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Specific Pathogen Free Macaque Colonies: A Review of Principles and Recent Advances for Viral Testing and Colony Management

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Abstract

Specific Pathogen Free (SPF) macaques provide valuable animal models for biomedical research. In 1989 the National Center for Research Resources (now Office of Research Infrastructure Programs ORIP) of the National Institutes of Health initiated experimental research contracts to establish and maintain SPF colonies. The derivation and maintenance of SPF macaque colonies is a complex undertaking requiring knowledge of the biology of the agents for exclusion and normal physiology and behavior of macaques, application of the latest diagnostic technology, facilities management, and animal husbandry. This review provides information on the biology of the four viral agents targeted for exclusion in ORIP SPF macaque colonies, describes current state-of-the-art viral diagnostic algorithms, presents data from proficiency testing of diagnostic assays between laboratories at institutions participating in the ORIP SPF program, and outlines management strategies for maintaining the integrity of SPF colonies using results of diagnostic testing as a guide to decision making.

Keywords

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Background

Specific Pathogen Free (SPF) macaques provide valuable, highly utilized, and well-characterized animal models for biomedical research. In addition to the value of SPF macaques to biomedical research, the derivation of SPF macaques over the past 25 years has contributed significantly to the improvement of overall breeding colony health. In 1989 the National Center for Research Resources (now Office of Research Infrastructure Programs ORIP) of the National Institutes of Health initiated experimental research contracts to establish and maintain SPF colonies with the goals of improving animal health and reproduction by elimination of potential animal pathogens to thereby, a) improve the quality of nonhuman primates (NHP) used in biomedical research by providing animals free of potentially confounding concurrent infections, and b) reduce or eliminate potential sources of human occupational exposure to selected NHP viruses (57). These goals have been successfully achieved by employing a test and removal strategy for the original four selected NHP viruses: 1) *Macacine herpesvirus* 1, formerly known as *Cercopithecine herpesvirus* 1 and also referred to as B virus or Herpes B virus (BV), 2) simian immunodeficiency virus (SIV), 3) simian betaretrovirus formerly known as simian retrovirus type D (SRV), and 4) simian T-cell lymphotropic virus (STLV-1). That strategy relied primarily on two basic requirements: 1) initial and ongoing surveillance testing to correctly identify all infected animals, and 2) a barrier management system to prevent direct and indirect contact between SPF and non-SPF or untested animals (55; 69). These approaches have been successfully applied to eliminate or characterize infection not only for the original four target viruses, but also for additional pathogens such as simian foamy virus, rhesus cytomegalovirus, rhesus rhadinovirus, simian virus 40, lymphocryptovirus, simian varicella virus, and measles virus (56).

This paper reviews the current state-of-the-art viral testing programs for deriving and maintaining NHP SPF breeding colonies. Included are descriptions of general principles necessary to ensure accurate detection of infection as well as examples for applying these principles to design efficient step-wise algorithms using well-validated, quality-controlled diagnostic tests. The importance of implementing a proficiency assessment program in the context of large multi-institutional SPF macaque breeding programs is also addressed. The conclusion of this report provides a brief description of how results of viral testing can be applied to the management of SPF macaque breeding colonies.

Laboratory tests for pathogen detection

When developing a comprehensive pathogen detection program for developing or maintaining SPF macaque breeding colonies, incorporating a two-tiered testing algorithm (screening and confirmatory assays) will ensure both accuracy and efficiency (34; 35). The performance of each test must be reproducibly validated by testing samples from known infected and uninfected monkeys (95). Where possible and to fully challenge diagnostic test sensitivity, it is advantageous to include positive samples from known infected monkeys at early stages of infection (i.e. with recent seroconversion) as well as at later stages of infection, and also from monkeys with clinical findings ranging from subclinical to overt disease. Specimens should also be tested from monkeys not infected with the virus under

investigation, but carrying other virus infections that would serve as specificity controls. It is important to include samples from all species of monkeys for which the test will be used.

For the screening phase of a pathogen detection program, the antibody tests must be exquisitely sensitive (>99%) with the goal of correctly identifying all infected animals. By definition, this level of sensitivity will result in a lower specificity (34; 95). Ideally a screening assay is rapid, high throughput, inexpensive, and extremely sensitive. Thus, the purpose of the screening test is to identify all true negative samples from uninfected animals while identifying a smaller group of true- and false- positive samples that would then require further testing with a more specific confirmatory test (34; 57).

Immunoassays using target antigens for antibody capture and subsequent detection using a secondary conjugated antibody for colorimetric enzyme-substrate or fluorescent detection platforms have been successfully used as screening tests. The performance characteristics of any given antibody test are highly dependent on the quality of the target antigen. Antigen quality is determined by the inherent immunogenicity, sensitivity and specificity of the epitope selected as well as the method of its production and purification. The assay format of the classical enzyme- linked immunosorbent assay (ELISA), also known as an enzyme immunoassay (EIA), continues to be a valid screening test. Endpoints for defining positive ELISA results are typically set at absorbance values 2.5–3 standard deviations higher than the mean optical density (OD) exhibited by negative controls (57, 95). If there are several agents of concern (i.e. BV, SIV, SRV, STLV-1), the newer simultaneous multiplex assays have been developed and proven to be at least as accurate and more cost effective than using single ELISA tests (48; 49; 59).

With the advent of multiplex testing, automated or semi-automated liquid and solid arrays using immunoassay platforms with colorimetric or fluorescent endpoints have made it possible to simultaneously screen for antibodies to numerous, different pathogens, improving the efficiency and economy of testing large populations of macaques. In the bead-based liquid array, a panel of microscopic beads are coated with antigens from different viruses, pooled and incubated with plasma from animals undergoing testing. After incubation to allow capture of antibodies, the beads are then washed to remove unbound antibodies and subsequently incubated with fluorescently labeled secondary antibody specific for nonhuman primate antibodies that are bound to the antigen-coated beads. After washing the beads again to remove unbound fluorescent secondary antibodies, fluorescence of beads is measured by flow cytometry to determine which antigen-specific primary antibodies were captured and present in the test specimen. Colony samples are tested along with known high and low positive and negative controls in order to accurately determine the cutoff for sample positivity. In the solid phase array platform, glass slides are coated with multiple, specific antigens. The slides are incubated with plasma from animals undergoing testing as well as positive and negative controls followed by a series of washes and incubations with secondary enzyme-conjugated antibodies specific for nonhuman primate antibodies and colorimetric substrate. The slides are then examined by digital image scanner to identify antigen-specific antibodies in the test specimens. Examples of commercially-available tests include the Luminex bead-based assay or Multiplexed Fluorometric Immunoassay (MFIA®) from Charles River (Wilmington, MA) and the slide array-based

assay from Intuitive Bioscience (Madison, WI). The read-out for these assays is a numerical value based on large number of normal uninfected as well as known positive samples that were tested to establish the normal ranges for negative specimens. Outlier results above the "normal" range are thus interpreted as positive in this screening test. Various statistical methods are used to determinate cut-off values (95) and in some cases, an indeterminate group of samples yielding high negative to low positive values is identified. The exact statistical method is often empirical, depending on the performance of the reagents, method, and samples. In general the goal is to maximize the signal to noise ratio (i.e. largest difference with least overlap between test result values generated from samples from infected and uninfected monkeys).

Confirmatory tests can be technically more complex by requiring additional time, equipment, and cost because they are used on a much smaller number of samples. By definition, confirmatory testing is necessary because the positive predictive value for screening is low in a low prevalence population, even if using a highly sensitive and specific test, producing false positive results (95). Indirect immunofluorescence antibody (IFA) and western blot (WB) immune-detection are two suitable confirmatory antibody-based test methods that can be performed on samples that were initially positive or indeterminate/ borderline by screening methods. Although these tests use immunoassay principles similar to the screening assays, they may apply different antigens or conditions for antibody capture to allow for visualizing unique and characteristic reactivity staining patterns to virus-infected cells (IFA) or to antigens separated by molecular weight after electrophoresis and transfer onto membrane blots. Western blots, for example, provide increased specificity of the humoral responses observed in the initial screen by allowing the detection of antibodies against specific sets of viral proteins, typically four-to-nine bands of reactivity. If the WB or IFA reactivity pattern detected in an individual test sample matches that seen with known positive controls it is confirmed as being positive. If reactivity is present, but it does not match the characteristic pattern seen with the positive control, it cannot be confirmed as virus specific and must be reported as indeterminate being neither positive nor negative. Alternatively, a second confirmatory test may be applied so that if either the IFA or WB is indeterminate, the other test may be helpful in further interpreting the results. If the confirmatory IFA and WB fail to detect reactivity to any viral target proteins, the sample test result is interpreted as being negative. The confirmatory test result supersedes the screening test result. If the confirmatory test is negative, the antibody result is negative, regardless of the screening test result.

Virus culture and nucleic acid amplification have been used for diagnoses, as well, but may be challenging in the presence of low numbers of infected cells and low virus copy numbers (53; 57; 114). Virus culture is labor intensive and identifying positive specimens highly dependent on the correct sample type and time of collection. The approximately six week turn-around time to definitively diagnose a negative result is a major obstacle (68) and delays colony management decisions. Amplification of DNA or RNA by polymerase chain reaction is a more time-efficient and sensitive alternative (53; 114). Special attention however, must be given to the selection of appropriate primers and probes to ensure broad reactivity against all virus variants of interest that could possibly be present in the colony under investigation, as well as be sufficiently stringent to exclude amplification of non-

specific DNA sequences. This is especially important for SRV PCR which can be complicated by the presence of endogenous retroviral sequences related to SRV. Several different PCR-based assays are typically used to detect viral DNA genomes in peripheral blood cells and include; endpoint PCR, nested endpoint PCR, competitive PCR, and real-time PCR also known as quantitative PCR (qPCR). Since the objective of SPF testing is to determine the mere presence of virus in participant macaques, a less expensive, qualitative real time or endpoint PCR is usually sufficient to diagnose potentially infected animals. However in rare cases, quantitative approaches using competitive or real-time PCR that incorporate both a virus-specific target and a host-specific target, may be warranted. Such an assay is useful when it is important to quantitate a lower limit of detection to determine with extraordinary confidence that a particular animal is not infected. PCR testing is run at least in duplicate, and both replicates must produce identical results for the presence or absence of amplicons to be interpreted at positive or negative, respectively. Reactivity in only some wells may require repeat sampling and / or testing and repeated variable results would be interpreted as indeterminate in the final report. As with antibody testing, the rigorous validation of the methods, reagents, and interpretative criteria in the population being tested is critical.

