Novel Serological Assay Using High-Throughput Chromogenic Microarray Technology for Screening SPF Nonhuman Primates

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Abstract

Routine surveillance programs that reliably detect the types of microbial agents present in non-human primate colonies are critical to maintaining colony health and safety, as well as ensure research is not compromised due to underlying health status. Serological testing is a crucial element of maintaining colony health, but current methods rely on slow, expensive, and low-throughput ELISA-based serological assays. Here we describe the development of a sensitive, specific, robust, and easily automated method for colony health screening programs. Specific antigens for Herpes B virus, STLV (simian T lymphotropic virus), SRV (simian retrovirus groups 1 and 5), SIV (simian immunodeficiency virus), and measles virus were arrayed onto clear nitrocellulose slides in a multiplex format. One microliter of sera from a large set of routinely tested pathogen-free rhesus macaques was quantified by viral antigen microarray analysis, utilizing anti-simian IgG antibodies detected by a novel silver deposition technology. ROC analysis was used to calculate cut-off values and determine the accuracy of this multiplex diagnostic test against the negative animals from the SPF colony at the NEPRC. Interestingly, we observed that SRV positive sera from a conventional colony may have confounding results for other viral infections due to the known immune suppression by SRV infection, which may cause false negative readings. Ongoing studies will better define cutoff values for colonies with unknown pathogen status. Our studies show that maintaining an SRV-free colony is critical for identifying other underlying infections, and that for routine surveillance of established SPF colonies this high-throughput method will aid in rapidly identifying compromised animals that threaten colony health.

Materials and Methods

Sera. Previously tested negative sera were obtained from the Specific Pathogen Free (SPF) colony of rhesus macaques at the New England Primate Research Center (NEPRC) in Southborough, MA. Sera with known test-positive reactivity to the tested viral antigens were obtained from the conventional colony of rhesus macaques at the NEPRC. Positive sera from other National Primate Centers were kind gifts from J. Yee (UC-Davis, Davis, CA), Dr. K. Andrews (Oregon Health Sciences University, Portland, OR) , and Dr. C. Coe (Harlow Primate Laboratory, Madison, WI).

Antigens. Viral antigens for simian retrovirus group 1 (SRV1), simian immunodeficiency virus (SIV), human T lymphotrophic virus (used as surrogate antigen for STLV), and measles (MS) were purchased from commercially available sources as inactivated viral lysates. Purified simian retrovirus group 5 (SRV5) was purchased from a commercially available source. For herpes B virus (BV), inactivated cell extract from herpes virus simplex-1 MacIntyre Strain infected cells was purchased from a commercially available source. Commercially available human IgG and goat anti-rhesus IgG, and BSA-biotin (Sigma-Aldrich) were used as positive controls. For a buffer only negative control, Print Buffer was used at 1X concentration (Gentel Biosciences, Madison, WI).

Virus Inactivation. To inactivate virus, 1.0 mL of 2.9 x 10^{10} virus particles/mL of SRV5 was inactivated using the method described by Tsung et al. 1996. Briefly, live virus was incubated with 100ng/mL Psoralen (Sigma-Aldrich, St. Louis, MO), and exposed to UV light at 365 nm for 60 minutes in a Stratalinker (Agilent, Santa Clara, CA).

Microarrays. Antigens for SRV1, SRV5, SIV, STLV, B virus, and Measles and controls are present on the APiX Simian Pathogen Array Version 1.0 (Gentel Biosciences).

Detection of Sera. Individual serum samples were assayed on microarrays using the APiX View™ Detection- Simian Pathogen (Gentel Biosciences).

Scanning. Images were acquired using the Gentel Proteomics Multi-System™ (Gentel Biosciences).

Data Analysis. Images were analyzed and spot intensities quantified using AthenaQuant® software package. The Relative Intensity Unit (RIU) generated by the report in AthenaQuant were imported into GraphPad Prism (GraphPad Software Inc, La Jolla, CA) for ROC analysis.

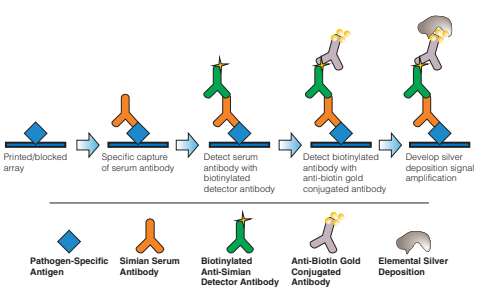

Figure 1. Principles of APiX View™ Detection using APiX Simian Pathogen Arrays. Antigens specific to SRV1, SRV5, STLV, B virus, and SIV are arrayed onto distinct location on the APiX Simian Pathogen Array V1.0 , and blocked. The array is assembled into SIMplex assay device, to which test sera added. If an animal has been exposed to a pathogen and mounted an immune response, specific IgG antibodies will bind to the antigen. A biotinylated anti-simian IgG is used to detect those bound IgG antibodies. An anti-biotin antibody conjugated with a gold particle is used to detect the antibody complex. Finally, elemental silver is precipitated by the gold conjugated antibody, allowing signal visualization.

