Published in final edited form as:

AIDS Res Hum Retroviruses. 2001 October 10; 17(15): 1455–1466. doi:10.1089/088922201753197123.

Titration of an SIVmac251 Stock by Vaginal Inoculation of Indian and Chinese Origin Rhesus Macaques: Transmission Efficiency, Viral Loads, and Antibody Responses

Marta L. Marthas^{1,2}, Ding LU¹, M.C.T. Penedo³, Andrew G. Hendrickx¹, and Christopher J. Miller^{1,2,4}

¹California Regional Primate Research Center, School of Veterinary Medicine, University of California, Davis, California 95616

²Department of Veterinary Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, California 95616

³Veterinary Genetics Laboratory, School of Veterinary Medicine, University of California, Davis, California 95616

⁴Center for Comparative Medicine, School of Veterinary Medicine, University of California, Davis, California 95616

Abstract

The purpose of this study was to determine whether rhesus monkeys of Chinese origin are suitable for studies of mucosal lentivirus transmission by comparing the relative ability of these animals and rhesus macaques of Indian origin to become infected by vaginal (IVAG) inoculation with SIVmac251. In addition, we sought to test the hypothesis that differences in viral load during the first few weeks after inoculation were due to the relative strength of the anti-SIV immune responses in the two populations of rhesus macaques. Significant difference was not observed between the number of Indian and Chinese origin monkeys that were infected after IVAG SIV inoculation in this study. For 8–9 weeks after infection there was considerable overlap in the range of viral loads among the Indian and Chinese animals and the variation among the Indian origin animals was greater than the variation among the Chinese origin monkeys. By 6 weeks postinfection, viral loads in SIV-infected Chinese origin monkeys tended to be at the lower end of the range of viral loads observed in SIV-infected Indian origin monkeys. The strength of the anti-SIV antibody response was also more variable in the Indian origin rhesus macaques, but at 6–8 weeks postinfection, Chinese and Indian origin rhesus macaques had similar titers of anti-SIV antibodies. Microsatellite allele frequencies differed between Chinese and Indian rhesus macaques; however, the majority of alleles present in Indian-origin animals were also found in Chinese macaques. Together these results show that host factors, other than geographic origin, determine the ability of a rhesus macaque to be infected after IVAG SIV exposure and that geographic origin does not predict the viral load of SIV-infected animals during the first 8–9 weeks after IVAG inoculation.

Introduction

The Use of Rhesus Monkeys in AIDS research has led to a growing demand for these animals. This demand has outstripped the supply of domestically bred animals, forcing

researchers to consider using monkeys from other sources. Most of the domestically bred rhesus macaques are derived from monkeys originally imported from India. Because it is no longer possible to import animals from India, importing monkeys from China seems to be the most reliable option for obtaining additional animals. However, there is some evidence that the clinical course of SIV infection is slower and more variable in Chinese origin monkeys compared with Indian origin monkeys.¹ The goal of this study was primarily to determine whether Chinese origin rhesus monkeys are suitable for studies of mucosal lentivirus transmission or whether they were more inherently resistant to infection after IVAG SIV inoculation compared with Indian origin rhesus macaques.

We observed no significant difference in the number of SIV-infected Indian and Chinese monkeys after vaginal SIV inoculation in our study. However, infected Chinese origin monkeys tended to have lower viral loads by 6 weeks postinfection compared with infected Indian origin monkeys. During the 8- to 9-week observation period there was considerable overlap in the range of viral loads among the Indian and Chinese animals. In addition, the variation in plasma SIV RNA levels and anti-SIV antibodies was greater among Indian origin animals than among Chinese origin monkeys at 6–8 weeks postinfection. Only 4 of 13 highly polymorphic microsatellite loci had allele frequencies that were sufficiently different between Chinese and Indian origin macaques to be useful in identifying the geographic origin of individual animals. We found no microsatellite alleles that were diagnostic for Chinese or Indian origin. Together these results support the notion that geographic origin neither determines the ability of a rhesus macaque to be infected after intravaginal (IVAG) SIV exposure nor predicts the viral load or anti-SIV antibody responses of SIV-infected animals during the first 8–9 weeks after IVAG inoculation. However, to estimate the complete range of differences in response to SIV infection between Chinese and Indian origin rhesus macaques will require long-term studies that assess viral loads and immune responses over the course of infection from the time of inoculation to the onset of immunodeficiency.