BV, SIV, and STLV-1 infections have been successfully identified using serological or antibody testing alone. Serology alone, however, is insufficient for detecting SRV infections and thus does not successfully serve for culling macaques from the breeding colonies. As will be further described, true SRV infection with undetectable antibody or virus at a given time point has been documented in monkeys. Thus, both specific antibody and virus detection assays are necessary to reliably diagnose SRV-infected rhesus macaques.

Proficiency testing and quality assurance

A cornerstone of the establishment and maintenance of specific pathogen free breeding programs is the requirement for regular assessment of the validity of detection assay results. Proficiency testing is particularly important for establishing integrity across large programs, such as the NIH SPF Macaque Breeding Colony Program, that comprises many participating laboratories using different assay platforms for diagnosing animals with a predetermined list of infectious agents. In 2008, the Breeding Colony Management Consortium (BCMC) of the National Primate Research Centers (NPRC) surveyed their members regarding SPF breeding colony diagnostic testing needs and available resources. The results of the collaboration led to the sharing of viral testing protocols and well-characterized serum panels provided by the Pathogen Detection Laboratory (PDL) at the California National Primate Research Center. The materials were made available to other NPRCs to be used to improve and validate the quality of testing performed in individual laboratories. That initial collaboration has led to the development of a more formal proficiency exchange panel comprised of serum or plasma, as well as DNA samples, donated by the participating institutions. Investigators at the PDL prepared and distributed the panels of specimens to investigators at the individual laboratories where tests were performed and results interpreted. Reports from the test sites were then submitted to investigators at the PDL where the overall cumulative results were collated, analyzed and shared with all participants.

At the time of this publication, three proficiency exchange panels have been completed by laboratories across the NPRC program. The first panel included well-characterized and clearly positive or negative serum or plasma samples for serological (antibody) testing. The second panel included both characterized serum/plasma samples for antibody testing as well as DNA for PCR-based virus detection. Due to technical issues related to insufficient DNA quantity and quality for virus detection, only the antibody test result data were analyzed. The third panel was designed to mimic practical, real world testing situations and included both characterized and unknown or "problem" serum or plasma and DNA. Specifically, this panel was comprised of 24 samples (DNA and plasma/serum specimens from 20 animals, serum/plasma only from three animals, and DNA only from one animal) and was distributed to the seven NPRC testing laboratories.

Over the course of these three panel testing comparisons, there was a noticeable quality improvement achieved based on an increased number of results in agreement with the correct or consensus antibody results from all laboratories. Serological results of the proficiency exchange panel 3 are shown in Table 1 and overall, performance across participating NPRCs was accurate. The four BV false-negative results were generated by two different laboratories using the same commercial reagent. The four SRV false-negative samples test results were generated in two different laboratories, where one used commercial reagents and the other used "in-house" reagents. As already mentioned, it is strongly recommended that SRV testing include both antibody and virus detection since one test may be insufficient for accurate diagnosis. Thus it is possible that the laboratories would have produced identical and more consistent results for these four animals if parallel serology and direct viral detection tests had been applied in this panel of specimens. No false-negative results were reported for SIV or STLV. Some of the apparent false-positive results reported by participants were based on only using screening tests because they do not have internal capacity for confirmatory testing and would normally refer any reactive screening tests to another facility for further testing. As previously discussed, false-positive results are expected during the screening phase when using exquisitely sensitive antibody assays and are often resolved by more specific confirmatory testing. Ongoing technical difficulties in providing adequate sample quantity as well as the small number of laboratories participating in the proficiency exchange DNA testing limited the amount of data derived to preclude arriving at statistically significant conclusions. This experience is being used to inform and continue improving quality assurance in the SPF testing platforms among the NPRCs.

Published methods, commercially available reagents, and reference laboratory services are now available for antibody and virus diagnostic testing of macaques in SPF breeding colonies. Regardless of whether testing is performed in house or contracted through an outside laboratory, it is important to validate the testing algorithm using samples from known infected and uninfected animals matched for species, age, sex, housing, immunization, background infections, clinical status, etc. to test the population (69; 95). Every test run should include process controls that mimic real test samples as closely as possible. At minimum, clearly positive and negative controls should be included in each test run. If available, it is advantageous to also include a low-reacting positive control, which might be adversely affected by minor technical or reagent fluctuations. Furthermore, regular proficiency testing, such as the BCMC exchange panels just described, provides a means to

monitor and ensure acceptable quality of testing algorithms, reagents, methods, equipment, and personnel. It may be necessary to adjust parameters of the individual tests (i.e. defining the positive / negative cut-off values) or the algorithm (i.e. management of indeterminate results) based on the specific characteristics of the population being tested, such as prevalence or housing configurations.

The following sections of this report apply the diagnostic principles and practices reviewed above to BV, SIV, SRV, and STLV-1, the four selected NHP viruses chosen for exclusion by the NIH in the NPRC SPF macaque breeding colony program. An overview, the currently utilized assays, and a proposed testing algorithm will be presented for each virus. These sections are followed by a consolidated discussion of result-based viral testing, and management practices to establish and maintain SPF colonies.

Herpes B Virus / *Macacine herpesvirus 1* / Cercopithecine herpesvirus 1 (BV)

Overview

Herpes B Virus, or BV, is a common virus producing latent and typically asymptomatic infection of Asian macaques. Viral isolation and/or serological techniques have documented infection in numerous macaque species including *Macaca mulatta* (rhesus), *M. fascicularis* (cynomolgus), *M. radiata* (bonnet), *M. nemestrina* (pig-tailed), *M. fuscata* (Japanese), *M. cyclopis* (Formosan rock), and *M. silenus* (lion-tailed) (4; 44; 100). Herpes B virus belongs to the subfamily *Alphaherpesvirinae* and genus *Simplexvirus* and is related to similar viruses infecting humans (*Human herpesvirus 1 and 2*), baboons (*Papiine herpesvirus 2*), African green monkeys (*Cercopithecine herpesvirus 2*), and squirrel monkeys (*Saimiriine herpesvirus 1*) (74; 75; 81; 106). While *Papiine herpesvirus 2*, *Cercopithecine herpesvirus 2*, and *Saimiriine herpesvirus 1* (73) have no known zoonotic potential (106), BV can cause disseminated viral infection and acute ascending encephalomyelitis in humans who become inadvertently infected through macaque bites, scratches, injury with contaminated fomites (e.g. needles, scalpel blade), or exposure of mucous membranes with infectious material (e.g. macaque blood, saliva, feces) (10; 42; 73; 111). Clinical signs in humans usually present within 30 days of exposure, and the incubation time can be as short as three to seven days (11). Clinical signs can include vesicular skin lesions at or near the site of exposure, localized neurologic symptoms (pain, numbness, itching) near the wound site, flu-like aches and pains, fever and chills, headaches lasting more than 24 hours, fatigue, muscular incoordination, and shortness of breath (11). Respiratory involvement and death can occur 1–21 days after onset of clinical signs (11). The case fatality rate for BV infection in humans is 70–80% without proper treatment and up to 20% even with drug intervention (90). Therefore, comprehensive prevention and control measures for BV infection are of primary importance in all institutions where macaques are maintained (72), and should comprise establishment of standard operating procedures (SOPs) for proper handling of macaques and their tissues, education and training of all personnel working with macaques or their tissues, appropriate use of personal protective equipment (PPE), ready access to first aid stations and to medical staff with experience evaluating and treating BV exposure, follow-up tracking of all NHP-related injuries, exposures, and potential infections, and periodic review and update

of procedures and policies (106). In addition to the increasing use of animals from specific pathogen free (SPF) macaque colonies that exclude BV, effective and widespread implementation of such comprehensive practices are presumed to have significantly reduced the prevalence of human BV infections, with fewer than 50 cumulative documented cases described to date (41).

Fatal disseminated disease from BV infection has been documented in various non-macaque species including: *Erythrocebus patas* (Patas monkey), *Colobus abyssinicus* (black and white colobus), *Cercopithecus neglectus* (DeBrazza's monkey), *Cebus apella* (capuchin monkey), and *Callithrix jacchus* (common marmoset) (32; 61; 100; 116). In addition, there was a report of a colony of capuchin monkeys that developed persistent and asymptomatic BV infection while housed near, but not in direct contact with rhesus macaques (13). The tremendous potential impact from BV infection in nonhuman primates other than macaque species dictates careful design and strict adherence to operational practices that minimize risk of cross-species infection to the lowest possible level.

The incidence of BV infection is low in immature non-SPF rhesus macaques, but increases rapidly after sexual maturity at three to four years of age, approaching 80–90% of animals in some colonies (5; 91; 112). In overcrowded or unsanitary conditions, animals may become infected at an earlier age and seropositivity rates may be higher (106). The primary route of transmission is through sexual contact via the oral or vaginal mucosa, with the highest risk of infection occurring during breeding season in adolescent macaques of two to three years of age (112; 113). Transmission also may occur through biting, grooming, and contact with fomites (112).

Infection in macaques is characterized by virus residing in the trigeminal and lumbosacral sensory ganglia that persists for the life of the animal, latent periods with no clinical evidence of the virus, and periodic viral shedding in oral and genital secretions (69). Clinical signs of BV infection in Asian macaques usually manifest during primary infection at the site of inoculation and at other locations during subsequent periods of recrudescence. Characteristic vesicular lesions develop on the oral and genital mucosae and/or adjacent haired skin that ulcerate and resolve within 10–14 days (106). Viral shedding occurs during this time and intermittently during asymptomatic periods, notably during breeding season (43; 112). Active viral shedding also occurs when macaques demonstrate clinical disease, so to avoid exposure, personnel should not attempt treatment or any other direct manipulation of infected macaques during this time (106).

Disseminated disease in macaques is rare and usually fatal (9; 96; 116). Infections in such cases produce a variable clinical course transitioning from acute to slowly progressive, and often are not initially suspected for BV thereby creating additional zoonotic risk if animals continue to be handled. The risk for disseminated herpesvirus infection increases in immunosuppressed humans (85), so there is a presumed similar risk for disseminated BV in macaques undergoing immunosuppressive drug regimens. Disseminated BV infection also has been documented in cynomolgus macaques with concurrent SRV infection (9). In addition, a respiratory form of BV infection has been documented in bonnet macaques (*M.*

radiata) that manifests with coryza, rhinorhea, cough, and conjunctivitis, and may transmit infection via aerosolized oral secretions (22; 92).

