commercial vaccines approved and licensed by USDA for rabies prophylaxis in horses (EquiRab, Intervet [Merck]; ImRab, Merial; and Rabvac3, Boehringer Ingelheim). Vaccine strains are lab adapted strains of various origins with many related to Pasteur's isolation and culture of a dog strain taken from a rabid cow from Paris, 1882 (Pasteur, Pitman-Moore), but

also includes a dog strain from a rabid human from Georgia, 1939 (Flury LEP) and a rabid dog from Alabama, 1935 (ERA). The rabies strain primarily used in laboratory methods to measure the immune response to vaccination is the Challenge Virus Strain-11 (CVS-11), which shares the highest degree of homology with the Pitman-Moore (PM) strain. All the vaccine strains and CVS-11 have been passaged thousands of times in rabbit or mouse brain and/or cell culture adapting them to production (Tordo, 1996). Vaccine strains have been proven to elicit cross-protection of strains with genotype 1, reduced protection with genotypes 2-5, and no protection with genotype 6 and 7(Smith, 1991). Glycoprotein (G) is an immunodominant protein, is less conserved between strains than the nucleoprotein (N) protein, and is responsible for the receptor binding that leads to infection. One of the two most immunogenic sites on the G protein (site III) is also the most critical site for determination of pathogenicity (Tordo, 1996; Benmansour et al., 1991; Johnson, McElhinney, Smith, Lowings, & Fooks, 2002). Response to vaccination is expected, based on titer testing of monoclonal antibodies, to be affected by antigenic differences between viruses (Benmansour et al., 1991). Several reports document variable reactivity of monoclonal antibodies with different vaccine and wild type rabies strains (Horton et al., 2010; Marissen et al., 2005; Sloan et al., 2007).

Current diagnostic methods to detect humoral immunity in horses and other domestic species consists of enzyme linked immunosorbent assays (ELISA) to detect anti-rabies glycoprotein binding antibodies or rabies virus neutralizing antibody (RVNA) assays to detect antibody that neutralizes virus and prevents infection of cultured cells. The virus strains used in these assays as the antigen are lab-adapted strains vaccine strains (or closely related). Microsphere-based immunoassays provide a new methodology capable of detecting multiple analytes (50 -100) simultaneously in a 50 to 96-well format (Kellar, Mahmutovic, &

Bandyopadhyay, 2006; Vignali, 2000). This technology (Luminex and Magpix) is widely

commercially available for human samples to measure cytokines, antibodies, tumor markers,

hormones, and many other analytes. The technology utilizes unique sets of magnetic

microscopes internally dyed with different fluorescent intensities that have unique spectral

properties separated by fluorescent emissions. Proteins, oligonucleotides, lipids,

polysaccharides, or small peptides can be absorbed or chemically coupled to the microspheres to

capture analytes that are measured by a fluorochrome-conjugated detection system. The power

of the technology and advantage over traditional immunoassays (i.e. ELISA) is that the analytes

can be multiplexed and analyzed in the same sample of smaller sample volume with more

reproducibility and less preparation than traditional ELISA assays (Dasso, Lee, Bach, & Mage,

2002). The automated systems (Luminex and Magpix) provide a high throughput platform and

recently have fully customizable bead sets for development. In veterinary research, multiplex

assays are beginning to be developed and implemented. Multiplex microsphere-based assays

have been tested and validated to measure cytokines in horses and cats (Wagner & Freer, 2009;

Wood, O'Halloran, & Vandewoude, 2011). Similar multiplex systems have been used to screen

antibodies to viruses in pigs and to quantitate immunoglobulins in human sera (Dasso et al.,

2002; Lin, Wang, Murtaugh, & Ramamoorthy, 2011). It is our working hypothesis that horses

vaccinated with the commercial rabies vaccine strain (PV) have weak antibody titers

(concentration) to other virus variants that occur in nature. Our objective is to determine the

antibody response using a novel multiplex immunoassay in vaccinated horses to rabies virus

variants including north central skunk, south central skunk, raccoon, and bat strains. The results

from the multiplex assay will be compared with traditional ELISA and neutralizing antibody

assays. The findings from this study will have important implications in the current vaccine

practices, particularly if the vaccine strain does not invoke strong antibody responses to terrestrial rabies virus strains.

To accomplish this objective, a multiplex-microsphere based assay was developed to quantify immunoglobulin G specific to the various rabies strains in horses vaccinated against rabies. The multiplex microsphere-based assay uses magnetic beads (up to 50 sets) that have a unique spectral profile. Through a chemical reaction vaccine proteins can be attached to each set. Protein-coated beads are then incubated with patient serum to allow for antibody binding. A fluorescent strept-avidin detection conjugate is added; and a biotinylated secondary species specific (equine) antibody is used to complete the reaction. The goals were to determine the antibody response in horses before and after vaccination, and to determine how well the rabies vaccine booster stimulates a humoral immune response to the vaccine strain and the terrestrial and bat rabies virus variants. The 7-plex assay was used to compare pre- and post-vaccinated antibody concentrations. Antibody concentrations were compared to a single-plex ELISA and RVNA assay.

5.3 **Materials & Methods**

5.3.1 *Horse serum samples*

Ten horses from the Kansas State University herds and eight privately owned horses were enrolled in the study. Thirteen of the 18 horses had a history of prior rabies vaccination; the remainder had unknown rabies vaccine history. All horses were rabies vaccinated (Equirab, Merck) at time of enrollment. Blood samples were drawn on the day of rabies vaccination and again 14 days after. Blood samples were allowed to clot and centrifuged to obtain the serum samples. The serum samples were aliquoted in 2 mL volumes into freezer vials and stored at -20°C until tested.

5.3.2 *Antigens*

The rabies viruses of the laboratory adapted strains (Challenge Virus Strain-11 [CVS-11], Flury-Low Egg Passage [Flury-LEP], and Evelyn-Rokitnicki-Abelseth [ERA]) were propagated in BHK-21 cells (WHO Laboratory Techniques in Rabies, 1996). The rabies viruses of the wild type strains (Eastern Pipistrelle, Tadarida, South Central Skunk and North Central Skunk) were adapted to MNA cell culture by initial inoculation of the cells with rabies-infected brain tissue taken from confirmed rabies positive animals by the Direct Fluorescent Antibody (DFA) test. The rabies virus in the brain tissues was genotyped by PCR/sequencing. The infected cells were passaged until an adequate viral titer was obtained in the supernatant (between passage 6 and 9) to produce enough virus for the bead assay. Identity of the cell passaged viruses was verified by N and G sequencing. The harvested supernatants were centrifuged for 30 minutes at 5000 g to remove cellular debris and filtered through a 0.22 μm PES filter. The virus stocks were inactivated by mixture with β propiolactone (Sigma Chemical Company, St. Louis, MO) at a ratio of 1:4000 and incubated at 37C for 2 hours, shaking very 10-15 minutes. Complete inactivation was determined by serial passage of MNA or BHK cells mixed with 0.5 mL of the inactivated virus suspension and microscopic examination on each day of passage for virus infected cells using fluorescent conjugated anti-rabies antibodies. No virus was detected in the cultured cells. The inactivated viruses were concentrated (approximately 100X) with PEG10 (PEG Virus Precipitation Kit, Abcam, Cambridge, MA). Each viral suspension was measured for protein (Pierce^{TM} BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA). The presence of rabies glycoprotein (G) in the virus preparations was verified by a silver stain of a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel.