Materials and Methods

Animal inoculations and virus stock

All the animals used in this study were adult, multiparous female rhesus macaques. The Indian origin rhesus macaques were colony bred at the California Regional Primate Research Center (CRPRC, Davis, CA). The Chinese origin rhesus macaques were imported from a primate center in Kunming Province, People's Republic of China. Before beginning the study, the animals were observed for 3 months at the CRPRC and regular menstrual cycles were documented. All animals were seronegative for HIV-2, SIV, type D retrovirus, and simian T cell lymphotropic virus type 1 at the beginning of the study. At the time of SIV inoculation the age range of animals was 3.9 to 16.1 years (Table 1) and their weight range was 4.0 to 9.2 kg. The animals were housed in accordance with American Association for Accreditation of Laboratory Animal Care standards. We adhered to the *Guide for Care and Use of Laboratory Animals*.² When necessary animals were anesthetized with ketamine hydrochloride (10 mg/kg; Parke-Davis, Morris Plains, NJ) injected intramuscularly.

Rhesus macaques were inoculated intravaginally with 1 ml of undiluted SIVmac251-5/98 virus stock containing 1×10^5 TCID₅₀ (50% tissue culture infective doses)/ml and 4×10^9 copies of RNA per milliliter. This SIVmac251 stock was produced by short-term expansion of an older virus stock on rhesus peripheral blood mononuclear cells (PBMCs). The technique used for intravaginal inoculation has been published.^{3,4} The animals were inoculated in experimental groups without regard for the stage of the menstrual cycle. In this study, and in retrospective analysis of a large number of rhesus macaques, we have not observed that stage of the menstrual cycle is associated with differences in the ability of

animals to be come infected after IVAG inoculation with SIV (C.J. Miller, unpublished results). Blood samples were collected into heparinized tubes at weeks 0, 2, 4, 6, 8, and 10 postinoculation.

***In vitro* titration of SIVmac251-5/98 stock on primary PBMCs from Indian and Chinese origin rhesus monkeys**

PBMCs were isolated from the blood of SIV-naïve rhesus monkeys as described above. The cells were cultured until the cell numbers had doubled (7–10 days) in complete RPMI 1640 containing recombinant human interleukin 2 (IL-2, 50 U/ml; Cetus, Emeryville, CA) and *Staphylococcus* enterotoxin A (0.5 µg/ml; Toxin Technologies, Sarasota, FL). We have demonstrated that this method reliably stimulates rhesus PBMCs as needed to propagate a variety of SIVmac isolates.^{5–7} To titrate the virus stock, the activated PBMCs were resuspended in RPMI at a concentration of 2×10^6 cells/ml and 100 µl of the cell suspension was added to wells of a 96-well plate. Serial 10-fold dilutions of the virus stock (100 µl) were added directly to wells in quadruplicate. The virus stock dilution series ran from 10^{-1} to 10^{-5} . Thus, on the basis of the TCID₅₀ of this stock in CEMx174 cells, the multiplicity of infection (MOI) in the dilution series used to infect primary PBMCs ranged from 0.5×10^{-2} to 0.5×10^{-6} . The cultures were maintained for 1 week without changing the medium and then aliquots of medium were assayed for the presence of SIV major core protein (p27) by antigen capture enzyme-linked immunosorbent assay (ELISA).^{7,8} Each well was scored as positive or negative for p27 and the TCID₅₀ of the stock in an individual animal's PBMCs was calculated by the method of Reed and Muench.⁹ PBMCs from five Indian origin and five Chinese origin rhesus macaques were tested in this assay. Note that the PBMC TCID₅₀ assay described above differs from the standard titration assay to generate the TCID₅₀ in CEMx174 cells. Although the MOIs tested were the same, for titration in CEMx174 cells, the culture volume is expanded to 5 ml in flasks and media are changed every 3–4 days for 4 weeks. Thus, the TCID₅₀ values generated in the PBMC assay and the CEMx174 assay are not directly comparable.