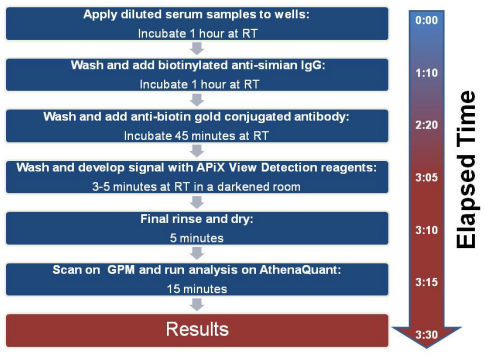

Table 1. Assay workflow. Standard assay protocol with incubation times listed. Total time from addition of samples to generation of results in AthenaQuant report about 3hr 30 min.

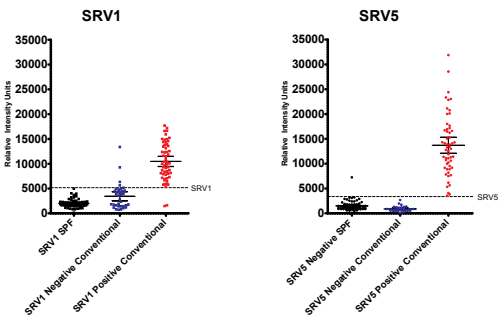

Figure 2. Results for SRV1 and SRV5 samples. RIU is measured as background subtracted signal and plotted on the y-axis. Sample group is labeled on the x-axis. Lines representing the calculated cut-off values for SRV1 and SRV5 are shown.

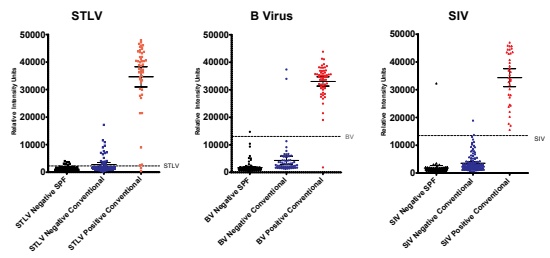

Figure 3. Results for STLV, B virus, and SIV samples. Relative Intensity Unit (RIU) is measured as background subtracted signal and plotted on the y-axis. Sample group is labeled on the x-axis. Lines representing the calculated cut-off values for STLV, B virus, and SIV are shown.

A.

Antigen	Cut-off	Sensitivity %	Specificity %	Negative Samples	Positive Samples
SRV1	5173	96.43	100.00	71	56
SRV5	3383	100.00	98.59	71	56
STLV	2274	95.83	95.71	107	48
BV	13069	98.25	99.57	107	57
SIV	13491	100.00	99.57	70	35

B.

Antigen	Cut-off	Sensitivity %	Specificity %	Negative Samples	Positive Samples
SRV1	5246	100.00	97.89	190	64
SRV5	3811	97.06	90.15	203	68
STLV	5475	98.15	94.12	289	54
BV	8970	100.00	97.74	266	61
SIV	15283	100.00	100.00	371	38

Table 2. Sensitivity and Specificity by Antigen.
A. The sensitivity and specificity for each antigen was calculated using a ROC analysis. The negative samples, or "controls," were specimens from animals at the NEPRC in a known SPF colony that have routinely tested negative by other measures. The positive samples, or "patient" samples, were collected from animals from the NEPRC conventional colony that have historically tested positive for one or more of the viruses on our panel by other methods (ELISA, MFIA, or PCR).
B. The sensitivity and specificity for each antigen was calculated using a ROC analysis. The negative samples, or "controls," were specimens from animals from several colonies that have routinely tested negative by other methods (ELISA, MFIA, or PCR). The positive samples, or "patient" samples, were collected from animals from several colonies that have historically tested positive for one or more of the viruses on our panel by other methods (ELISA, MFIA, or PCR). Cut-off values highlighted in yellow are significantly different that those established in the single-colony experiments.

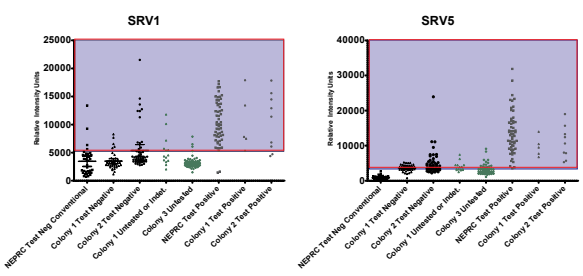

Figure 4. Testing Established Cut-off Values With Other Colonies. Samples from 3 NHP colonies were tested on the APiX Simian Pathogen Array V1.0 and evaluated using the established cut-off values. The negative samples were specimens from animals that have previously tested negative for SRV-D (Type 1, 2, or 5) by other measures (ELISA, MFIA, or PCR). The positive samples were specimens from animals that have previously tested positive for SRV-D (Type 1, 2, or 5) by other methods (ELISA, MFIA, or PCR). Relative Intensity Units are graphed on the y-axis, and colony name displayed on the x-axis. The blue shaded box represents the cut-off value established using the NEPRC data. The red box illustrates the cut-off value if using positive samples from the 3 other colonies.
A. Samples for SRV-D sera evaluated on the SRV1 antigen.
B. Samples for SRV-D sera evaluated on the SRV5 antigen.