5.3.3 *Coupling of rabies virus antigens and anti-IgG to beads*

Coupling of the antigen to the microspheres was achieved through a generic two-step carbodiimide coupling with sulfo-N-hydroxysulfosuccinimide (NHS) and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) (xMap kit, Luminex) according to the manufacturer's instructions. Rabies whole virus was used as the antigen, produced as described above; 5 µg of each virus preparation was covalently coupled to 1.0×10^6 carboxylated paramagnetic microspheres (beads) (MagPlex microspheres, Luminex, Austin, Texas). Confirmation of the binding was determined by testing the coupled beads with mouse anti-rabies glycoprotein, (clone 0.N.541, 1:500 in PBS-1%Tween, Abcam, Cambridge, MA) and mouse anti-rabies nucleoprotein (clone C18-62-143-2, 1:500 in PBS-1% Tween, Light Diagnostics/Millipore, Darmstadt, Germany), and detected with R-PE-goat anti-mouse IgG (H+L) 2 µg/mL (Columbia Biosciences, Frederick Maryland) http://stores.columbiabiosciences.com/goat-anti-mouse-h-l-surelight-r-pe /.

5.3.4 *Bead-based serology*

The assay was performed on a MAGPIX instrument (Luminex). Pilot experiments were performed to determine an optimal serum dilution and detection system concentrations for the measurement of equine antibody specific for the rabies virus strains. For each assay an equal volume (calculated volume dependent upon the number of tests to be performed so that there was at least 100 beads for each set) beads with a specific rabies virus strain were aliquoted to each well. Each test serum was serially diluted (1:125, 1:500, 1:2000, and 1:8000). For the assay, 50 µL of each serum dilution was combined with 50 µL of the bead mix (2.5×10^6 beads/ mL) in wells of a 96 well reaction plate, incubated overnight in the dark at 4 º C on a shaker (200 rpm). After incubation, the contents of the well were washed with PBS-1% Tween twice and

resuspended in 50 µL PBS-1% Tween with aid of a magnetic plate separator per the kit

instructions. Then 50 µL of the detection antibody, biotinylated goat anti-equine IgG (heavy and

light chain) (Jackson ImmunoResearch) at a 2 µg/mL concentration was added to each well,

incubated for 60 minutes at room temperature on a shaker followed by a wash as described

above. Phycoerythrin-strepavidin (50 µL) (BioLegend, 4 µg/mL, in PBS) was added to each

well, the plate was covered and incubated for one hour on a plate rotator (250 rpm) at room. For

each sample 50-75 µL was analyzed on the Luminex MagPix instrument (xPonent, Luminex) to

obtain the mean fluorescence intensity. The optimal serum dilution was determined to be 1:2000.

5.3.5 *ELISA assay*

The serum samples were analyzed with the Bio-Rad Rabies Platelia Kit II (Bio-Rad,

Marnes-la-Coquette, France) an indirect ELISA method. The Bio-Rad Evolis instrument was

utilized for the test performance per the manufacturer's instructions. The kit contains strips of

wells coated with rabies glycoprotein (G protein of the lab-adapted Pasteur Virus [PV] strain) for

use as the antigen. The secondary (detection) system was an enzyme conjugated Staphylococcus

aureus protein A/substrate color reaction. The results were reported in EU/mL (anti-rabies

glycoprotein level) calculated by comparison of the sample optical density reading against a

standard curve of positive standards supplied in the kit.

5.3.6 *RFFIT assay*

The serum samples were tested in the RFFIT assay as previously described (Velleca &

Forrester, 1981). The established RFFIT assay uses CVS-11 as the challenge virus strain. For the

modified RFFIT assays, the following strains were used: Eastern Pipistrelle, Tadarida, and Flury

LEP. RVNA titer values were standardized to IU/mL values by comparison with the Standard

Rabies Immune Globulin (SRIG) (WHO 1st international RIG/Lot R-3 FDA/CBER).

5.3.7 *Statistical analysis*

After serological testing was completed, the MFI (bead assay) IU/mL (RFFIT assay) and EU/mL (ELISA assay) results were analyzed for comparison of the assays. Result comparison by assay and by time point was performed by ANOVA (Analyse-It Software). Method comparison tests (Kappa Analyse-it Software) were used to compare the multiplex bead assay with the single-plex ELISA and RFFIT assays and to compare the MapPix results between rabies strains (Passing-Bablok fit, Analyse-it Software). Assay precision over a range of analytic concentration of equine IgG was determined. The coefficient of variation was calculated for 4 replicate tests of three samples tested in the same assays for intra-assay precision and eight samples tested in 2 independent assays for inter-assay precision analysis.

5.4 **Results**

5.4.1 *Silver Stain of SDS-PAGE*

The SDS-PAGE gel was silver stained to identify the presence of the glycoprotein in the rabies antigen preparations. The gel (see Figure 5-1) indicates bands at molecular weight 65 consistent with the G protein, the immunodominant protein of the rabies virus.
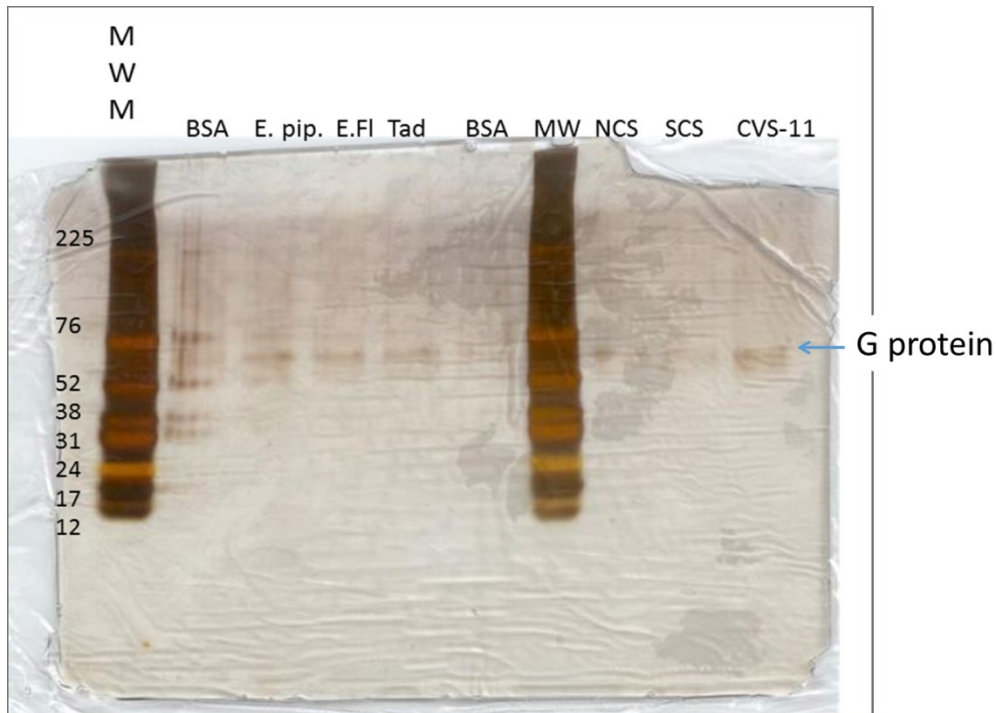
Figure 5.1 Silver stain of SDS-PAGE to identify the glycoprotein in the rabies virus antigen preparations.

5.4.2 *Confirmation of microsphere antigen coating*

Confirmation of G and N protein from the rabies virus strains binding to the microspheres by MagPix assay using the coupled beads and monoclonal antibodies to G and N proteins, both monoplex and triplex (performed in duplicate), indicated that both proteins were coupled to the beads and that the presence of the other bead sets in the triplex did not interfere with the binding of antibody to the proteins. The mean fluorescent intensities (MFIs) for each protein were similar in the monoplex and the triplex formats demonstrating compatibility for the multiplex format (see Table 5-1).

Table 5.1. MFI readings of monoclonal anti-rabies G and N antibodies against rabies antigen coated beads tested in monoplex and triplex assays.