Virus isolation

PBMCs were isolated from whole blood of SIV-inoculated animals by Ficoll gradient separation (lymphocyte separation medium; Organon Teknika, Westchester, PA) and cocultured with CEMx174 cells as previously described.⁸ Aliquots of the culture medium were assayed regularly for the presence of SIV major core protein (p27) by antigen capture ELISA.^{7,8}

SIV provirus PCR analysis

Nested polymerase chain reaction (PCR) was carried out on genomic PBMC DNA in a DNA thermal cycler (Perkin-Elmer Cetus) as previously described.¹⁰ Briefly, cryopreserved PBMCs isolated from whole blood of each monkey in the experiment were washed three times in Tris buffer at 4°C and resuspended at 10^7 cells/ml. Ten microliters of the cell suspension were added to 10 µl of PCR lysis buffer (50 mM Tris-HCl [pH 8.3], 0.45% Nonidet P-40 [NP-40], 0.45% Tween 20) with proteinase K (200 µg/ml). The cells were incubated for 3 hr at 55°C, followed by 10 min at 96°C. Two rounds of 30 cycles of amplification were performed on aliquots of plasmid DNA containing the complete genome of SIVmac1A11 (positive control) or aliquots of cell lysates under conditions described elsewhere.¹⁰ The primers used specifically amplify SIV *gag*. DNA from uninfected CEMx174 cells was amplified as a negative control in all assays to monitor potential reagent contamination. β-Actin DNA sequences were amplified by two rounds of PCR (30 cycles per round) from all PBMC lysates to direct potential inhibitors of *Taq* polymerase. After the second round of amplification, a 10-µl aliquot of the reaction product was removed and run

on a 1.5% agarose gel. Amplified products in the gel were visualized by ethidium bromide staining. Blood samples for PCR analysis were collected at the times indicated in Table 1.

Assessment of plasma SIV RNA levels

SIV RNA in plasma and in the SIVmac251-5/98 stock were quantified by an SIV-specific branched DNA (bDNA) signal amplification assay.¹¹ This assay is similar to the Quantiplex HIV RNA assay except that target probes were designed to hybridize with the *pol* region of the SIVmac group of strains including SIVmac251 and SIVmac239. SIV *pol* RNA in plasma samples was quantified by comparison with a standard curve produced with serial dilutions of cell-free SIV-infected tissue culture supernatant. The SIV RNA in tissue culture fluid was quantified by comparison with a standard curve of purified DNA-free, *in vitro*-transcribed SIVmac239 *pol* RNA. SIV RNA associated with viral particles was measured after concentration from 1 ml of heparinized plasma by centrifugation ($23,500 \times g$ for 1 hr at 4°C). The lower limit of detection of the assay was 1500 copies of SIV RNA per milliliter of plasma.

Detection of plasma SIV-specific antibodies

Plasma samples were collected at 0, 6, and 8 weeks postinfection and tested for the presence of anti-SIV IgG antibodies; SIV IgG antibody titers were then determined for positive samples as described.¹²