The pathogenesis of BV infection in macaques is similar to that of *human herpesvirus 1* (HHV1) (106). Primary infection results in an initial round of replication at the inoculation site that is histologically characterized by ballooning degeneration of keratinocytes and progression to vesiculation with multinucleated syncytial cells and eosinophilic to basophilic intranuclear viral inclusions. Necrosis of endothelial cells with intranuclear viral inclusions and inflammatory cells may be found within vesicles, epidermis, and subjacent dermis. If lesions are equivocal, immunohistochemistry can be used to demonstrate viral antigen in lesions for definitive diagnosis. In disseminated disease, there is widespread, hemorrhagic necrosis within the liver, lung, brain, adrenal gland, and lymphoid organs (4; 9; 22; 96).

Aggressive SPF programs that exclude BV have been associated with a nearly 20-fold reduced risk for BV exposure as reported by Hilliard and Ward (39). Specifically, the cumulative rate of non-negative results from six SPF colonies changed from 0.132 to 0.036 after one year of aggressive SPF management, and then declined further to 0.018 in year two and 0.004–0.006 in years three to six (39). Management and occupational health safety practices dictate that all macaque species should be treated as though they are infected with BV regardless of SPF status or seroreactivity due to the imperfect nature of all current serologic tests, as well as the potential for zoonotic transmission with other infectious agents that macaques harbor. Reduction in the true prevalence rate of BV infection in SPF macaque colonies is expected to yield further decreases in associated zoonotic infection rates, improvements in health and well-being of macaque and non-macaque species, and an optimization of the macaque for use as a research model.

Currently-utilized assays

Reliable antemortem diagnosis of BV is challenging due to false negative test results in animals that are latently infected yet are seronegative, immunologically unreactive, or that express low antibody levels during the early stage of infection (109). Seroconversion usually occurs 14–21 days after primary infection and is associated with the resolution of clinical signs. However some animals fail to seroconvert until several years after entering SPF colonies and after producing multiple false-negative test results, and such animals represent the greatest threat to maintaining the integrity of any SPF program (109).

Standardized screening strategies to detect antibodies to BV have utilized enzyme- linked immunosorbent assay (ELISA) and western blot immunodetection as confirmatory tests at the National B Virus Resource Center (107–109). Homologous BV is the ideal antigen for use these tests, but has been problematic to produce in the past because generating large quantities of infectious BV posed considerable biohazard concerns, and required use of BSL-4 facilities plus CDC approval for this HHS Select Agent. The more recent availability of BV recombinant glycoproteins, however, has reduced these biohazard risks. Several ELISA tests for detecting BV antibody also have utilized HSV-1, HSV-2, *Papiine herpesvirus 2*, or *Cercopithecine herpesvirus 2* antigens due to their close phylogenetic and immunologic relationships with BV (46; 76; 98; 99; 117). HSV-1-antigen ELISAs are used commonly for in-house testing due to their lower cost and wide use in human medicine to

detect HSV-1 infections. The signal-to-noise ratio can be improved by using NHP alpha-herpesvirus antigens, making the HSV-1-based ELISAs only slightly less sensitive for detection of BV in monkeys (76). In addition, there is variability in the cross-reactivity between BV and HSV-1 among the monoclonal primary and/or secondary conjugated antibodies used in the HSV-1-based ELISAs and this can sometimes be circumvented by using commercial HSV-1 plates but incorporating in-house buffers and secondary conjugated antibodies specific to NHP immunoglobulins (H. Palmer, personal communication, 4/11/14). BV recombinant glycoproteins have recently become available for incorporation in these tests for improving the diagnostic potential and screening standardization. ELISAs utilizing multiple recombinant glycoprotein antigens (gB, gC, gD, and membrane-associated gG) were reported to provide the highest sensitivity and specificity (26; 80; 82). In addition, the gG antigen was useful in discriminating between BV and other closely related alphaherpesviruses (21). Importantly, recombinant BV antigens are relatively economical to produce for safe use in the clinical laboratory setting, unlike the production of antigen from whole BV cultures.

In addition to the established use of antibody-based serologic tests (e.g. ELISA, western blot), a number of strategies for BV identification using various PCR-based methodologies have been proposed and applied for adjunct screening (40; 65; 77; 81). PCR testing of clinical (antemortem) samples is considered more sensitive than viral culture methods (108). A significant limitation of PCR or viral isolation, however, is that positive results are only produced when latent virus infection reactivates and when virus is shed in oral and genital secretions. During virus latency in sensory ganglia, no virus will be circulating and diagnostic testing by PCR or viral isolation will be falsely negative (112). Thus, PCR exhibits decreased sensitivity and utility relative to serologic tests as a screening test (106). Trigeminal and sacral ganglia of macaques culled from an SPF group of animals can be examined *postmortem* by PCR testing, however, to improve overall surveillance for BV. Such postmortem screening is particularly useful in well-established colonies, where the expected true prevalence of BV is relatively low. At a minimum, all culled SPF animals that have had non-negative and/or ambiguous antibody tests in the past should undergo postmortem PCR testing on sensory ganglia tissue (109).

BV testing algorithm

Guidelines for the establishment of BV-free SPF colonies have been published by the National B Virus Resource Center at Georgia State University (39; 107–109). Those macaques that have generated serially negative test results by these standards can be incorporated into the SPF breeding colony after which animals should be tested for delayed seroconversion annually or semi-annually. Seropositive macaques have been detected as late as seven years after introduction to an SPF colony (39). As an SPF colony becomes established, the positive predictive value of screening tests is expected to decrease in proportion to the reduced prevalence of disease thereby increasing the chances of false-positive test results. Any seropositive animal should generally be quarantined while awaiting results from confirmatory testing by more specific ELISAs and/or western blots performed onsite or at an off-site service laboratory for the critical importance of identifying these positive animals. An important guiding principle regarding screening for BV in both SPF

and non-SPF macaque colonies is that a single test result is of limited value (106). A comprehensive strategy is required for optimal screening of BV and maintenance of SPF colonies that relies on; 1) examining patterns of sero-reactivity in individual animals rather than relying on a single test; 2) using both in-house and external confirmatory testing of clinical samples; and 3) postmortem analyses on animals culled from the SPF colony.

Each institution should adapt the above guidelines to meet their own particular requirements based upon the size of their SPF and non-SPF colonies as well as financial and time constraints. In addition, SPF colonies in different geographic locales and facilities will vary in the degree of background cross-reactivity in serologic assays for BV and other targeted infectious agents in a particular SPF program (C. Curbelo, personal communication, 5/1/14 and J. Yee, personal communication, 5/1/14). Such variation is presumed to result from exposures to differing non-SPF endemic diseases (e.g. dengue virus) and/or institutional interventional practices (e.g. vaccinations). Thus, the process for adapting the above guidelines to each institution's own requirements will naturally require adjustment and optimization of individual assays to account for such background cross-reactivity.

Figure 1 illustrates an example algorithm based on principles required for establishing a successful BV testing strategy. Animals in the "pretest" group are tested by either HSV-1 ELISA or rELISA annually. Following two consecutive negative test results, the animals may be entered into the SPF program whereas animals producing non-negative (positive or indeterminate) results should be retained and retested. Specimens from animals producing two consecutive non-negative test results should be sent to the B Virus Reference Laboratory for confirmatory testing. If the confirmatory test results are negative, the animals are allowed to enter SPF whereas confirmatory test-positive animals should be culled or utilized for non-SPF research. Herpes virus papio, type 2 is another antigen that has been used successfully as a sensitive ELISA or multiplex target in other alternate algorithms (48, 76).

Maintenance of SPF macaque colonies that exclude BV adds complexity to such practices, but those efforts provide ample return on investment through greater occupational health and safety, NHP health and well-being, and optimization of the macaque as a research model.

Simian immunodeficiency virus (SIV)

Overview

SIV is a retrovirus of the genus *Lentivirus* that infects nonhuman primates. More than 40 species of nonhuman primates in sub-Saharan Africa are naturally infected in the wild with species-specific variants of SIV (36; 94). Multiple cross-species transmissions of the SIV variants that infect chimpanzees (SIVcpz) and sooty mangabeys (SIVsmm) are responsible for the HIV-1 (Group M) and HIV-2 epidemics in humans, respectively (36). While SIVcpz infection of chimpanzees results in an increased mortality rate for certain populations of chimpanzees (47), it is thought that most of natural SIV infections of nonhuman primates in the wild are non-pathogenic due to a long history of host-virus coevolution (36). However, these viruses typically induce an AIDS-like illness when transmitted to a non-natural host such as humans or an Asian macaque (16; 58; 70). Thus, SIV infection of Asian macaque

species (primarily Indian rhesus and pig-tailed macaques) represents the current best pre-clinical experimental model for understanding immunodeficiency virus pathogenesis and for evaluating the effectiveness of new vaccines and novel treatment regimens (24).

Like HIV, SIVs primarily infect activated CD4+ T cells and macrophages (16). Upon entry of the virus, the viral genome reverse transcribes and is transported to the nucleus where it integrates into the host genome. Viral genomes that fail to integrate will sometimes circularize to form terminal forms with either 1-LTR (self-primed) or 2-LTR (blunt-end ligated) episomes. The turnover of these virologic dead-ends is thought to be relatively rapid and their presence may be indicative of ongoing viral replication (71). In SIV-infected macaques, virus genomes (either RNA or DNA) are detectable by PCR as early as 3–14 days later and sometimes longer after infection depending on the route of inoculation. Serological antibody responses can take three to six weeks to develop (97). SIV was first identified in a rhesus macaque at the New England Primate Research Center in 1985 (16). Through extensive molecular epidemiological studies, this virus was eventually traced back to the California National Primate Research Center in the 1970's when experiments attempting to develop a monkey model of prion disease are thought to have facilitated the transmission of SIVsmm into both rhesus macaques (SIVmac) and stump-tailed macaques (SIVstm) (6; 7; 70). Thus, SIVmac and SIVsmm are the primary sources for most of the SIV strains currently used to experimentally infect nonhuman primates for use in AIDS research (24). Specifically, SIVmac251 (an uncloned swarm named after the rhesus macaque from which it was derived) and SIVmac239 (a cloned virus resulting from serial passage of the virus from the same source as SIVmac251) represent the most frequently-used vaccine strains of SIV (38). These viruses are highly pathogenic due to sequential passage through multiple macaques (38). Recently, another uncloned swarm, SIVsmmE660, has come into more regular use as a challenge virus in vaccine studies due to its longer course of infection that more realistically replicates the course of HIV infection in humans (24). Natural infections with SIV in wild populations of macaques has not been noted. Transmission of SIV to captive adult macaques has been observed only after experimental inoculation (16) but this virus is capable of establishing transmission chains that are associated with increased prevalence of immunodeficiency disease (62). SIVsmm, for example, is highly prevalent in both wild and captive natural host sooty mangabeys (28; 89) in which infections occur primarily upon sexual maturation and are thus likely transmitted sexually as well as through bites and scratches (36). Group housed sero-discordant monkeys, however, have been known to transmit their virus (J.Yee, personal communication, 8/23/15). Therefore, SIV-infected rhesus macaques are housed in single-animal cages and separated from non-SIV-infected animals to minimize risk of transmission to nonhuman primates in breeding colonies. Upon the completion of studies employing SIV-infected animals, the monkeys should be housed without contact with uninfected animals or culled from the colony. These methods should help decrease the risk to extremely low levels for exposure of SPF colonies to SIV-infected animals.