	Antigen		
	CVS-11	ERA	Flury
Monoplex Anti-G	78 MFI	53 MFI	41 MFI
Monoplex Anti-N	88 MFI	38 MFI	40 MFI
Triplex Anti-G	81 MFI	52 MFI	40 MFI
Triplex Anti-N	67 MFI	32 MFI	33 MFI

5.4.3 *MagPix Assay*

The optimal sample dilution was determined by evaluating the ability to detect antibody levels across the range of samples. As seen in Figure 5-2, the 1:2000 serum dilution was better able to distinguish differences in the specificities of the antibodies than 1:500 for three horses with antibody levels in the high, moderate and low range of the assay. The precision of the Mean Fluorescent Intensity (MFI) readings in MagPix assays was determined to range from 2.7% to 12.2% for intra-assay and from 0.0% to 29.9% for inter-assay (see Table 5-2) for two independent assays. It was noted that, as expected, the lowest precision was seen in the samples with the lowest MFI readings. Also, noted a third independent assay results were outside this precision range.
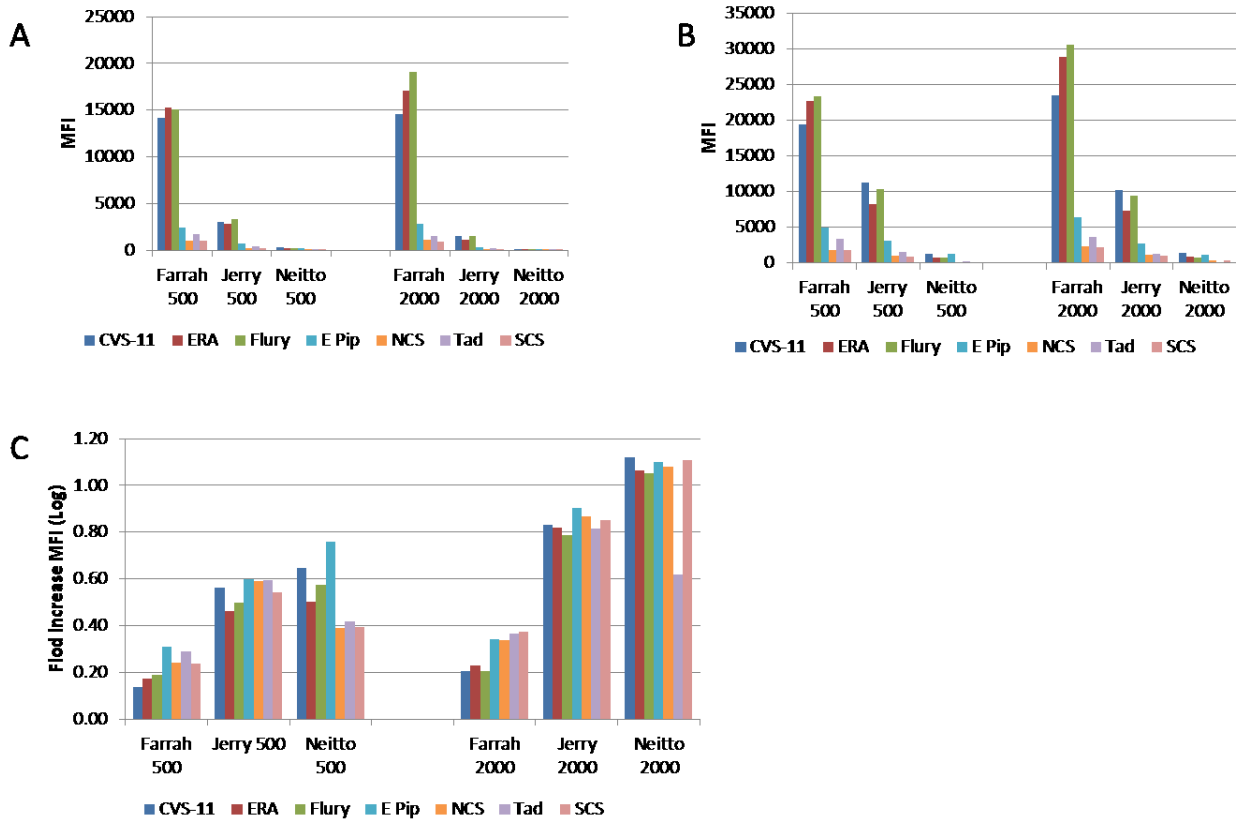
Figure 5.2. MFI readings for three horses (Farrah, Jerry, and Neitto) pre-booster (panel A) and post-booster (panel B) samples; and the fold increase MFI (Log) for each (panel C).

The MFI readings for each horse serum sample (pre and post booster vaccination) for the microspheres coupled with the wild type rabies strains (Eastern Pipistrelle, North Central Skunk, Tadarida, and South Central Skunk) and the lab-adapted rabies strains (ERA, Flury-LEP, and CVS-11) varied by rabies strain and by individual horse (see Figure 5-3). The post booster antibody responses as measured against the wild type bat and skunk strains in the MagPix assays were lower (5-10 fold less) compared to the lab-adapted strains (see Figure 5-3, panel B). One horse (Neitto) was found to have a very low antibody response to all the strains in both its pre and post serum sample (see Figure 5-3, panels A and B). Examination of the fold-increase in MFI between pre and post booster serum samples indicates a non-uniform pattern in the

responses to the different strains. Just over half of the subjects (10 horses or 64%) had the

highest fold increase in response (MFI) to the lab adapted/vaccine strains; the fold increase was

highest to ERA strain in most of those (7 horses). Of the horses with their highest rise in rabies

antibody response specific for the wild type strains, no one strain was prevalent. (See Figure 5-

4). Of the horses with the highest fold antibody level increases to rabies antigens overall (greater

than 2-fold rise), 75% had their highest fold increase to the lab-adapted/vaccine strains.

Table 5.2. Intra- and inter-assay precision of MFI (log transformed) readings.

|	Sample	CVS-11	ERA	Flury-LEP	E. Pip.	NCS	Tad.	SCS
Intra-assay precision (CV%)	Luke Pre 1:2000	2.9	3.7	3.1	4.0	6.6	7.6	7.0
	Reggie Pre 1:2000	5.3	7.4	6.7	3.3	5.2	2.7	5.4
	Neitto Pre 1:2000	12.2	11.7	12.0	2.7	5.8	5.6	5.2
Inter-assay precision (CV%)	Luke Pre 1:2000	4.4	3.5	2.9	1.0	4.8	3.9	4.7
	Luke Post 1:2000	6.3	5.9	5.3	3.0	7.5	14.3	7.8
	Reggie Pre 1:2000	1.2	1.0	1.9	16.0	4.0	13.9	6.7
	Reggie Post 1:2000	0.1	0.3	1.1	6.8	1.3	3.3	2.1
	Neitto Pre 1:2000	11.1	15.0	14.3	29.0	27.4	27.4	29.9
	Neitto Post 1:2000	0.6	2.9	2.1	3.5	3.1	12.0	2.8
	Janus Pre 1:2000	4.1	5.2	5.9	14.2	8.6	11.0	10.5
	Janus Post 1:2000	0.5	0.3	0.3	8.5	0.4	1.3	0.0