Genetic assessments

We screened rhesus macaques for the presence of the major histocompatibility complex (MHC) class I alleles *MamuA*01* and *MamuB*01*, using a PCR-based technique.^{13,14} Genetic variation in the Chinese and Indian origin macaques was assessed by DNA testing of 13 microsatellite loci distributed throughout 10 of the 21 chromosomes of the rhesus macaque genome. Standard, multiplexed PCR-based DNA genotyping methods were used. 15 Macaque microsatellites were assayed from genomic DNA extracted from whole blood collected in anticoagulant EDTA. Heterologous human primers for loci *D3S1768*, *D6S276*, *D6S291*, *D6S1691*, *D7S513*, *D7S794*, *D8S1106*, *D10S1412*, *D11S925*, *D13S765*, *D16S403*, *D17S804*, and *D18S572* were used for PCR amplification. Primers were obtained from Research Genetics (Huntsville, AL). For purposes of multiplexing markers in PCR tests, primers for *D6S276* (Z16711), *D11S925* (Z17002), *D17S804* (Z17033), and *D18S72* (Z17153) were redesigned from human clone sequences deposited in GenBank (available from the Internet: URL <http://www.ncbi.nlm.nih.gov/>) (M.C.T. Penedo, unpublished). Numbers in parentheses are GenBank accession numbers. Genotypes for each locus were used to calculate number of alleles detected, frequency of most common allele and heterozygosity. This panel of microsatellite markers has been validated by using rhesus macaques of known pedigree.^{16–18}

Statistical analysis

To assess differences in susceptibility among Chinese and Indian rhesus macaques to SIV infection after one or two intravaginal inoculations, logistic regression analysis was performed for the number of SIV-infected animals in each of the two categories (geographic origin and number of intravaginal inoculations). All tests of the levels of SIV RNA in plasma were made on log-transformed values of SIV RNA copy number for each animal. Comparisons of viral load in plasma over the entire observation period between SIV-infected Chinese and Indian rhesus macaques were performed with a mixed model, repeated measures analysis of variance (ANOVA). Comparisons of viral load in Indian and Chinese origin animals at specific times after SIV infection were done by using *t* tests with Welch's correction.^{19,20}

For the statistical analyses of the outcome of SIV inoculation, results were considered significant if the probability (p) of obtaining a result at least as extreme as the observed result was less than 0.05 (i.e., $p < 0.05$). The probability values reported are for two-tailed tests. The SAS (version 8.0; SAS, Cary, NC) and InStat (version 2.03 for the Mac; GraphPad Software, San Diego, CA) statistical software packages were used for these analyses. One-tailed paired-sample t tests²¹ were used to test the significance of differences between Chinese and Indian monkeys for average number of alleles per locus and heterozygosity.

Results

PBMCs from Chinese and Indian origin rhesus macaques are equally susceptible to SIV infection *in vitro*

In an *in vitro* study, the SIVmac251-5/98 stock replicated in PBMCs from all six Indian origin and all six Chinese origin rhesus monkeys (Fig. 1). None of these 21 animals was subsequently inoculated IVAG with SIV. There was considerable variability in the viral p27 antigen levels produced and the p27 concentration was dependent on the specific PBMC donor used. However, the variability and range of p27 concentration produced by PBMCs from the Indian and Chinese origin monkeys were similar. Thus, there was no evidence of any difference in the levels of SIV replication in PBMCs from Chinese or Indian rhesus monkeys *in vitro*.

IVAG inoculation is equally efficient in Chinese and Indian rhesus macaques

After a single IVAG inoculation with 1 ml of the undiluted SIVmac251-5/98 stock, 3 of 6 Chinese origin rhesus monkeys (50%) and 8 of 12 Indian origin rhesus monkeys (67%) became virus isolation positive (Table 1). After two IVAG inoculations (spaced 4 hr apart) with 1 ml of the undiluted SIVmac251-5/98 stock, seven of eight Chinese origin rhesus monkeys (88%) and eight of eight Indian origin rhesus monkeys (100%) became virus isolation positive (Table 1). The results of PCR for proviral SIV *gag*, using genomic DNA extracted from PBMCs, uniformly confirmed the virus isolation results (data not shown). Although the results of IVAG inoculation could be interpreted to suggest a trend toward a slightly decreased ability to be SIV infected in Chinese origin monkeys compared with Indian origin animals, the difference is not statistically significant ($p = 0.290$). SIV transmission was significantly more efficient in all rhesus monkeys, Chinese and Indian, IVAG inoculated twice compared with monkeys inoculated only once ($p = 0.036$).