Bonferroni's Multiple Comparison Test	Mean Diff.	Significant
NEPRC Negative SPF vs NEPRC Negative Conventional	607.0	No
NEPRC Negative SPF vs Colony 1 Negative	-2122	Yes
NEPRC Negative SPF vs Colony 2 Negative	-3388	Yes
NEPRC Test Negative Conventional vs Colony 1 Negative	-2729	Yes
NEPRC Test Negative Conventional vs Colony 2 Negative	-3995	Yes
Colony 1 Test Negative vs Colony 2 Test Negative	-1267	No

Table 3. Comparison of SRV5 results from several colonies. Pairwise comparison of the SRV5 values was performed using the Bonferroni's Multiple Comparison Test. The Mean Difference is calculated as the mean of the "comparison" colony (listed second) subtracted from the mean of the "control" colony (listed first). If the Mean Difference value is negative, the mean value for the colony is higher from the "comparison" colony. The Significance is measured as P value <0.001.
*Colony 2 had several animals with high signals from the test negative group.

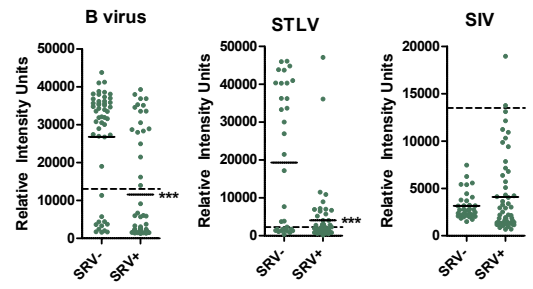

Figure 5. Seropositive status for SRV-D influences results for other viruses.
A. The mean signal for B virus in SRV-D positive animals significantly differs from those negative for SRV-D.
B. The mean signal for STLV in SRV-D positive animals significantly differs from those negative for SRV-D. C. The mean signal for SIV in SRV-D positive animals does not significantly differ from those negative for SRV-D. *** signifies p-value<0.001

Conclusions

Establishment of specific pathogen free (SPF) colonies of non-human primates (NHP) began over two decades ago to decrease health risks associated B virus positive animals, and to prevent confounding results in research animals that have been exposed to pathogens. For many viruses that infect NHPs, serology is considered to be the gold standard for studying the prevalence of these viruses in a population[1]. Our experiments show that the **APiX Simian Pathogen Array is effective for highly sensitive screening of SPF animals for B virus, STLV, SRV, and SIV in a fast and efficient manner by labs equip to run serological tests.** Like all other serological screening tests, the multiplex antigen array method will also require confirmatory testing by other methods like PCR. Our results show that for some viruses there will be unique cut-off values for each colony (Table 2, A) based on the baseline value of confirmed negative animals (Table 3). For example, the NEPRC had a significantly lower mean RIU for SRV5 in test negative animals when compared to three other colonies, and by excluding these values from the ROC analysis we could increase the specificity and sensitivity of the assay for the three other colonies to 100.0% and 98.5%, respectively (data not shown).

Our results also confirm that **SRV positive animals have a significantly lower mean antibody response to other pathogens, like B virus and STLV (Fig. 5), as has been previously observed[2].** Based on these results, screening known SRV positive animals with a different set of cutoff values than SRV negative animals could increase the sensitivity of multiplex analysis using serology. It is currently recommended that screening methods for SRV begin with testing for antibody presence using a serological method, followed by detection of virus by isolation or PCR to detect SRV-infection in antibody negative carriers[1]. For animals that have already been tested negative for SRV by PCR, the serological method for antibody detection proposed here is likely sufficient to detect those animals whose status would change due to a new infection, particularly if monitored at regular intervals[2]. **Our results support the current practice of maintaining SRV-free colonies to enhance overall colony health, and to allow accurate surveillance of colonies of unknown viral status.**

Overall, we have shown that the APiX Simian Pathogen Array multiplex platform can be used qualitatively by setting rigorous cut-off values based on colony testing and providing a positive/negative/indeterminant value, or in a quantitative format by examining the changes in RIU over routine sampling[3]. **This method is fast and affordable, and will be a useful tool for SPF colony health screening programs. Moreover, this method is flexible to accommodate additional antigens to multiplex screening for other types of pathogens that are known to elicit an antibody response, offering a wealth of data for colony health management.**

References.

1. A. Voevodin and P. Marx (2009). Simian Virology. Wiley-Blackwell (2): 48-49.
2. N. Lerche and K. Osborn (2003). Simian Retrovirus Infections: Potential Confounding Variables in Primate Toxicology Studies. Toxicol Pathol 31: 103.-110.
3. J. Simmons (2008). Development, Application, and Quality Control of Serology Assays Used for Diagnostic Monitoring of Laboratory Nonhuman Primates. ILAR 49(2):157-169.