A
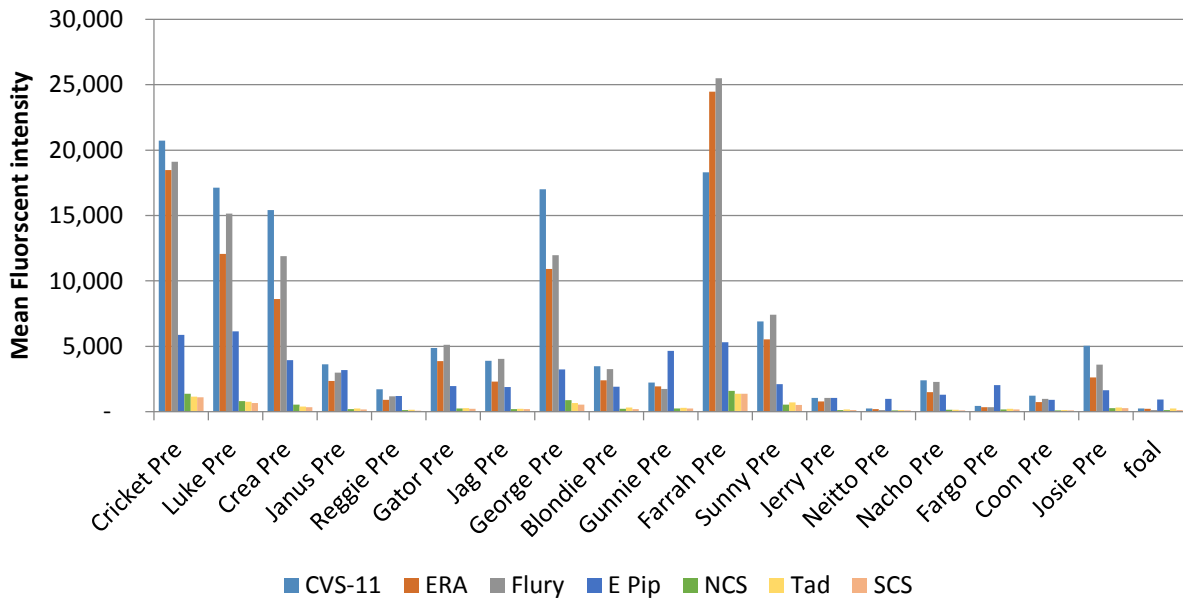
B
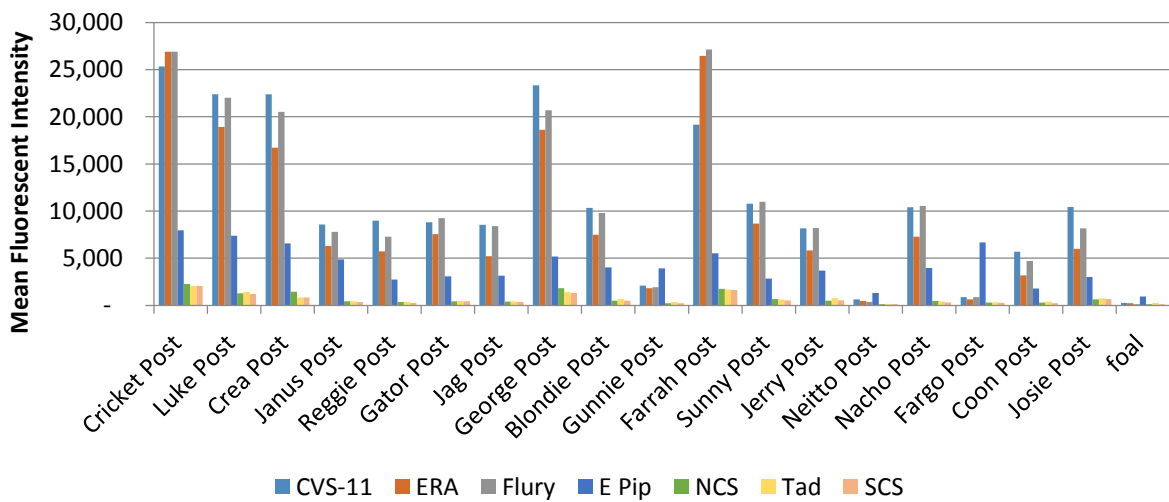
Figure 5.3. Mean fluorescent intensity readings of the microspheres coated with different rabies strains (CVS-11, ERA, Flury-LEP, Eastern Pipistrelle, North Central Skunk, Tadarida, and South Central Skunk) tested against the horse sera from 18 horses drawn pre (A) and post (B) rabies vaccination.
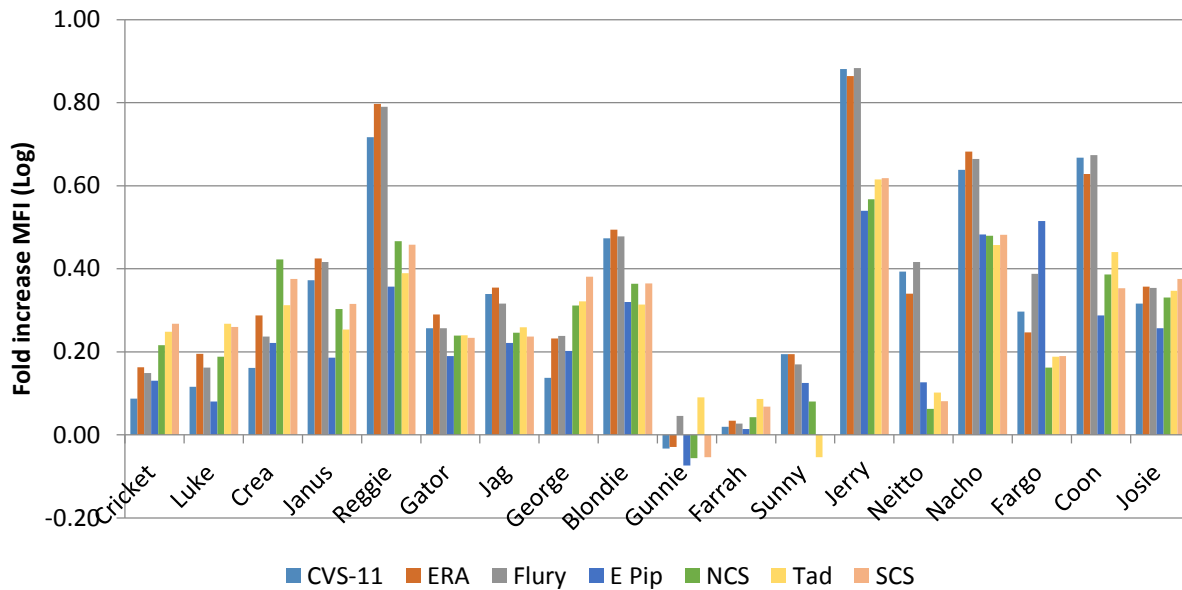
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Figure 5.4. The fold-increase (log), defined as the post booster MFI measurement divided by the pre booster MFI measurement , for each Magpix assay.

5.4.4 *Assay comparison*

The assays were compared by the ability to detect specific antibody response to rabies vaccination; both the individual measurements and the fold increase values were analyzed. Table 5-3 displays the results of the MagPix assay (CVS-11, ERA, and Flury-LEP as the target antigens), RFFIT results (CVS-11 as the challenge virus strain), and ELISA (PV as the target antigen). For most horses all three assays were able to detect rise in antibody response (89% of the horses had a 10% or greater rise in response by all the assays); for 8 of the 18 horses the RFFIT measurement fold-increase was highest, for 7 horses the ELISA measurement fold increase was highest, and for 3 horses the MagPix measurement fold increase was highest (see Figure 5-5). Of the horses with the highest fold antibody response increase across the different assays, the RFFIT fold increase was the highest.

The multiplex MagPix assay results are reported in MFI and were not converted to standardized units as is done with the RFFIT and the BioRad ELISA antibody measurements.

The results for each assay were categorized as positive or negative by the following cut-off

levels 0.5 IU/mL for RFFIT (as recognized by WHO and OIE for indication of adequate

response to vaccination), 0.125 EU/mL for ELISA (per the ELISA kit instructions) and MFI 332

(the mean of all background, and rabies antibody negative control sample MFI readings plus 3

standard deviations). Using the kappa test for the analysis of agreement between the assays

(RFFIT, ELISA, and MagPix with CVS-11 as the antigen), categorizing the results as positive or

negative, produced the following results: RFFIT versus ELISA had a kappa statistic 0.72

(p<0.0001), RFFIT versus MagPix had a kappa statistic 0.48 (p=0.0008), while the ELISA

versus MagPix comparison produced a kappa statistic of 0.30 (p=0.0116).