Since SIV is not endemic to rhesus macaque populations in the wild or in captivity, its exclusion in SPF macaque populations is related to monitoring for the possibility of inadvertent transmission from experimentally infected or naturally infected host species. Given that SIV infection induces detectable antibody responses within weeks of infection

and SIV genomes are detectable in peripheral blood CD4+ cells sometimes within days of infection, a testing regimen that combines both serological and molecular assays can be used to both reliably monitor SPF colonies for outbreaks and to evaluate the transmission risks of potential breaches in SPF colony sequestration. Thus, in addition to regular SIV testing, an SPF rhesus macaque colony should be structured such that animals enrolled in studies involving SIV infection never come into contact with those that are housed in the SPF breeding colonies. Furthermore, in the course of selected SIV vaccine studies, some animals are vaccinated but not challenged and thus may be positive by serological screening despite not being SIV-infected. Toward this end, special care should be taken to sequester SIV-positive, SIV-challenged, and SIV-vaccinated rhesus macaques from the general SPF macaque population. Animals that are removed from SPF colonies for performing medical procedures and for use in non-SIV studies should be housed in facilities completely separated from facilities where SIV-positive animals are housed. These structural barriers to SIV transmission are by far the most effective way to prevent SIV transmission to animals housed in an SPF colony. Furthermore, given the potentially long incubation period for SIV-induced immunodeficiency to become obvious in an infected individual, extreme care and caution must be taken to report all potential exposures for monitoring and testing.

Currently-utilized assays

Detection of SIV in SPF rhesus macaques is straightforward and adapted from principles of HIV diagnosis in humans (45; 57). The development of SIV-specific antibodies early during infection allows for the indirect screening of animals using relatively inexpensive and sensitive methods. These tests are considered reliable for both human and nonhuman primate immunodeficiency virus infection testing. The most common tests in use for SIV serology are enzyme immunoassays (ELISA), multiplexed liquid microbead or slide arrays, and western blot immunoassay using peptide, recombinant, or purified virus antigens.

Confirmatory western blot immunoassays are performed on specimens that tested positive or indeterminate/ borderline by screening methods. Western blot diagnosis of SIV infection in macaques typically follows established parameters for those performed in humans (45). A positive result is defined as antibody reactivity against two or more viral proteins that must include reactivity against p24, gp41, and either gp120 or gp160. Antibodies against the viral envelope spike (gp120, gp41 and/or its uncleaved precursor gp160) are typically among the earliest detectable antibody responses expressed against SIV and thus facilitate early detection of SIV infection. Indeterminate western blot results are defined as antibody reactivity to only one viral protein, reactivity to lower molecular weight viral proteins (e.g. p24 and p17) but not against the viral envelope spike (gp120 or gp160), or non-specific reactivity that does not correspond to a specific viral protein. Although most animals with an indeterminate western blot are likely uninfected, the result could be indicative of incomplete seroconversion. In such cases, repeat testing at a later time point or molecular testing (e.g. by PCR) may resolve determination of the infection status. The SIV genome readily infects circulating CD4+ T cells, integrates into the host cell's genome and is readily detected even in individuals undergoing multi-drug antiretroviral therapy (18). Thus, PCR primers are typically designed to target the highly conserved *gag* or LTR regions of the virus to facilitate detection of the wide variety of SIVs used in pre-clinical AIDS research.

SIV testing algorithm

Figure 2 illustrates the screening and confirmatory algorithm principles used to detect SIV and is similar to that applied to diagnosing other SPF retroviruses (69). Briefly, macaque plasma samples are screened for antibody reactivity against viral antigens using either an ELISA or multiplex array with endpoint cut-off values based on responses using known high and low reactive samples in addition to multiple negative control specimens. Rhesus macaque samples with positive or borderline levels of SIV reactivity are subjected to a more specific SIV western blot assay. Positive and negative western blot results are considered definitive because the banding pattern in SIV-infected animals is well-defined. Indeterminate samples are subjected to molecular testing using a highly specific and SIV-sensitive PCR designed to amplify a highly conserved region of the SIV genome. The PCR assay is qualitative and definitive. Finally, care should be taken to account for the range of viruses currently in use for studies on vaccination and pathogenesis of SIV infection. Both serological and molecular assays should be designed to account for the genetic and antigenic diversity presented by the several SIVmac, SIVsm, and hybrid SHIV strains that are used to infect rhesus macaques. While this may not be as important for serological testing, care should be taken to design PCR primers located in highly conserved regions across the SIV genome.

Simian betaretrovirus / Simian Retrovirus, type D (SRV)

Overview

Both exogenous and endogenous simian *Betaretroviruses* are members of the *Retroviridae* family of the genus *Betaretrovirus*. Since these genetically closely-related enveloped RNA viruses exhibit a type D retrovirus morphology of an icosahedreal capsid composed of an envelope-associated outer shell and an inner ribonuceloprotein core, they were previously known as Simian Retrovirus, type D (68). As is typical for retroviruses, the SRV genome is organized simply into four genes: *gag*, which codes for the group specific antigen, *Prt*, which encodes the viral protease, *Pol*, which codes for the polymerase and endonuclease/integrase enzymes, and *Env*, which encodes the external envelope spike and the transmembrane glycoproteins. SRV has a broad cellular tropism and readily infects lymphoid, monocytic, and epithelial cells present in a wide range of tissues and fluids (68; 104). To replicate, the virus transcribes its RNA genome into double-stranded DNA by using a magnesium-dependent reverse transcriptase enzyme. The DNA integrates as provirus into a host DNA chromosome in the nucleus. Provirus RNA is transcribed into both genomic RNA as well as mRNA from which viral structural proteins are made to then express the retrovirus. The viral capsid is assembled as an intracytoplasmic A particle before migration to the plasma membrane from which a virion buds (68; 104). The exogenous serotypes have been associated with active infection and disease in their natural hosts, while the endogenous types have not.

Asian macaques commonly used in biomedical research are among the natural hosts of exogenous SRV. The first reported prototype SRV was isolated from rhesus monkey mammary carcinoma tissue in 1970 and is known as Mason Pfizer monkey virus or SRV-3 (68). Since that time and as listed in Table 2, at least five genetically closely-related

serotypes have been isolated from macaques and at least partially sequenced (119). SRV serotypes 1, 3, and 5 tend to predominate in Indian and Asian origin *M. mulatta* and SRV-2 is most common in Southeast Asian *M. fascicularis* and *M. nemestrina* (69). Sequence data for SRV-4 isolated from cynomolgus macaques in captive US colonies and SRV/D-T isolated from Asian cynomolgus macaques show a high degree of homology (37; 119). Although the five SRV serotypes share at least some serologic cross-reactivity and tissue culture characteristics, each can be distinguished by antibody neutralization and nucleic acid sequencing (e.g. of PCR amplicons) (69; 114). Closely related endogenous retrovirus sequences in macaques however, can potentially confound detection of these exogenous SRVs. Other betaretroviruses such as baboon, langur and squirrel monkey endogenous viruses and exogenous SRV-6 and SRV-7 also have been reported (68; 104; 119). Cross reactivity between these somewhat less closely-related viruses and SRV serotypes 1-5 is limited, so current methods used for testing SRV serotypes 1-5 may not detect the less closely-related viruses. However, to date only SRV serotoypes 1-5 have been reported in captive macaques and targeted for elimination in SPF colony development.

Both natural and experimental SRV infections can result in a wide spectrum of diseases ranging from subclinical to severe immunodeficiency with associated opportunistic infections. Common clinical findings include anemia, granulocytopenia, lymphopenia, thrombocytopenia, diarrhea, weight loss, splenomegaly, and lymphadenopathy (31; 52; 68; 104). SRV-induced perturbations of immune responses include suppression of T and B lymphocyte function leading to the down regulation of MHC Class II antigen expression, reduced mitogen-induced proliferation, decreased immunoglobulin production, and other functional defects. Co-infections with retroperitoneal fibromatosis herpesvirus and Epstein-Barr virus-related lymphocryptovirus have been reported to result in the SRV-associated tumors such as cutaneous fibrosarcoma-retroperitoneal fibromatosis and B cell lymphomas (52). While hematologic and immunologic defects may be the most pronounced features of persistent SRV infection, the greatest concern to damaging overall colony health has been the stealth or undetected, subclinical infections that provide opportunities for transmission between infected and uninfected animals. Prolonged intervals of time between infection and expression of overt disease as well as a true asymptomatic carrier state during which virus shedding occurs have been documented (69). Although numerous instances have gone unpublished for various reasons, there are multiple accounts in the scientific literature describing studies that were compromised or lost because of undetected SRV infection in the research animals (55).

Direct animal to animal horizontal contact is the most common route of transmission. High numbers of virus particles can be found in saliva, urine, blood, lacrimal secretions, cerebrospinal fluid and breast milk (52). Contact with contaminated equipment such as tattoo needles, gavage tubes, dental instruments, and transfer boxes have also been implicated in transmission (68). Transplacental transmission has also been documented (68). The route, dose, and frequency of exposures, as well as host factors, all contribute to the variable lengths of time reported between exposure until seroconversion occurs under both natural and experimental settings (54; 104). Some animals with high proviral DNA loads in peripheral blood mononuclear cells do not appear to readily transmit the virus to other

animals whereas other animals with high levels of virus or viral RNA do so more easily. This led to the suggestion that animals with high proviral DNA loads are able to more effectively neutralize the virus through cellular or humoral immune responses (69). The prevalence rates of SRV infection in macaque colonies range from 0% – 50% and can be greatly influenced by the geographic origin of the monkeys, testing program, management, and husbandry practices (68; 104).