Plasma viral RNA levels differ in SIV-infected Chinese and Indian rhesus macaques

Regardless of geographic origin, peak plasma SIV RNA levels in SIV-infected animals occurred at week 2 postinfection in 25 of 26 monkeys with samples available at all study time points (Fig. 2). In one Chinese origin animal (31410) SIV RNA did not peak in plasma until week 4 postinfection (Fig. 2D). The range in peak plasma levels was greater for Chinese origin animals (1.1×10^5 to 1.2×10^8 copies of SIV RNA per milliliter) than for Indian origin animals (1.1×10^7 to 3.4×10^8 copies of SIV RNA per milliliter). Although the mean peak level of SIV RNA in plasma was 2-fold higher for Indian origin animals (5.0×10^7 copies of SIV RNA per milliliter) than for Chinese origin animals (2.5×10^7 copies of SIV RNA per milliliter) (Fig. 3A), these means were not significantly different (Table 2, $p = 0.094$).

The range in plasma SIV RNA levels at 6 weeks postinfection was greater for Indian origin animals (1.3×10^5 to 5.9×10^7 copies of SIV RNA per milliliter) than for Chinese origin animals (5.4×10^3 to 8.2×10^5 copies of SIV RNA per milliliter). In addition, the mean level of SIV RNA in plasma at 6 weeks postinfection was 25-fold higher for Indian origin macaques (1.5×10^6 copies of SIV RNA per milliliter) than for Chinese origin animals (6.3

$\times 10^5$ copies of SIV RNA per milliliter) (Fig. 3A); these means were significantly different (Table 3, $p < 0.001$). Viral RNA levels in plasma of one Chinese origin monkey (31410) never exceeded 5.1×10^5 copies/ml (Fig. 2D); this pattern of low plasma viral load was not observed for any of the SIV-infected Indian origin macaques.

As previously observed for SIV-infected rhesus macaques, SIV RNA levels in plasma decreased from 2 weeks to 6 weeks postinfection in most animals (Figs. 2 and 3B). A greater range of decline in SIV plasma RNA levels from 2 weeks to 6 weeks postinfection was found among Indian origin macaques (Fig. 3B). However, the mean reduction in SIV plasma RNA levels from 2 weeks to 6 weeks postinfection (Fig. 3B) was significantly greater (Table 3, $p = 0.020$) for animals of Chinese origin (~100-fold decrease) compared with animals of Indian origin (~25-fold decrease). Thus, Chinese origin macaques tended to have lower levels of SIV RNA in plasma at 6 weeks postinfection compared with those of Indian origin animals. Nevertheless, the lower mean levels of SIV RNA observed in plasma of SIV-infected Chinese origin macaques at 6 weeks postinfection cannot be used to infer that viral loads several months later in infection would also have been lower compared with SIV-infected Indian origin animals.

Antibody responses to SIV infection

Of the 10 Chinese origin rhesus macaques that became SIV infected, 9 developed detectable serum anti-SIV antibody responses by 8 weeks postinfection (Table 1). Similarly, 15 of 16 of the SIV-infected Indian origin rhesus monkeys had detectable serum anti-SIV antibody responses by 8 weeks postinfection (Table 1). The range of antibody titers in SIV-infected Indian origin rhesus monkeys was highly variable (200–800,000), spanning a 4000-fold range. Rising titers of SIV-specific antibodies levels were not observed for two SIV-infected Indian origin animals during the course of this study. From 6 weeks to 8 weeks postinfection SIV antibody levels in plasma remained low (200) for monkey 28336 and declined 10-fold (32,000 to 3200) for monkey 28418.

Compared with Indian origin animals, the anti-SIV antibody titers among the SIV-infected, Chinese origin rhesus macaques during the first 8 weeks postinfection were more consistent (16,000–800,000), differing by 10- to 50-fold. The two SIV-infected monkeys that failed to mount anti-SIV antibody responses rapidly developed severe clinical AIDS. Chinese origin monkey 31415 was killed at 6 weeks postinfection with severe nonresponsive enterocolitis and wasting, and Indian origin monkey 31168 was killed at 12 weeks postinfection with *Pneumocystis* pneumonia. Thus, rapid progression to AIDS can occur in both Chinese and Indian origin monkeys that fail to develop anti-SIV immunity.