Table 5.3. Assay results (RFFIT in IU/mL, ELISA in EU/mL, and MagPix in MFI) for the horse serum samples drawn pre- and post-booster vaccination.

| | | Assay and Rabies Virus Strain (Antigen) | | | | |
| | | RFFIT | ELISA | | MagPix | |
ID	Time-point	CVS-11	PV	CVS-11	ERA	Flury-LEP
Cricket	pre-booster	0.1	0.0625	20,717	18,477	19,112
Cricket	post-booster	100.0	18.261	25,344	26,884	26,909
Luke	pre-booster	22.0	1.549	17,137	12,060	15,158
Luke	post-booster	93.0	4.192	22,371	18,907	21,997
Crea	pre-booster	11.9	0.671	15,416	8,626	11,894
Crea	post-booster	125.0	3.639	22,368	16,733	20,512
Janus	pre-booster	16.0	1.835	3,641	2,369	2,985
Janus	post-booster	28.0	3.252	8,585	6,302	7,779
Reggie	pre-booster	0.1	0.0625	1,723	913	1,183
Reggie	post-booster	5.8	0.439	8,972	5,722	7,293
Gator	pre-booster	8.0	1.625	4,884	3,877	5,119
Gator	post-booster	22.0	2.971	8,816	7,556	9,243
Jag	pre-booster	100.0	3.618	3,905	2,305	4,056
Jag	post-booster	95.0	15.133	8,534	5,215	8,396
George	pre-booster	68.0	8.006	17,005	10,916	11,966
George	post-booster	113.0	14.722	23,346	18,620	20,699
Blondie	pre-booster	24.0	2.888	3,472	2,396	3,263
Blondie	post-booster	37.0	3.208	10,326	7,477	9,809
Gunnie	pre-booster	6.0	0.623	2,246	1,943	1,741
Gunnie	post-booster	26.0	3.778	2,084	1,817	1,934
Farrah	pre-booster	20.0	3.253	18,305	24,461	25,479
Farrah	post-booster	37.0	12.296	19,153	26,447	27,123
Sunny	pre-booster	26.0	3.899	6,905	5,539	7,425
Sunny	post-booster	29.0	7.067	10,796	8,665	10,970
Jerry	pre-booster	11.9	1.698	1,073	799	1,072
Jerry	post-booster	21.0	10.008	8,162	5,836	8,191
Neitto	pre-booster	0.1	0.0625	253	214	143
Neitto	post-booster	0.5	0.0625	626	469	373
Nacho	pre-booster	0.5	0.0625	2,396	1,513	2,282
Nacho	post-booster	107.0	3.635	10,412	7,272	10,529
Fargo	pre-booster	12.3	0.939	444	360	362
Fargo	post-booster	120.0	17.529	879	635	885
Coon	pre-booster	2.9	0.899	1,228	752	999
Coon	post-booster	93.0	5.189	5,706	3,195	4,715
Josie	pre-booster	105.0	7.435	5,038	2,634	3,610
Josie	post-booster	120.0	17.315	10,435	5,988	8,156
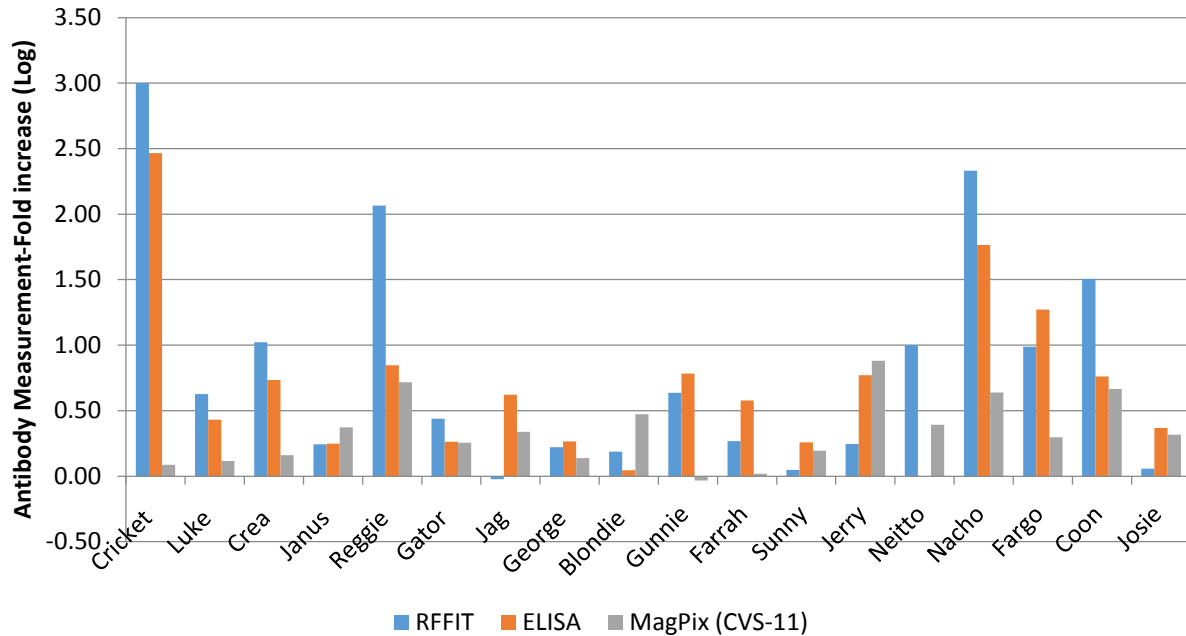
Figure 5.5. The fold increase (log) in antibody measurements for RFFIT (IU/mL), ELISA (EU/mL), and MagPix (MFI), calculated for each horse by dividing the post-vaccination result by the pre-vaccination result.

Taking all the assay results into consideration (MagPix with all 7 strains, RFFIT, and ELISA), there is significant difference between pre-booster and post-booster results (MFI, IU/mL and EU/mL, respectively) and between assays, $p<0.0001$ (2-way ANOVA, Analyse-It Software). No significant difference was found between MagPix results (MFI) of CVS-11, ERA, and Flury-LEP assays comparing all measurements (pre and post booster vaccination), $p=0.6396$, though a significant difference between the antibody measurements of all samples with wild-type strain MagPix assays was found, $p=0.0001$ (1-way ANOVA analysis, Analyse-It Software). By pairwise (Tukey test at 95% confidence interval) analysis, a significant difference in the results between the Eastern Pipistrelle assay and each of the North Central Skunk, Tadarida, and the South Central Skunk assays was found. No differences between the assays North Central Skunk

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versus Tadarida, North Central Skunk versus South Central Skunk, and Tadarida versus South Central Skunk pairwise comparisons were determined.