The detection and elimination of SRV is one of the primary goals of SPF colony development. Since 1989, diagnostic testing and removal strategies have been employed and proven effective in reducing and eliminating this exogenous retrovirus in nonhuman primate colonies. Accurate testing to initially identify and then regularly survey all animals for infection is required and must be complemented with a management system that prevents any direct or indirect contact between uninfected and infected or untested animals (57). Testing for SRV infection requires assays that both detect antibody and that detect virus because these biomarkers (i.e. antibody and virus) wax and wane over time, often in opposition to each other and are not always detectable throughout the course of infection (57). In documented cases of SRV infection, there may be individual time points at which either antibody or virus is undetectable, as illustrated in Figure 3. It has been suggested that high levels of virus and proviral DNA with concurrent low levels of antibody could be a result of immune tolerance (69). Various colonies have successfully developed and maintained SPF colonies using this strategy of strict barrier maintenance and accurate testing. Early in SPF colony derivation when the history of negative SRV tests is short or whenever new animals are introduced, follow-up testing should be performed at approximately six week intervals. After the colony has remained closed with no incidence of SRV for several years the surveillance testing interval can be extended to as long as two years. Ideally an SPF group is closed to new introductions, however if new pre-tested members are added, the entire group should remain isolated from direct or indirect contact with any other SPF animals until all members are tested negative at least twice over a minimum interval of 6 weeks (69).

Currently-utilized assays

The betaretrovirus genome is comprised of four genes: *gag*, *pro* (prt), *pol* and *env* which code for proteins that could potentially be used for diagnostic antigens. Currently only the *gag* and *env* gene products have been validated and widely used as antigens in serological tests (69; 104). Specifically, these comprise the major capsid gag protein, p27, and the transmembrane glycoprotein, gp20-gp22, that are the highly conserved immunodominant targets for SRV antibody detection and must be represented among the target antigens to ensure acceptable levels of sensitivity in the serologic diagnostic test (69). Minor capsid proteins (p55, p14, p10, p7), envelope protein (gp70), and polymerases (p51, 31) also may be included as antigens, but are less conserved and therefore induce less cross-reactive antibodies between serotypes. In the recent testing of serum panels exchanged by the Breeding Colony Management Consortium (BCMC), the participating laboratories reported equivalent, accurate test results using carefully chosen and validated recombinant and / or viral lysate proteins. Contamination from host cell antigens or other artifacts produced during virus antigen propagation however, may contribute to poor specificity caused by the

detection of non-specific antibodies so careful interpretation is required that also depends upon comparisons between results of test specimens and those of the positive and negative controls.

Several immunoassay platforms have been successfully used as SRV antibody detection screening tests. Earlier work primarily used the classical ELISA methods, and these continue to be valid screening tests. However, as additional infectious agents were included for elimination from the SPF colonies, the newer simultaneous multiplex detection assays such as the liquid or solid phase arrays, have proven to be at least as accurate as the single ELISA tests as well as more efficient and cost effective (48; 49; 59). IFA and WB methods are two suitable choices for confirmatory antibody tests. If a WB test result does not detect reactivity to any viral proteins the sample is interpreted as seronegative. If there is reactivity to both the core p27 and env gp20-22 or gp70 (although true gp70 in the absence of gp20 is rare) it is interpreted as antibody positive. Intermediate reactivity patterns may be due to either true infection or non-specific cross reactivity and cannot be interpreted as either positive or negative. The most common indeterminate pattern is reactivity to only p27 gag proteins, and true infection has very rarely been documented from such samples. Complete band pattern reactivity indicative of infection has been documented in subsequent samples from animals whose initial sample only demonstrated gp20-22 reactivity. The confirmatory test result supersedes the screening test result. For example, if the confirmatory test is negative, the final antibody test result is considered seronegative, regardless of the screening test result.

Virus culture and nucleic acid gene amplification have been used for virus detection (53; 57; 114). Low numbers of infected cells, low virus copy numbers, and a six week turn-around time to ascertain negative test results are major challenges to diagnose SRV infection by virus culture methods (57). Amplification of DNA or RNA by PCR thus is considered a faster and more sensitive alternative (114). Some validation work has been performed and good sensitivity has been achieved by PCR using pools of 8 to 10 samples. If any signal is detected in a pooled sample, each specimen in that group would then require individual testing. This technique, however, can only be recommended for stable closed groups with a long history of no infections. As with antibody testing, the rigorous validation of the methods, reagents, and interpretative criteria in the population being tested is critical.

SRV testing algorithm

An updated version of a previous, successful testing algorithm (57) is illustrated in Figure 4. All samples initially should be screened for antibody using a single target or multiplex immunoassay. Using a cut-off or endpoint result based on optimized maximum sensitivity, all non-reactive results falling below the cut-off value are interpreted as antibody negative and those samples must then subjected to PCR testing. If reactivity is detected in the initial antibody screening, confirmatory antibody testing by WB or IFA is required. If the confirmatory test is negative, the antibody result is considered seronegative and the specimen of that animal, along with specimens from all the antibody-screened negative animals then undergo PCR testing. If the confirmatory test for SRV antibody is positive, that animal is considered antibody seropositive indicating infection and is excluded from the SPF

colony. If the confirmatory test is indeterminate, antibody reactivity is neither confirmed nor ruled out and the most conservative interpretation would be to also exclude that animal from the SPF colony. However, if there are no contra-indications in the animal's clinical or exposure history, and since infection in antibody-indeterminate and PCR-negative animals is extremely rare, it is reasonable to retain the animal in the colony and continue PCR testing surveillance. At the PCR testing stage, all antibody-positive monkeys have already been excluded. Samples from the antibody-negative (and possibly indeterminate) monkeys are then tested by PCR. Monkeys with a PCR-positive result are excluded from the SPF colony (regardless of their antibody result). Monkeys with indeterminate PCR results should either be excluded or may be kept isolated and introduced back into the SPF colony following two complete sets of serological and PCR tests performed at a minimum interval of six weeks that produce uniformly negative results. If both the final antibody result (either based on a negative screen or a negative confirmatory test) and PCR results are negative for SRV there is no infection and the animals may be included in the SPF colony. An SRV testing algorithm requires inclusion of both antibody and virus detection tests as exemplified in this example.

Simian T-cell lymphotropic virus 1 (STLV-1)

Overview

Simian T-cell lymphotropic virus 1 (STLV-1) is a Deltaretrovirus and a C-type member of the oncornavirus subgroup of retroviruses (30; 105;). STLV-1 was discovered in the early 1980's after the isolation of human T-cell lymphotropic virus 1 (HTLV-1). STLV-1 and HTLV-1 are antigenically and genetically closely related, and are referred to collectively as primate T-cell lymphotropic viruses (PTLVs) (27; 103). Each PTLV species (PTLV 1, 2 and 3) includes both human and simian viruses, which are classified as isolates/strains and include HTLV-1, HTLV-2, HTLV-3 and STLV-1, STLV-2 and STLV-3. Morphologically, the PTLVs are indistinguishable (105). The most extensively studied nonhuman primate PTLV is STLV-1 (105). More than 25 different Old World nonhuman primate species including baboons (*Papio spp*), chimpanzees (*Pan troglodytes*), African green (*Cercopithecus aethiops*), cynomolgus macaques (*Macaca fascicularis*), and rhesus macaques (*Macaca mulatta*) have been documented with STLV-1 infections (72; 86; 103). The seroprevalence in wild and captive populations has been reported to vary considerably from 0–80% and 3–12%, respectively (17; 57; 63).

STLV-1 is highly cell-associated and demonstrates a tropism for CD4+ and CD8+ T cells (30). Cell-free virions do not play a significant role in transmission (79). Transmission is primarily through transfer of infected cells, which may occur through transfer of semen or cervical secretions during breeding, breast milk to infants, or iatrogenically through blood transfusions (27; 30; 79). Transmission increases as animals age to sexual maturity, and STLV-1 infection is predominantly seen among sexually mature animals (15; 23; 33; 67; 79; 105). In addition, transmission has been associated with wounding during aggressive social interactions in baboons (15, 105). Based on reports of stable seroprevalence in STLV1-infected colonies, horizontal and vertical transmission is not readily achieved in closed

colonies of rhesus and cynomolgus macaques (79). Maternal infant transmission does occur, but is infrequent and thought to be through breast milk (79).

STLV-1-induced overt clinical disease is extremely rare or nonexistent in macaques, but has been linked with lymphoma and lymphoproliferative disease in African nonhuman primate species (27; 72; 110; 115). Lymphoproliferative disease in African species occurs after prolonged infection (78; 83). In one report, cross species STLV-1 transmission between rhesus macaques and baboons resulted in a significant increase in the incidence of T-cell leukemia-lymphoma in baboons, which was higher than the rate seen in baboons infected with baboon strains of STLV-1 (105).

Retroviral infections, including STLV-1 infections, may manifest as latent or subclinical infections and may be reactivated or cause clinical disease after experimental procedures (93). Loss of experimental data due to latent STLV-1 infections may occur as a result of lost research subjects, increases in morbidity and mortality, confounding viral-induced clinical abnormalities and histologic lesions, alteration of physiologic parameters, and interference with in vitro assays and destruction of primary cell cultures (55; 102, 118). STLV-1 infection may affect immune responses, and even in the absence of clinically manifested disease, these altered immune responses can confound experiments and impact interpretation of results (57). Previous reports have found no significant differences in populations of cells expressing CD3, CD4, CD8, CD25, CD28, CD38, and HLA-DR in STLV-1 infected animals when compared to uninfected animals (8). Concerns about the impact of clinically silent STLV-1 infection on the pathogenesis of other viral infections have been raised in the context of animal health as well as for animals used as models in infectious disease research, particularly retroviral infection studies. Dual infection with SIV and STLV-1 has been shown to potentiate STLV-1 related disease, but appears to have no effect on SIV burden or disease progression (28; 29; 101). In STLV-1 and SRV-2 coinfections in cynomolgus macaques, SRV-2 proviral burden was increased and marked increases in immunopathological changes were seen in mesenteric lymph nodes and spleen (66). Subtle effects of subclinical viral infection may include alterations of cytokine profiles, cell surface markers, and clinical laboratory assay results (25). For example, STLV-1 infection induced the release of TNF, GM-CSF, FGF and IL6 in transformed cell lines (51). In another study, levels of IFN-gamma secreted from cultures of peripheral blood mononuclear cell (PBMC) cell cultures from STLV-1 seropositive macaques were significantly higher than from PBMC cultures from STLV-1 seronegative macaques. In addition, IL2 secretion was increased in a subset of STLV-1 infected animals when compared to controls (118). These observations of the potential impact of STLV-1 infection on immune response were primary factors in the selection of STLV-1 as one of the four viral agents targeted for exclusion in the NIH SPF breeding program for macaques (55; 57; 69).