Genetic diversity among rhesus macaques from different ancestral countries and impact on SIV infection

Screening for the presence of two well-characterized alleles of rhesus macaque MHC class I region, *MamuA*01* and *MamuB*01*, was done to verify reports that these alleles are rare or absent in rhesus macaques of Chinese origin. In populations of Indian origin rhesus macaques the frequency of *MamuA*01* is 20 to 25% and the frequency of *MamuB*01* is 25 to 50% (D. Watkins, personal communication; and M.L. Marthas and M.C.T. Penedo, unpublished results). In this study, *MamuA*01* was not detected in 10 Chinese origin macaques and was present in only 3 of 16 Indian macaques; *MamuB*01* was found in 1 Chinese origin animal and 7 Indian origin animals (Table 4). These observed frequencies of *MamuA*01* (19%) and *MamuB*01* (44%) were similar to expected frequencies for Indian origin macaques. The *MamuA*01* and *MamuB*01* alleles were rare in Chinese origin macaques, consistent with previous findings (D. Watkins, personal communication). In addition, there was no association of resistance to infection after IVAG SIV inoculation or

low plasma viral load after SIV infection in animals carrying *MamuA*01* or *MamuB*01* alleles. The one Chinese origin macaque positive for *MamuB*01* (31406) had a peak viral load (3.8×10^7 copies of SIV RNA per milliliter) that fell within the range of peak viral load for the seven *MamuB*01*-positive Indian origin macaques (Table 1 and Fig. 1D). The three Indian origin *MamuA*01*-positive animals (28209, 28349, and 28418) became SIV infected and maintained high viral loads in plasma (Fig. 1A).

Microsatellite marker loci were used to evaluate the degree of genetic similarity between macaques of Chinese and Indian origin chosen for this study (Table 4). Differences in allelic composition were found between the two groups. The average number of alleles per locus was significantly higher in Chinese (9.85 ± 2.88) than in Indian (7.77 ± 3.17) macaques ($t_{12} = 2.46$, $p < 0.025$). Similarly, the proportion of heterozygotes per locus was significantly higher in Chinese (0.79 ± 0.16) than in Indian (0.74 ± 0.23) macaques ($t_{12} = 3.60$, $p < 0.0025$). These results are in agreement with findings from another comparison of genetic diversity among Chinese and Indian rhesus macaques based on a set of 15 microsatellite loci, only 1 of which (*D17S804*) was common to both studies.²²

The genotype of an animal at the four loci with alleles that were unique to either Chinese or Indian origin (*D6S276*, *D18S72*, *D16S403*, and *D17S804*; Table 4) could correctly identify the ancestral country of the 30 individuals in this study. However, the genotype of an animal at these four microsatellite loci may not be a reliable predictor of the ancestral country of origin for other rhesus macaques. Also, this panel of 13 microsatellite loci could not be used to identify individual rhesus macaques of mixed Chinese/Indian descent, that is, animals with ancestors of both Chinese and Indian origin.

Discussion

The major goal of this study was to determine whether rhesus macaques of Chinese origin were resistant to SIV infection by IVAG inoculation. We found that there was no significant difference among Indian and Chinese origin rhesus macaques with regard to the number of animals that were infected after IVAG inoculation with either one or two doses of SIV. Thus, monkeys from both countries are useful for SIV studies. We also found no difference in the *in vitro* ability of PBMCs to become SIV infected, based on the geographic origin of the donor monkey. However, we did find that two IVAG doses of SIV resulted in significantly more SIV-infected macaques than one IVAG inoculation; this finding is consistent with previous reports for inoculation with IVAG SIVmac251.^{23,24}