Passing and Bablok comparisons between the antibody measurements (MFI) of all samples (pre and post booster vaccination) of selected pairs of MagPix assays were performed designating the Mag-Pix CVS-11 as the reference assay (see Figure 5-6, panels A to D). The best fits obtained were between the lab adapted strains assays. Contrasts between lab-adapted strains (ERA and Flury-LEP) and the wild-type strains (E. Pipistrelle and Tadarida, North Central Skunk and Tadarida) indicate that though the fit between all lab-adapted strains is better than between lab-adapted and wild-type (CVS-11 versus Easter Pipistrelle and versus Tadarida), the fit between wild type strains is more varied (see Figure 5-6, panels E, F and G).
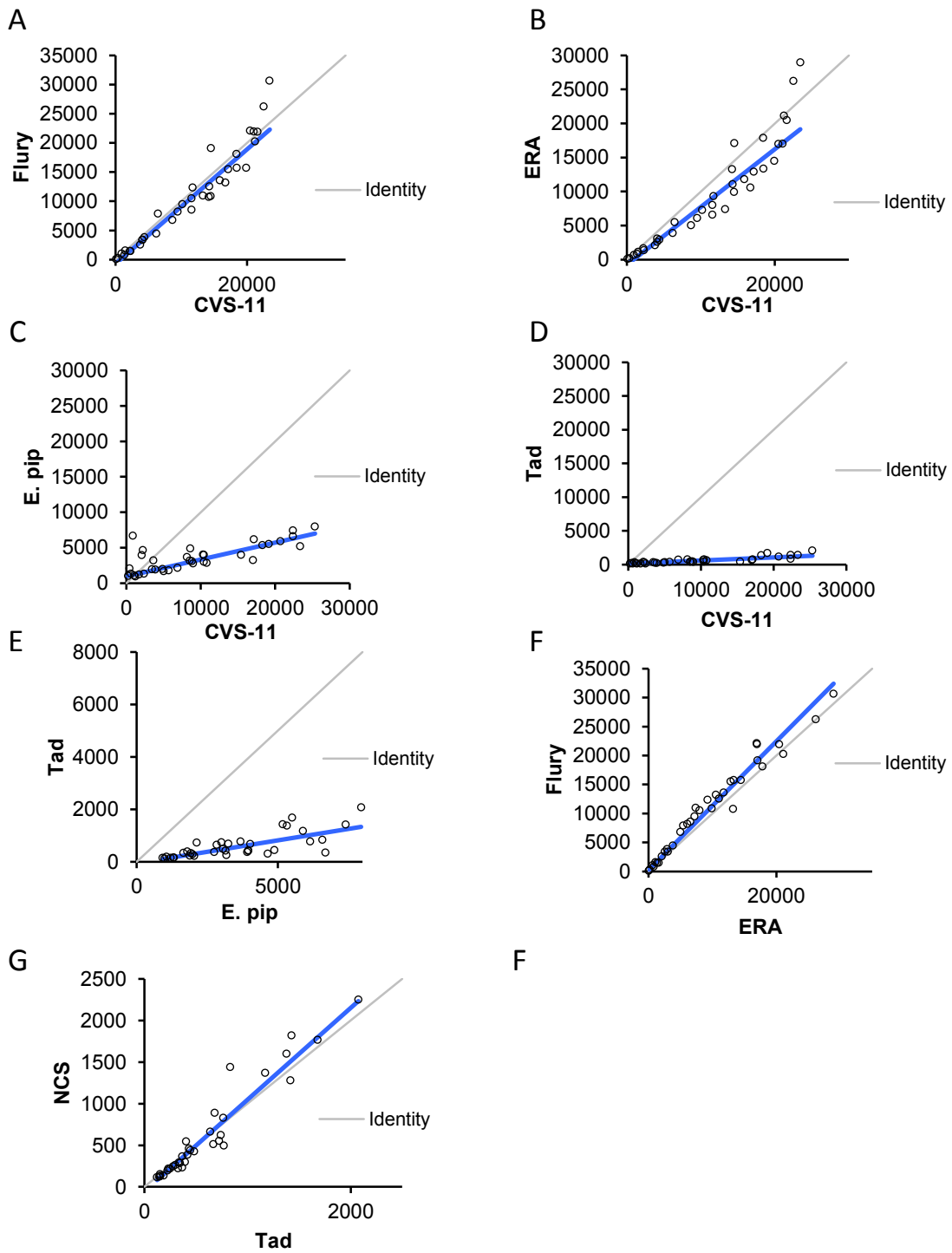
Figure 5.6. Scatter plot with Passing & Bablok fit of MFI measurements of selected MagPix assays.

5.4.5 *Comparison of strain difference in neutralizing antibody measurement*

The paired samples (pre and post booster vaccination) from six horses were evaluated for RVNA level in modified RFFIT assays using one lab-adapted, vaccine strain and two wild-type strains in place of CVS-11 as the challenge virus. The results obtained were compared against the results (IU/mL) obtained for the established RFFIT assays using CVS-11 for differences or trends between viruses, between horses, and between assays. In the modified RFFIT assays, though all horses but one (Crea), in both pre and post booster samples, had highest IU/mL results to the Flury-LEP strain and all except one (Josie) had lowest IU/mL results to Tadarida (see Table 5-4), the fold increase in results were quite varied. No established pattern of strain with the highest or lowest rise in RVNA was identified (see Figure 5-7). In addition, evaluation for trends between the modified RFFIT assay results and the MagPix assay results with the different rabies virus strains demonstrated that for some horses the relationship of the log transformed fold increase (highest to lowest responses to the strains) in the two assay types were different, see Figure 5-8, Luke and Crea; while for other horses similar fold increases were measured for the strains in both assays, see Figure 5-8, Neitto and Josie.

Table 5.4. RVNA (IU/mL) results obtained from modified RFFIT assays using different rabies strains as the challenge virus for six paired horse samples.