PTLVs cannot be isolated using classical procedures such as the infection of a permanent cell line with cell-free material containing virus (105). Additionally, isolation of virus has no practical diagnostic value for purposes for establishment or maintenance of STLV-1 infection-free macaque breeding colonies. Instead, testing algorithms using ELISA, WB, and PCR appear to be adequate for identifying macaques infected with STLV-1 (57; 60). Antibody testing alone has been successfully utilized to establish and maintain STLV-1-free

breeding colonies of rhesus macaques when the algorithm incorporates a regularly-scheduled testing strategy (57). Because of its antigenic and genetic similarity, HTLV has been utilized as a surrogate antigen for the detection STLV-1 in clinical samples. Early serological screening tests for detection of STLV-1 infections by ELISA using whole cell lysates as antigen have been improved by incorporating recombinant HTLV p21e antigen which increased sensitivity over HTLV- infected cell lysates alone (55). PCR testing provides benefit when testing at a single time point to determine STLV-1 infection status in seronegative or seroindeterminate animals or in those that have not been screened regularly by ELISA (60).

STLV-1 infection-free colonies have been established and maintained by removal of antibody seropositive and/or virus- or provirus-positive animals. The cell-associated nature of this virus and low level of transmission enhance abilities to eliminate this agent from macaque breeding colonies (69). In some cases, however, relatively long periods of time have been noted between infection with STLV-1 and seroconversion (55). In addition, PCR studies in humans and nonhuman primates infected with PTLVs have shown that seroconversion does not occur, in some cases, despite detection of virus by PCR (20; 88; 120). In some cases, STLV-1 and SIV co-infected animals have remained STLV-1 antibody negative, but PCR positive for more than 43 months (60). Delayed seroconversion after infection may rarely result in infections that become apparent months to years after exposure and these animals may be missed if only antibody tests are used for surveillance. Thus, STLV-1 biology requires that consideration be given to incorporating PCR testing in newly-established STLV-1-free colonies, or those applying infrequent or irregular antibody screening.

As discussed earlier, STLV-1 is a cell-associated virus which has implications for efficiency of transmission (27; 30; 69; 79). Cases of STLV-1 antibody seroconversions in follow-up testing of seronegative animals in primary screening are very rare (<0.01%) (57). Use of repetitive antibody screening by ELISA and confirmatory testing using WB have been effective tools in establishing and maintaining STLV-1-negative macaque colonies (55; 57; 69; 93). The addition of PCR enhances detection of infection especially in antibody-indeterminate cases and in seronegative animals that are only infrequently assessed for seroconversion. The low efficiency of transmission for STLV-1 minimizes the chances of transmission to direct contacts, and as such, "hot" outbreaks are not likely to be seen with this agent in colonies that have been undergoing regular testing for STLV-1. Because STLV-1 has the potential for delayed seroconversion after infection, it may be advantageous to test all contacts by PCR for one or more rounds of testing after exposure to an infected animal.

Currently-utilized assays

The presence of specific antibodies to STLV-1 is considered a reliable indicator of infection (84; 105). Thus, serological testing, especially if repeated at regular intervals (e.g. six months) is considered sufficient for diagnosing infected animals for establishing and maintaining STLV-1 infection-free nonhuman primate colonies (55; 105). Serological tests commonly applied for routine diagnosis of infection in macaques and baboons include

ELISA used as a screening test, and WB and IFA as confirmatory tests. Since STLV types I and II share approximately 90–95% homology with HTLV types I and II, commercial HTLV-I/II ELISA and WB tests with synthetic peptides and recombinants to *env* and *gag* proteins have been applied (14; 50; 55; 57; 60; 87). STLV-1-infected cell lysates and recombinant proteins (e.g. p21e) now are available for serology and have been applied in multiplex serology platforms, as well (12; 59). Seropositive, but not type-specific specimens, exhibit antibody reactivity to at least one *env* protein (e.g. gp21, gp46 or gp62/68) and one *gag* protein (e.g. p19, p24 or p53) (3; 19; 57; 105). Reactivity to *gag* gp19-I and *env* gp46-I are specific for HTLV/STLV-I and reactivity to *env* gp46-II is indicative of HTLV/STLV-II infection. Incomplete WB reaction profiles are considered indeterminate, but may also result from infection with STLV-III or another PTLV.

Virus isolation in tissue culture is no longer routinely used for definitive diagnosis due to lower sensitivity and time constraints (55) so PCR is used instead for definitive diagnosis (55; 105). PCR to detect provirus in peripheral blood mononuclear cells (PBMC) typically targets the *tat* region, but may alternatively target and amplify *env* or *pol* gene sequences (19; 55; 60; 64; 105). PCR also is useful as a confirmatory test to resolve indeterminate serologic results, or under conditions of delayed seroconversion, particularly due to co-infection with other immunosuppressive viruses such as SIV and SRV (29; 66). It should be noted that PCR for STLV-1 only detects a specific sequence in STLV-1 and as such cannot be used to detect other PTLVs unless the detected sequence is conserved.

STLV testing algorithm

A testing algorithm for detection of STLV-1 infections to establish and maintain an STLV-1 infection-free animal colony is initiated by screening serum specimens by ELISA using antigen that includes envelope (either native or recombinant p21e) (Figure 5). Env antigen is considered necessary to provide additional sensitivity not seen with gag antigen alone (55). Commercially-available multiplex antibody detection assays employ both STLV-1-infected cell lysate and STLV-1 rp21e antigens in multiplex testing platforms that are used by several of the National Primate Research Centers for surveillance screening of their SPF colonies (1; 2). Results are defined as positive, negative, or indeterminate (borderline) relative to positive, negative, and reagent controls established by the manufacturers of these multiplex assays. Animals that produce negative serological responses may be placed into the SPF colony and should be repeat tested by serology at regular intervals of approximately every 6–12 months to verify continued STLV-1 negative status.

If any of the controls fail, or if a specimen tests positive or borderline to one or both antigens, the specimen is subjected to repeat testing. Specimens that continue to test positive or borderline are then subjected to confirmatory testing, typically via WB immunoassay. Animals that test negative by WB may be included in the SPF colony, but should continue to be tested by serology at frequent intervals. Animals that produce a positive STLV-1 WB reaction profile should be excluded from the SPF colony while those that produce an incomplete western blot reaction profile are considered indeterminate. IFA is available for testing on fixed STLV-1-infected cells to confirm infection. Animals that test positive on confirmatory tests should be excluded from the SPF colony. Animals with indeterminate

confirmatory test results should either be excluded from the SPF colony or tested by PCR to detect STLV-1 provirus in PBMC. PCR may be applied for confirmatory testing instead of WB and as an alternative in the absence of repeated multiple antibody testing. PCR testing is useful in cases where STLV-1 infection status is being determined from a single test and in cases where infrequent antibody testing is being performed. Antibody screening, confirmation, and PCR steps as described here must be incorporated into an STLV testing algorithm.

Management of SPF Macaque Breeding Colonies

The key to a successful SPF breeding colony management plan is to obtain and interpret diagnostic test results in a standard, consistent manner to accurately reflect and improve the overall characterization and health of the colony. This can be accomplished by using various assays, which follow the principles embedded in standardized testing algorithms described here for each virus. Whereas diagnostic testing is only half of the strategy required to successfully develop a SPF colony, barrier management practices also are required to prevent any direct or indirect contact between SPF and non-SPF or untested animals.

General management principles proposed to develop and maintain macaque SPF breeding colonies indicate that all animals in the SPF colony should continue to be tested for viruses once housed in stable groups or single housing (55; 57; 69). The testing frequency (quarterly, semi-annually, annually) should be determined by several factors including the prevalence of infection in the colony in general, length of time after any potential direct or indirect exposure to a non-SPF or unknown status / untested animal, status of animals introduced from outside of the colony, and the extent of practices employed to prevent infection in the colony. Managers should be aware of the potential sources of contamination should an animal seroconvert while in an SPF colony. In addition to direct animal to animal contact, potential sources of contamination that could contribute to inadvertent transmission include biological materials such as feces, urine, saliva, blood, fomites used in treatment procedure, and husbandry or operational procedures related to transport, treatment, or housing spaces.

Ideally, SPF colonies will have adequate fecundity to be self-sustaining and not require introduction of new animals from outside colonies. Dams and infants from established SPF colonies should undergo testing within the first year after birth. Colonies being derived from non-SPF stock may require earlier testing (69). In this scenario, infants at weaning should be screened before being moved to separate housing and once again four to six weeks after rehousing (115). Colonies that practice derivation from conventional nonhuman primate stocks and importation assume greater risks for infection and transmission than established, closed colonies. Thus, when unavoidable, imported or conventional animals should be appropriately quarantined and tested prior to introduction into an established SPF colony. When importation is required to increase production or genetic diversity, animals from well-characterized, validated SPF breeding colonies should be utilized.

Approaches to confirm and manage animals producing non-negative viral test results (positive or indeterminate) is an exercise in risk assessment, and should be based on several

criteria, including the transmission biology of the virus, sensitivity/specificity of the assays used for screening and confirmation, configuration of animal housing, opportunities for animal-to-animal contact, frequency and results of prior testing, and whether the colony is closed or is at risk for exposure through importation of animals. Approaches for addressing infection should be tailored based on these criteria and may vary in detail from one facility to another.

Survey findings among the NPRC SPF colonies indicate that BV, SIV, SRV, and STLV-1 uninfected animals are readily available and that the occurrence of new infections is rare. Under these circumstances a conservative approach can be taken when an exposure may have occurred. The infected animal should be immediately removed and excluded from further direct or indirect contact with other SPF animals. All other animals potentially exposed to that index animal should then be completely isolated from any direct or indirect contact with other SPF animals and retested at regular intervals until no new positive results are detected in at least two subsequent rounds of testing for at least six-to-eight weeks before re-inclusion to the SPF colony (69). If infection is suspected but not confirmed (indeterminate result), it may be possible to isolate the animal in question along with its contacts and then subject all these animals to the same testing scheme using the negative test criteria described for contacts of an infected animal. However, considering the logistical and financial costs of providing isolation and testing, as well as the potential gravity of studies lost to missed infections, it may prove cost effective to remove that entire group from the SPF program if infections continue to be suspected or detected in a group (52).

A less conservative approach may be more appropriate if animals are housed in small groups, or the results of confirmatory tests can be obtained quickly. In some cases removing an animal that is important to the social order of the group may pose a greater risk to stability of the group than if the animal remains in the colony enclosure. Thus, it is important to consider the social needs of the individual nonhuman primates in the context of the colony groups while evaluating the risk of transmission.