In addition, we sought to determine whether the level of viremia in SIV infection is markedly different in Chinese and Indian origin rhesus monkeys during the first 8–9 weeks of infection. As previously reported, viral load among SIV-infected macaques was variable and the variation was greater among the Indian origin rhesus monkeys than among the Chinese origin rhesus monkeys. The high SIV RNA levels at 2 weeks postinfection found in plasma of Chinese and Indian origin animals were not significantly different. However, by 6 weeks postinfection the plasma SIV RNA levels tended to be lower in Chinese compared with Indian origin rhesus macaques; this difference was statistically significant despite substantial overlap in the range of viral loads found in monkeys of Indian and Chinese origin (Fig. 1). This result is consistent with earlier observations from smaller numbers of Chinese and Indian rhesus macaques inoculated parenterally with SIV.¹ The observation that SIV-infected animals with similar levels of plasma viremia at 2 weeks postinfection can have substantially different plasma viral levels at 6–8 weeks postinfection has been reported in a previous study of Indian origin rhesus macaques inoculated IVAG with uncloned SIVmac251.²⁵ The SIVmac251 isolate used for IVAG inoculation in this study is composed of a genetically heterogeneous population of viral variants²⁵ (M.L. Marthas, unpublished

results). We have shown that the genetic complexity of SIVmac251 variants transmitted by IVAG inoculation varies substantially among rhesus macaques.²⁵ This study was not designed to evaluate IVAG transmission of SIV variants. However, on the basis of our previous findings, it is possible that plasma SIV RNA levels during the first 6 weeks postinfection among the SIV-infected animals of Chinese and Indian origin were modulated by the population of viral variants transmitted IVAG25 as well as by the immune response of individual macaques to SIV infection. Finally, it is important to note that the differences observed in plasma SIV RNA levels at 6 weeks postinfection between Chinese and Indian origin monkeys in this study cannot be used to infer that viral loads several months later in infection would also differ.

The range of SIV-specific antibody titers in plasma at 6–8 weeks postinfection was similar among Chinese and Indian origin macaques; however, anti-SIV antibody levels were more consistent among SIV-infected Chinese origin animals compared with Indian origin animals. Because this study was not designed to evaluate cell-mediated immune responses to SIV infection, it is possible that cell-mediated immunity to SIV might differ between Chinese and Indian origin macaques. In addition, it is possible that the levels or breadth of SIV-specific cell-mediated immunity might be associated with the lower plasma viral loads observed in Chinese origin compared with Indian origin animals.

Indian rhesus monkeys that fail to make an antibody response to SIV or SHIV infection have a rapid disease course.^{26–29} We now show that rapid disease progression also occurs in SIV-infected Chinese rhesus monkeys and is associated with the failure to make an anti-SIV antibody response. The frequency of rapid progressors in the two monkey populations was similar: 1 of 16 Indian origin monkeys and 1 of 10 Chinese origin monkeys developed terminal AIDS within 3 months of SIV infection. Although our study was not designed to compare the long-term clinical course of SIV disease in Chinese and Indian origin rhesus monkeys, the similar frequency of rapid progressors in the two populations provides some circumstantial evidence that the clinical course of SIV infection is similar.

A panel of 13 microsatellite markers was used to assess the degree of genetic similarity between monkeys of Chinese and Indian origin. Consistent with other studies of rhesus macaque populations, microsatellite marker allele frequencies differed between Chinese and Indian origin rhesus macaques; however, the majority of alleles present in Indian origin animals were also found in Chinese origin macaques.²² Our findings that the Chinese macaques were genetically more diverse than the Indian macaques as measured by average number of alleles per locus and average heterozygosity were also in agreement with previous studies.²² Even though significant differences were observed between the Chinese and Indian origin macaques used in this study, additional studies are needed to examine the reliability of this panel of microsatellites to predict the ancestral country of origin for all rhesus macaques and to identify animals of mixed descent.