| ID	Time-point	RFFIT CVS-11	modified RFFIT/Challenge Virus		
			Flury-LEP	E. Pipistrelle	Tadarida
Cricket	pre-booster	0.1	0.4	1.0	0.1
Cricket	post-booster	100.0	390.9	252.9	38.2
Josie	pre-booster	105.0	467.5	163.0	65.4
Josie	post-booster	120.0	955.9	598.1	250.0
Crea	pre-booster	11.9	16.4	19.3	7.7
Crea	post-booster	125.0	93.5	64.5	22.4
Gator	pre-booster	8.0	73.2	34.4	10.7
Gator	post-booster	22.0	61.2	40.9	13.1
Neitto	pre-booster	0.1	0.1	0.1	0.1
Neitto	post-booster	0.5	3.4	2.5	0.5
Luke	pre-booster	22.0	38.2	37.9	13.6
Luke	post-booster	93.0	109.9	82.5	62.7
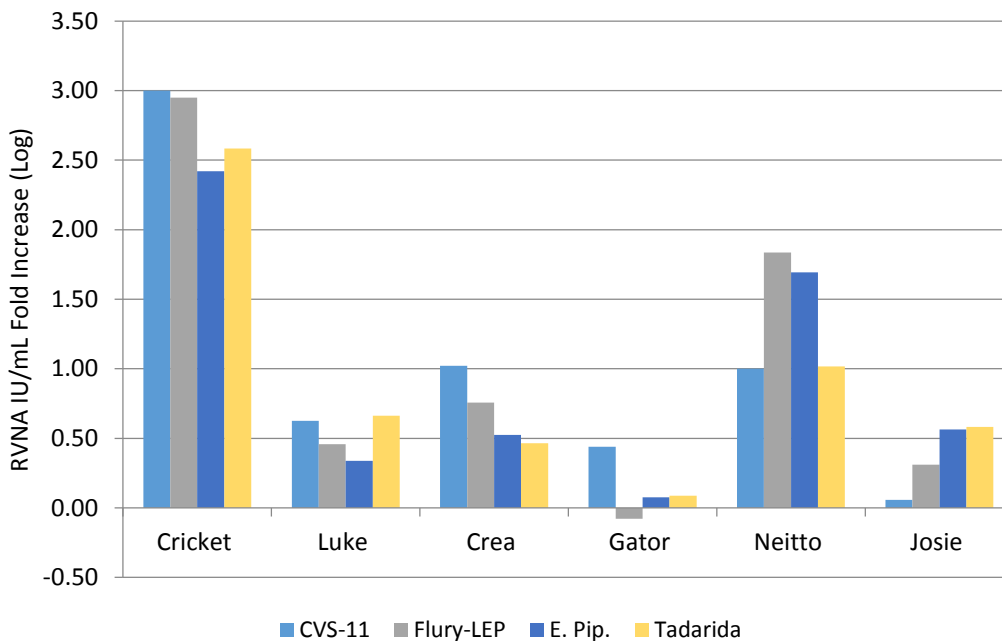
Figure 5.7. Fold increase in IU/mL results in modified RFFIT assays using different rabies strains as the challenge virus.
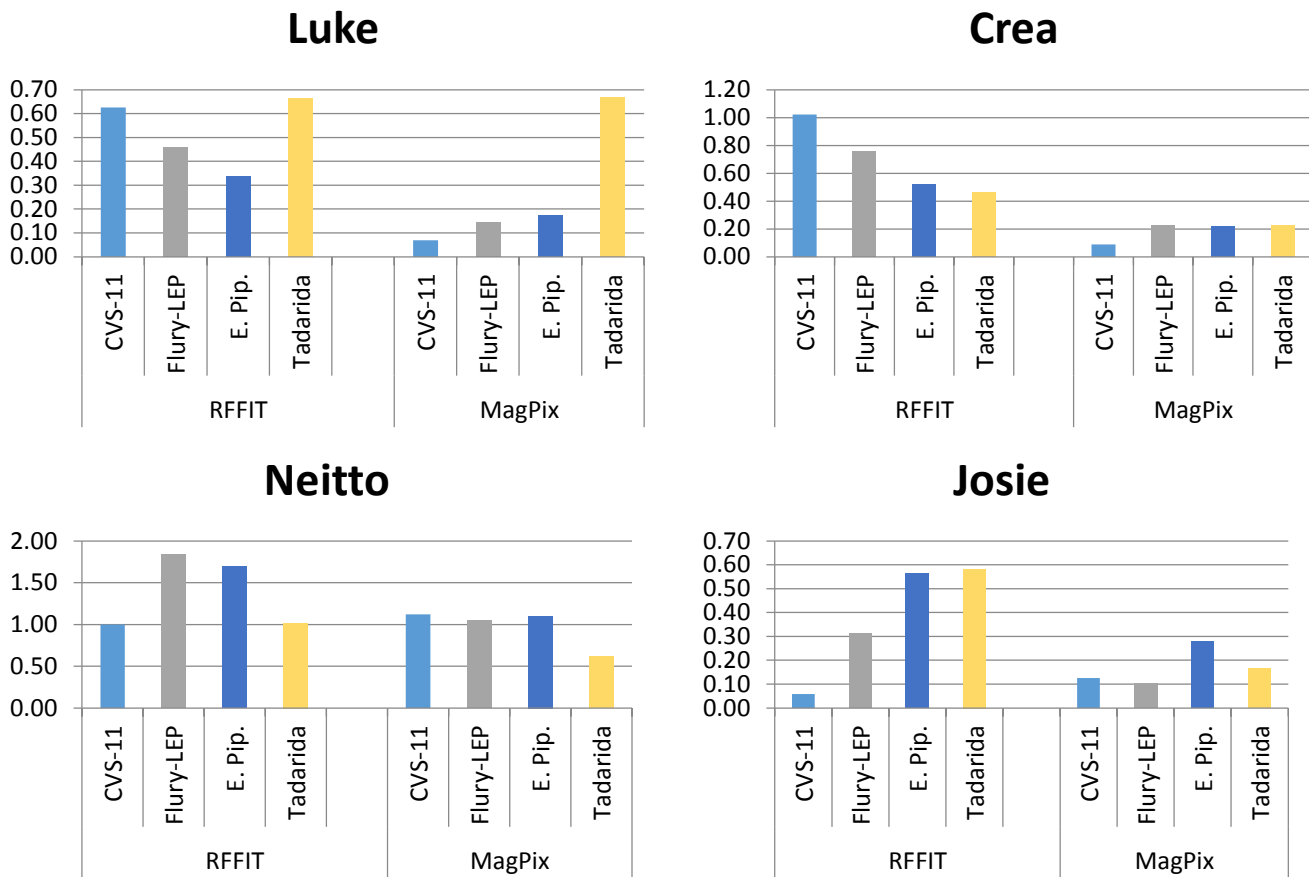
Figure 5.8. Fold increase in IU/mL (RFFIT) and MFI (MagPix) after log transformation for different rabies strains as the challenge virus or antigen, respectively.

5.5 **Discussion**

Horses, as are all mammals, are susceptible to rabies. Although the number of rabies cases in horses in the United States is low, the potential for human exposure from a rabid horse makes rabies vaccination a recommended precaution for all horses. Control of rabies in domestic animals by rabies vaccination coverage has resulted in the majority of reported rabies cases in the U.S. to be found in wildlife species. The incidence of rabies in domestic species follows that of wildlife species; when there are high numbers of rabies in wildlife there is greater

probability of rabies in domestic species. The majority of horses live outside exposed to wildlife species. Horses are naturally curious, investigating wildlife in their surroundings. Rabies vaccination produces a polyclonal response known to neutralize *in vitro* and protect against *in vivo* the different rabies virus strains within rabies geneotype 1 (classical rabies), including variants found in wildlife within the United States (Lodmell, Smith, Esposito, & Ewalt, 1995). Even so, a dissimilarity in antibody response and effective protection between species, mice and guinea pig, given a standard rabies vaccine and challenged with the street rabies virus variants has been demonstrated (Wiktor, 1985). Also, a variation immune response conferred by two rabies vaccine parent strains, PM and ERA, was found in mice after challenge with a Polish Bat strain of the rabies virus (Dietzschold, Tollis, Rupprecht, Celis, & Koprowski, 1987). Though these experimental findings indicate a potential variance in protection afforded by vaccine strains to street (or wild-type) strains no evidence exists for true vaccine failures (Smith, 2002). For rabies biologics, the ability to provide/stimulate effective immunity to cover all rabies virus variants present in the environment for the target species is paramount.

Measurement of the immune response to vaccination can be undertaken by various laboratory techniques. The use of microarray assays is particularly attractive to investigate antibody responses to multiple antigens because of the reduction of sample volume and time requirements compared to conventional antigen-antibody assays. Microarray assays such as bead-based multiplex assays have been used for serology for various infectious agents (Andrade et al., 2014; Michel, Pawlita, Boeing, Gissmann, & Waterboer, 2014). In this study, the utility of a microarray (MagPix) assay for investigation of specific antibody response to rabies virus variants, both vaccine (lab adapted) and wild-type strains as generated by routine rabies booster vaccination in horses was evaluated and compared to established procedures, RFFIT and ELISA.