A team of professionals familiar with the SPF breeding colony program including veterinarians, epidemiologists, information technology/animal records staff, behavioral management professionals, animal colony program managers, geneticists, and veterinary pathologists should be involved in the process of establishing and maintaining SPF colonies. Epidemiologic data including regular colony health benchmark data (morbidity/mortality, production rate), specific health surveillance data related to the excluded agents, and physical configuration of the colony should be collected and analyzed regularly to ensure that the SPF program is performing as desired to meet the needs of the resource. The analysis of these data will provide objective information that is invaluable for assuring the success and refinement of the program. Data should be evaluated at regular intervals to evaluate long-term trends, identify the effects of management changes, and to ensure that long-term goals are being met. The plan should be assessed regularly and revised based on advancements reported in the scientific literature, improvements in best practices, and changes to program parameters.

Conclusion

Programs to develop and maintain SPF macaque colonies have existed for over 20 years. During that time, much has been learned about the biology of viruses that infect nonhuman primates, and the reliability of diagnostic assays to detect these viruses has significantly increased. These advances led to improvements in the characterization and health of macaques used for biomedical research and contributed to a decreased risk for transmission of pathogenic viral agents to personnel who work with nonhuman primates. Many SPF colonies have matured and are self-sustaining, obviating the need to derive research animals from colonies of animals with varying viral infection status.

A successful, multi-institutional, comprehensive macaque SPF breeding program is comprised of several critical components including: 1) regular use of sensitive and specific pathogen detection assays, 2) use of standardized algorithms that incorporate a combination of screening and confirmatory tests, 3) regular validation of detection assays across institutions, 4) consistent management of derivation, testing, and disposition of indeterminate and positive cases, and 5) frequent review of colony health through assessment of epidemiologic data by a team of experts representing several disciplines.

This review expresses the current philosophy and set of practical guidelines for establishing/ deriving and maintaining an SPF macaque colony based on a collaborative effort among members of the NPRCs. During the initial formation of an SPF colony there is a clear rationale to exclude any monkey with non-negative test results. Over time, the interpretation of a rare non-negative result becomes more complex. Certainly, removing any suspect animal is the most conservative approach to maintaining an SPF colony, but answers to additional questions must also be carefully considered: How long has the animal been housed in the SPF colony? What is its testing history? What is its potential exposure history? Is the colony truly closed with no possibility of direct or indirect exposure to a non-SPF or untested animal? Has the observed reactivity been confirmed by multiple tests or on multiple dates? What is the animal's social rank in the group and will removing it de-stabilize the group? How much risk can be tolerated? Are the costs justified? Is a positive test result truly indicative of infection that threatens the SPF status of the group? Each potential exposure incident requires a careful, specific analysis and interpretation of the answers to these questions using the guidelines presented herein.

Acknowledgments

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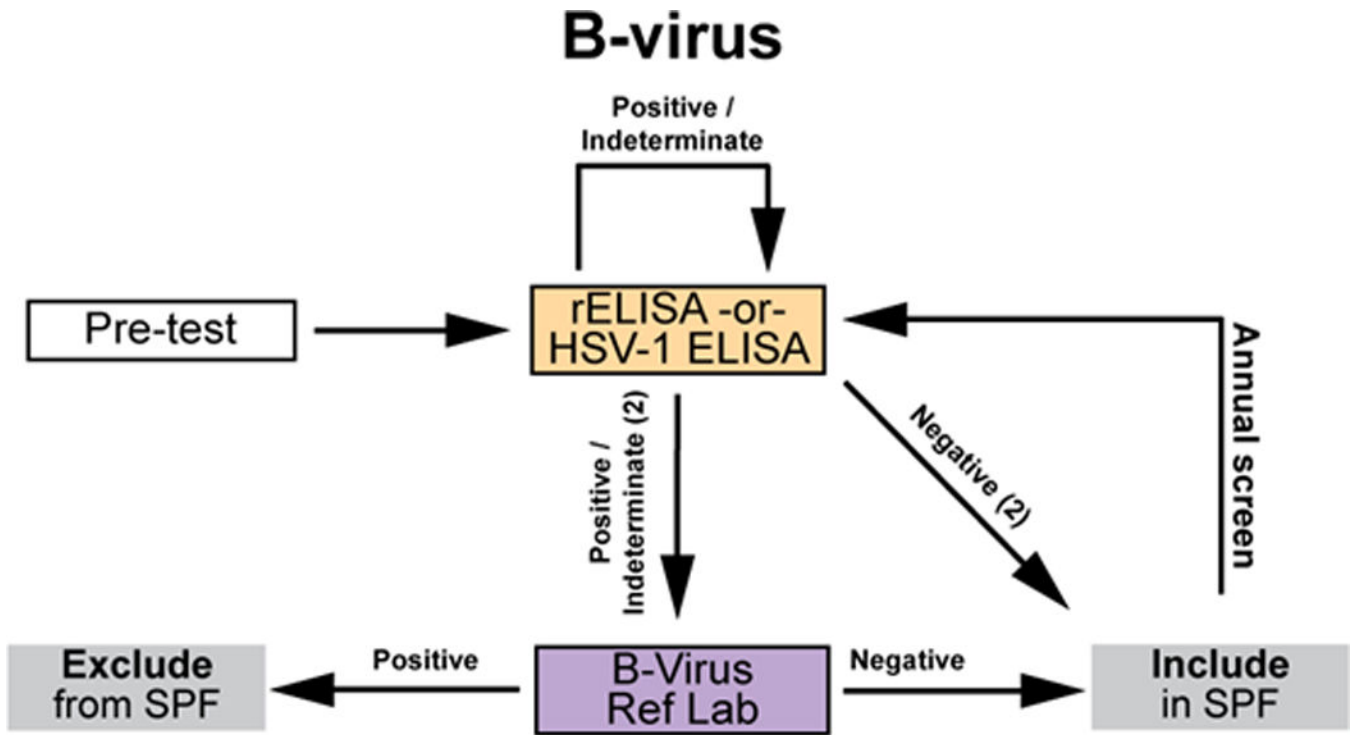
Figure 1.
B virus testing algorithm. This example uses rELISA which includes *Macacine herpesvirus 1* recombinant antigen or HSV-1 ELISA which includes human *Herpes simplex virus 1* antigen. An alternate algorithm successfully uses Herpes virus papio type 2 in either an ELISA or multiplex format. Neg/Pos(2) indicates two consecutive negative or positive tests.
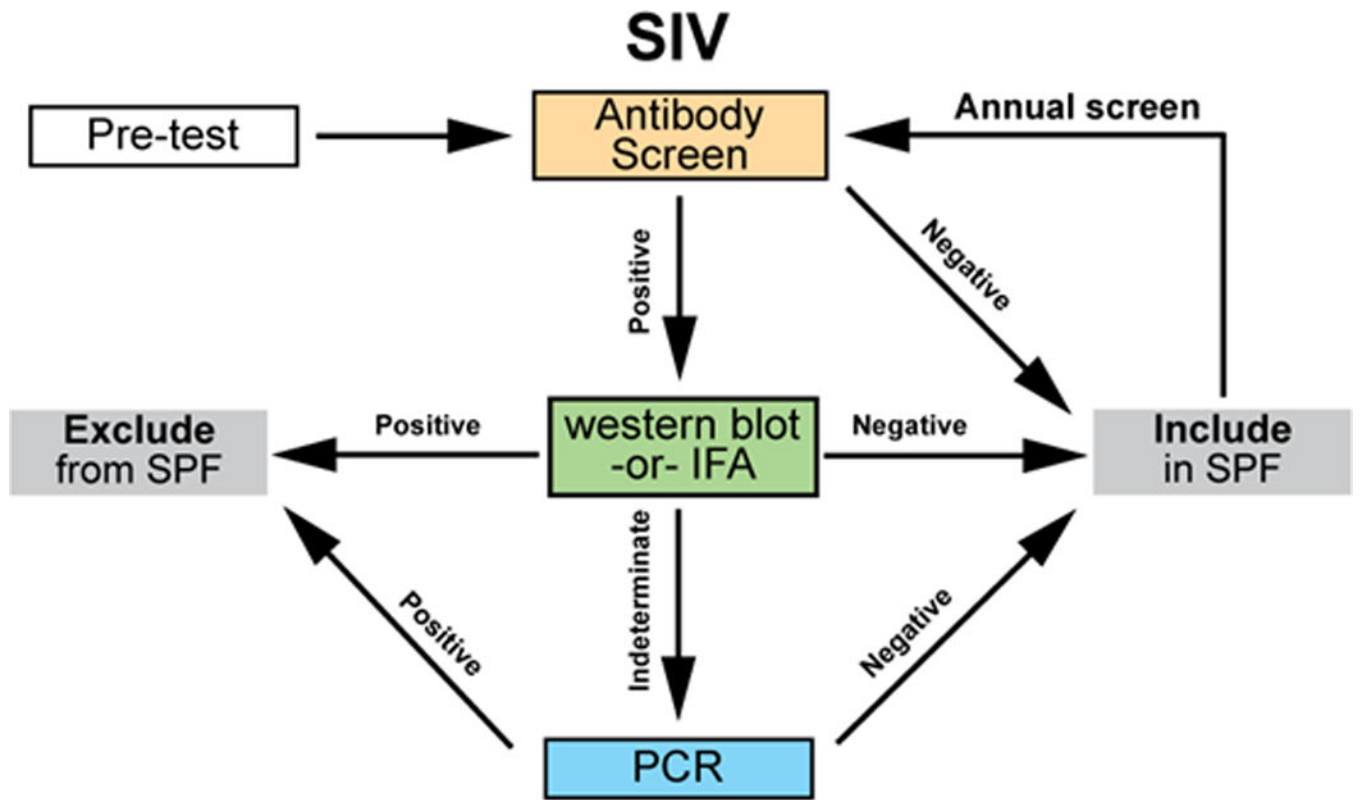
Figure 2.
SIV testing algorithm

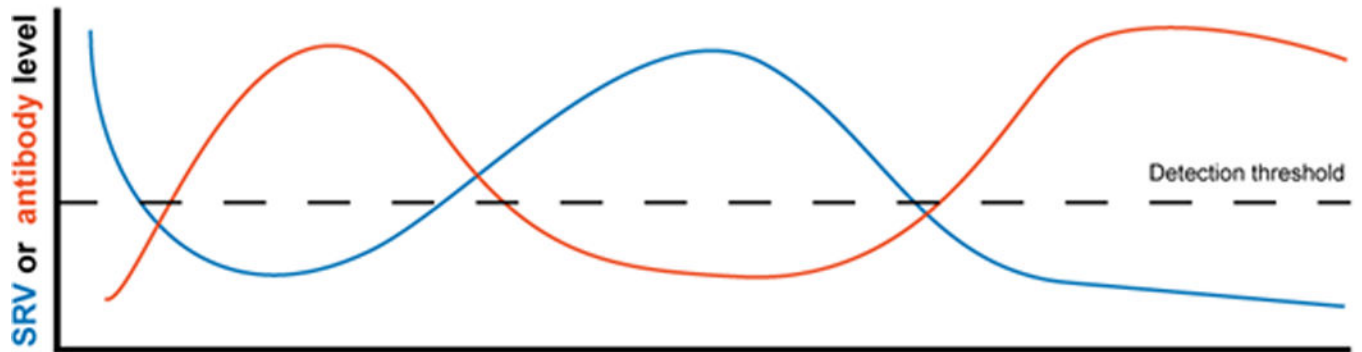| Collection Date	10/92	8/93	11/93	12/93	3/97	12/97	3/99
Antibody	(−)	(+)	(+)	(−)	(+/−)	(+)	(+)
Virus Isolation	(+)	(+)	(−)	(+)			
PCR				(+)	(+)	(−)	(−)

Figure 3.
Example of temporal SRV test results shifts in an infected pig-tailed macaque.
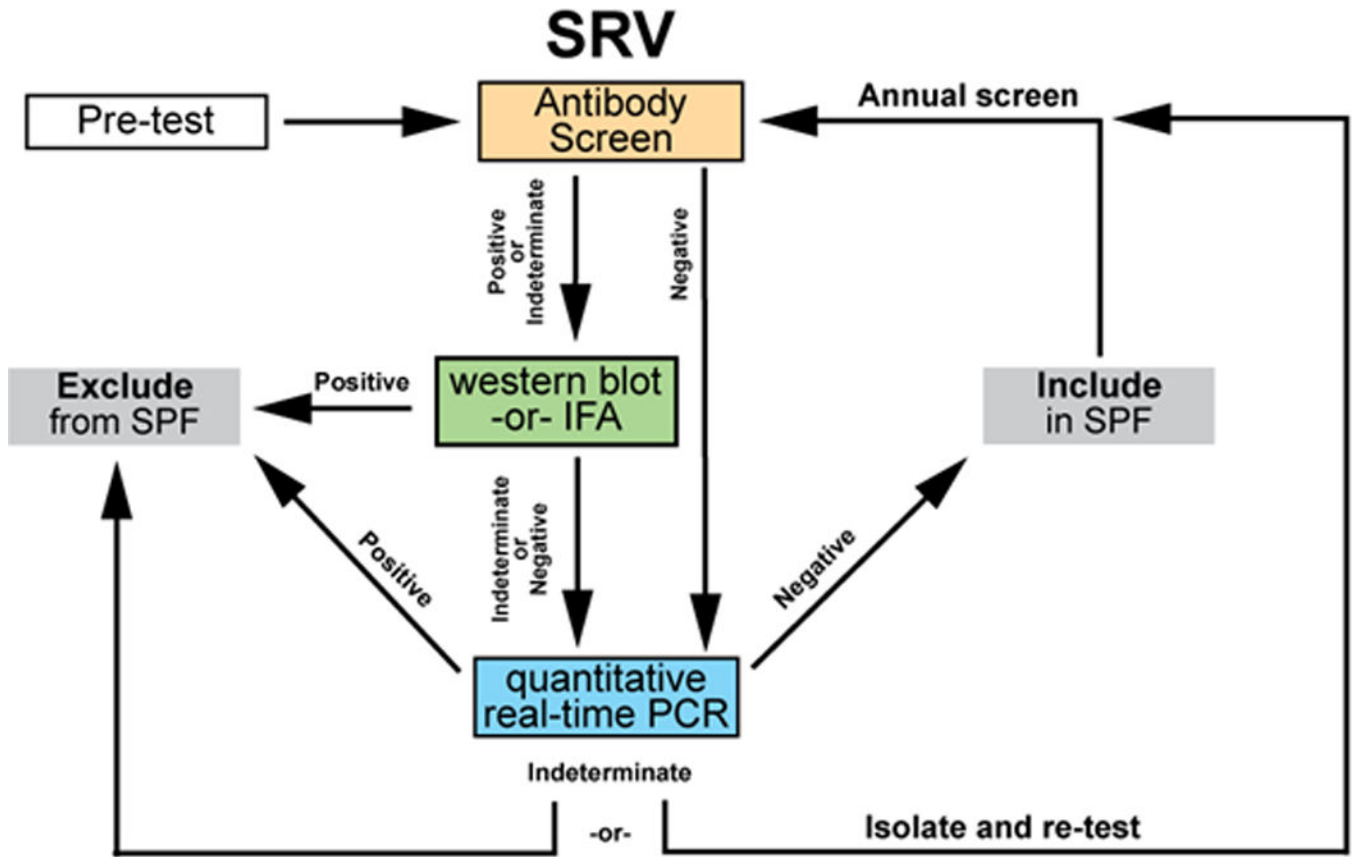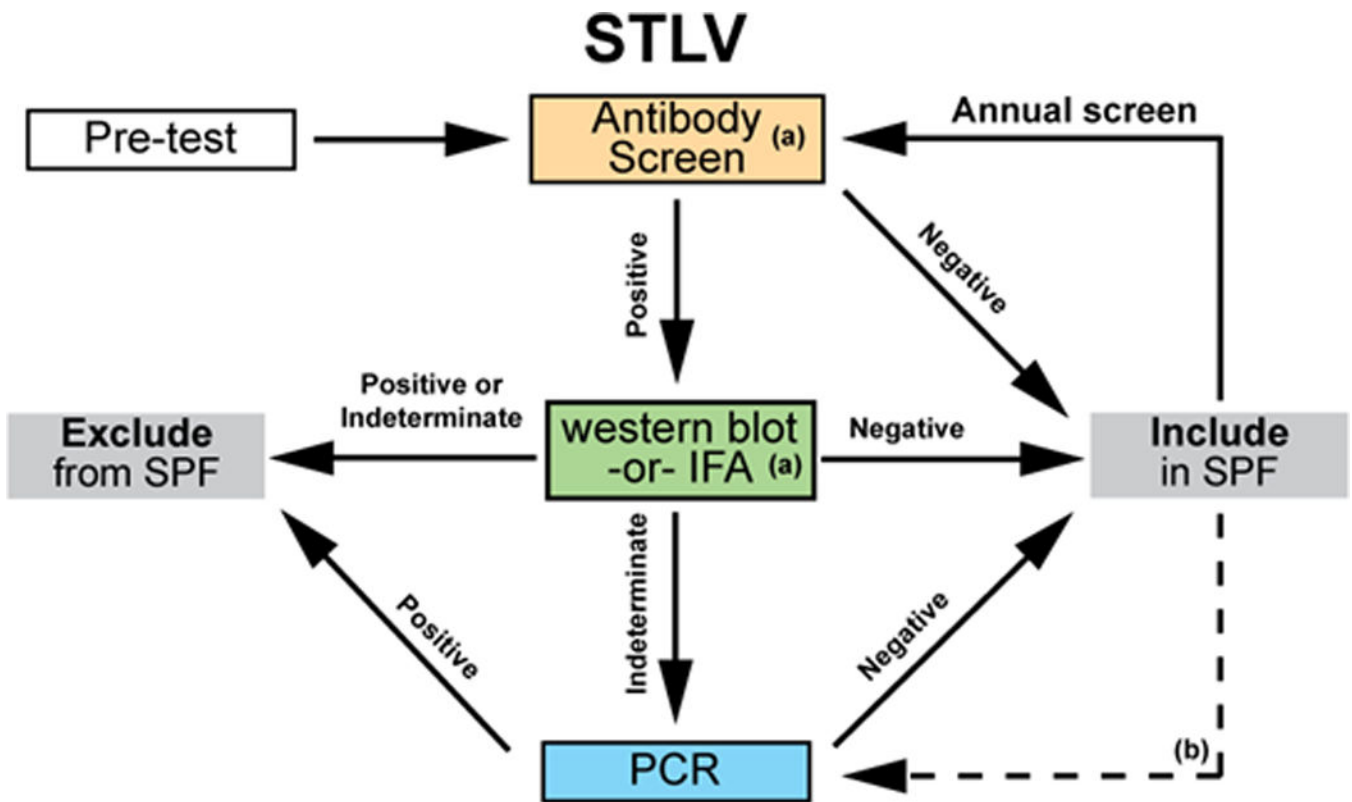
Figure 4.
SRV testing algorithm

Figure 5.
STLV-1 testing algorithm. a) Testing for antibody (EIA, western blot) is enhanced when using assays containing HTLV recombinant p21e antigen. Early detection of STLV1 infections by EIA is improved by incorporating recombinant HTLV p21e antigen, which demonstrates increased sensitivity over testing that uses HTLV-infected cell lysates without recombinant antigens. b) PCR may be used to augment serology or when serology is not performed frequently or when a single serologic test is being used for selection for inclusion into a SPF colony. Seroconversion may not occur for months to years after infection so PCR testing of seronegative and seroindeterminate samples is warranted in the absence of antibody testing at multiple time points. c) In this example WB or IFA is used as the confirmatory test for non-negative antibody screening tests. To reduce non-specific viral lysate reactivity concerns, some algorithms have preferred and successfully incorporated PCR at this step. (Adapted from: References 55 and 57).

Table 1

Aggregate BV, SRV, SIV, and STLV antibody testing results from NPRC SPF Laboratory Proficiency Testing Program (Proficiency Exchange Panel 3). Samples were characterized as either positive or negative based on the information provided by the submitting participant or by the consensus results reported from all participants. The number of tests performed per virus and per sample varied in each laboratory.

	Virus			
	BV	SIV	SRV	STLV-1
Number of Positive Samples	11	6	7	6
Total Number of Test Results	88	48	56	48
Number of Positive Test Results	84	48	52	48
Number of Non-positive Test Results	4	0	4	0
% Agreement	95	100	93	100
Number of Negative Samples	12	17	16	17
Total Number of Test Results	96	136	139	136
Number of Negative Test Results	94	118	130	133
Number of Non-negative Test Results[1]	2	18	9	3
% Agreement	98	87	94	98

[1] Includes screening results without confirmatory testing in some cases, as not all participants had in-house confirmatory testing capabilities

Table 2

SRV serotypes and their common natural hosts and geographic origins.

Serotype	Host	Geographic Origin
SRV1	*M. mulattta*	Southeast Asia, India
SRV2	*M. fascicularis*	Southeast Asia
	M. nemestrina	
SRV3 (MPMV)	*M. mulatta*	India
SRV4/ SRVD-T	*M. mulatta*	Asia
SRV5	*M. mulatta*	Southeast Asia
SRV6	*S. entellus*	India
SRV7	*M. mulatta*	India

In addition to the exogenous viruses listed above, endogenous betaretroviruses have also been reported in macaques, baboons, langurs, and squirrel monkeys,