The ability of the MagPix assay to detect a rise in specific antibody response to both the vaccine strains and the wild-type strains was demonstrated. The horses were given a booster vaccination with a vaccine strain (PV) closely related to ERA (see Figure 5-9).
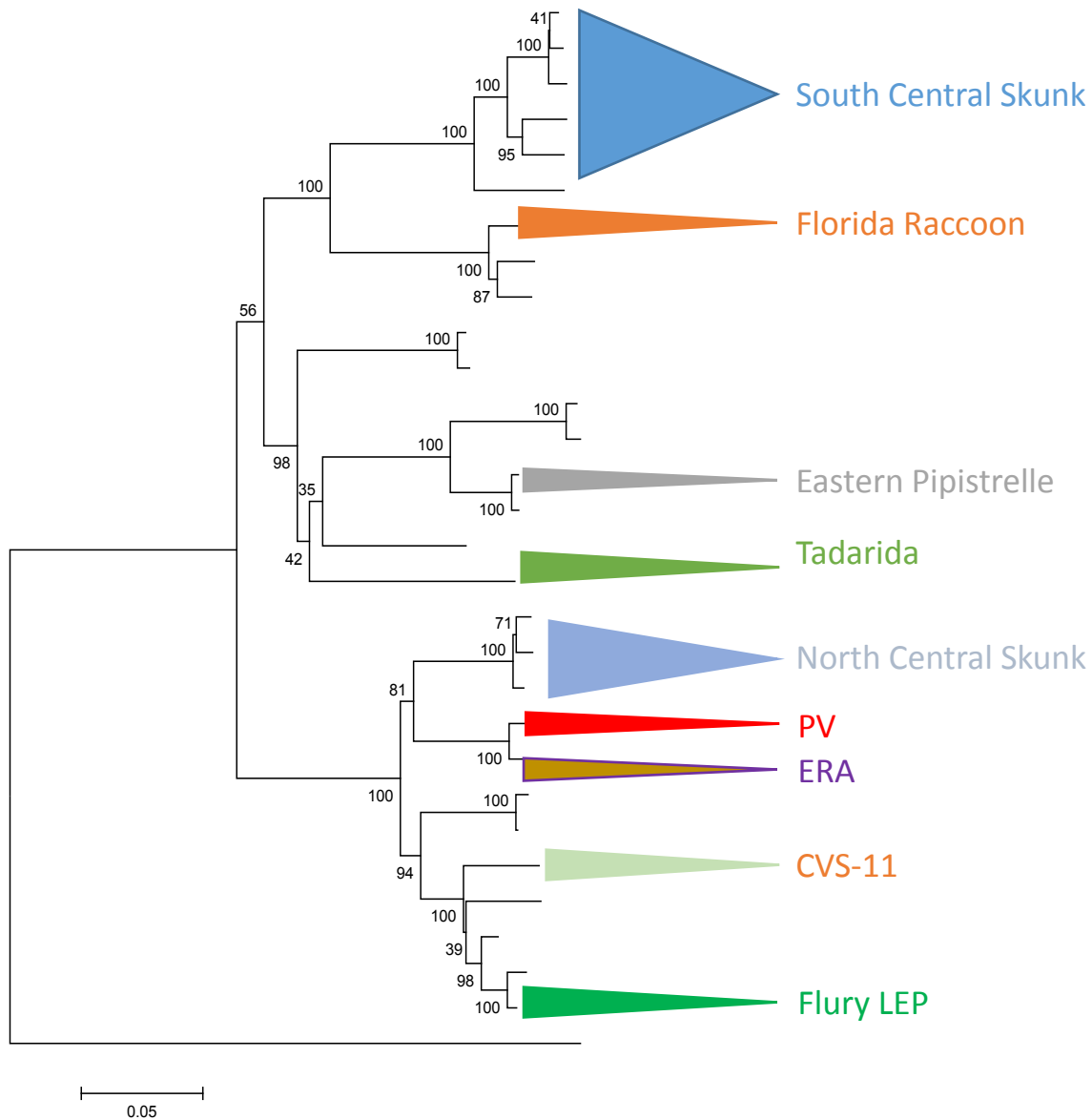

Figure 5-9. A phylogenetic tree of the G protein homology relationships between the vaccine/lab adapted and wild-type rabies virus strains.

The G protein relationships do not entirely explain the variations and magnitude of the antibody responses measured against the virus strains among all horses in the MagPix assays.

Clearly there was a lower response against the wild-type strains than the lab adapted strains in this assay. It has been found that pathogenic (wild-type) strains differ from apathogenic or attenuated strains in the expression of glycoprotein; pathogenic strains express less G protein than attenuated strains which allows the pathogenic strains to effectively spread through synaptic junctions and to avoid apoptosis (Morimoto, Hooper, Spitsin, Koprowski, & Dietzschold, 1999; Yan et al., 2001). Infection in mice with apathogenic rabies virus generates higher titers of RVNA than pathogenic rabies virus (Wiktor et al., 1978). A study to determine the effect of increasing or decreasing the G protein expression while maintain the same G protein sequence by codon optimization or de-optimization, respectively found that a 2-fold increase in expression resulted in decrease pathogenicity in mice indicating that degree of expression, not just sequence of the G protein affects actions of the virus (Wirblich & Schnell, 2011). It is possible that the difference in G protein expression of the wild-type viruses in the MagPix assays caused the decrease in measurement of specific antibody at least in part or combination with the difference in G protein epitopes.

Analysis of the MagPix assays results by Passing and Bablok demonstrate a closer agreement between the results obtained with the lab adapted strains, CVS-11, Flury-LEP, and ERA, as expected from the sequence relationships (see Figure 5-6 and 5-9). The agreement between the results obtained in the Eastern Pipistrelle and Tadarida MagPix assays is not as close given their less homologous G sequence relationship. Conversely, the agreement of response as measured by the Tadarida and North Central Skunk MagPix assays was nearly as good as between the lab-adapted strains indicating that homology in genetic relationship may not be the best indication of the antigenic relationship. It is interesting to note that for the majority of horses the highest increase in antibody response was measured in the ERA MagPix assay and that the

ERA strain is most closely related to the PV strain of the vaccine used for the booster vaccinations (see Figure 5-9). Evidence for G sequence variation or epitope difference resulting in a quantitatively immune response difference is difficult to distinguish. An effort to associate antigenic distances as determined by rabies serology to rabies virus glycoprotein sequence was undertaken by Horton et al (Horton et al., 2010); the findings conclude that the genetic and antigenic distances did not correlate as consistently as expected. Using sequence data to determine effectiveness of a vaccine strain in eliciting protective antibodies against wild-type strains is not entirely warranted. An interesting finding in the Horton study was that the same antigenic variation was determined whether the sources of antibodies were human, rabbit, or mouse signifying that species difference was not a factor.

The results of vaccine response measurement against different rabies strains by MagPix compared to RFFIT, underscores that the functional action of the antibody response cannot be interpreted from the quantification of the response by level of binding antibody. In all the horse sera measured for vaccine response by both modified RFFIT and MagPix, there was a smaller difference in neutralizing antibody measurement values (IU/mL) between the lab adapted strains and the wild-type strains than the binding antibody measurement values (MFI) by MagPix between the two types of strains. This indicates that though there was 5-10 fold less binding antibody (as detected by the MagPix assays), the ability to neutralize the virus was not diminished to the same degree. In at least two samples (Cricket and Crea, pre-booster samples) the neutralizing ability was higher to a wild-type than to a lab adapted strain, see table 5-4.

This study had a number of limitations. The lack of complete vaccination history for all horses limited the evaluation of the relationship of vaccine strain to the primary specificity of the antibodies produced as well as the effect of multiple vaccinations or use of different rabies

vaccines strains used in primary and subsequent vaccinations. The use of purified rabies glycoprotein rather than whole rabies virus as the antigen on the microspheres may have allowed better discernment of the antibody specificities and eliminated the possible confounding factor of degree of G protein expression. In addition, purified antigen or establishment of stable antigen preparation may increase the precision of the assay. Quantitative measurement of the antibody responses by relating the MFI to total IgG in the serum would standardize the results for enhanced evaluation of the response over time, between horses, and between assay runs, and allow assessment of the assay accuracy.

5.6   **Conclusions**

The results of this study demonstrate the utility of using a multiplex MagPix assay to measure the antibody response to rabies booster vaccination. The ability to separately analyze the specificities involved in the polyclonal response for evaluation of the vaccination coverage of rabies variants existing in nature was also verified. The assay has adequate precision as is expected for ligand based assays (2001). Agreement of results to established methods, RFFIT and ELISA is good as defined by the kappa test for this set of samples, while at the same time differences based on the particular aspect of the antibody response is measured by each assay was evident. The ELISA assay and the MagPix assay both detect binding antibody of the IgG subclass, while RFFIT measures the neutralizing ability of antibodies of all subclasses that may be produced to rabies vaccination and present in the serum (primarily IgG and IgM).

Vaccinated horses have succumbed to rabies, in some cases the horses had a RVNA level below 0.5 IU/mL as in a challenge study (Hudson, Weinstock, Jordan, & Bold-Fletcher, 1996) and in other instances the RVNA level was unknown as in a review of rabies cases in horses from 1970 to 1990 (Green, Smith, Vernau, & Beacock, 1992). The ability to easily check the

antibody response level in vaccinated horses would ensure individual horse maintains adequate protection from rabies exposure in their environment. Because the most likely exposures are to come from encounters with rabid wildlife, laboratory tests that can differentiate antibody specificities produced after rabies vaccination and identify weak responses to a particular strain would alert owners and veterinarians to a possible risk of rabies infection.

5.7 **Acknowledgements**

5.8 References

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