In summary, we observed no significant difference between Chinese and Indian rhesus macaques in their ability to become SIV infected after IVAG inoculation. However, compared with Indian origin animals, monkeys of Chinese origin that become SIV infected after IVAG inoculation, on average, exhibited lower plasma virus levels by 6 weeks postinfection. Together, these results suggest that the difference in viremia detected between Indian and Chinese origin rhesus macaques early in infection may reflect genetic differences in host response to SIV infection between these populations. Most importantly, because there is more variation in response to SIV infection among individuals within a group of outbred Indian origin or outbred Chinese origin macaques than between the two groups, it is not possible to predict the virus load for any individual macaque on the basis of the animal's ancestral country of origin. To evaluate the full range of differences in the pathogenesis and

natural history of SIV infection in Chinese and Indian origin rhesus macaques will require long-term studies of larger numbers of animals that assess viral loads and cellular and humoral immune responses over the course of infection from the time of inoculation through the development of fatal immunodeficiency.

Acknowledgments

We are grateful for the expert technical support of Steven Joye, Yichuan Wang, Judith Torten, Kristen Bost, Linda Hirst, David Bennet, and Wilhelm Von Morgenland, whose assistance was crucial to the successful completion of these studies. Dr. Neil Willits (Statistical Laboratory, UC Davis) provided expert assistance for statistical analyses. This work was supported by Public Health Science grant RR00169 from the National Center for Research Resources; grants AI39109 (MLM), AI39435 (CJM), and AI35545 (CJM) NO1 AI65312 from the National Institute of Allergy and Infectious Diseases; and Elizabeth Glaser Scientist award #8-97 (MLM) from the Elizabeth Glaser Pediatric AIDS Foundation.

References

1. Joag SV, Stephens EB, Adams RJ, Foresman L, Narayan O. Pathogenesis of SIVmac infection in Chinese and Indian rhesus macaques: Effects of splenectomy on virus burden. *Virology*. 1994; 200:436–446. [PubMed: 8178433]
2. Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources. Guide for Care and Use of Laboratory Animals. National Resource Council; Washington, D.C.: 1998.
3. Miller C, Marthas M, Torten J, et al. Intravaginal inoculation of rhesus macaques with cell-free simian immunodeficiency virus results in persistent or transient viremia. *J Virol*. 1994; 68:6391–6400. [PubMed: 8083977]
4. Miller CJ, Marthas M, Greenier J, et al. In vivo replication capacity rather than in vitro macrophage tropism predicts efficiency of vaginal transmission of simian immunodeficiency virus or simian/human immunodeficiency virus in rhesus macaques. *J Virol*. 1998; 72:3248–3258. [PubMed: 9525652]
5. Banapour B, Marthas M, Ramos R, et al. Identification of viral determinants of macrophage tropism for simian immunodeficiency virus (SIVmac). *J Virol*. 1991; 65:5798–5805. [PubMed: 1920617]
6. Unger R, Marthas M, Padrid P, Pratt-Lowe E, Padrid PA, Luciw PA. The *nef* gene of simian immunodeficiency virus SIVmac1A11. *J Virol*. 1992; 66:5432–5442. [PubMed: 1501282]
7. Marthas M, Ramos R, Lohman B, et al. Viral determinants of simian immunodeficiency virus (SIV) virulence in rhesus macaques assessed using attenuated and pathogenic molecular clones of SIVmac. *J Virol*. 1993; 67:6047–6055. [PubMed: 8371353]
8. Lohman B, Higgins J, Marthas M, Marx P, Pedersen N. Development of simian immunodeficiency virus isolation, titration, and neutralization assays which use whole blood from rhesus monkeys and an antigen capture enzyme-linked immunosorbent assay. *J Clin Microbiol*. 1991; 29:2187–2192. [PubMed: 1939569]
9. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. *Am J Hyg*. 1938; 27:493–497.
10. Miller CJ, McChesney ML, Lü X, et al. Rhesus macaques previously infected with simian/human immunodeficiency virus are protected from vaginal challenge with pathogenic SIVmac239. *J Virol*. 1997; 71:1911–1921. [PubMed: 9032322]
11. Dailey, PJ.; Zamrout, M.; Kelso, R.; Kolberg, J.; Urdea, M. 13th Annual Symposium on Nonhuman Primate Models of AIDS. Monterey, California: 1995. Quantitation of simian immunodeficiency virus (SIV) RNA in plasma of acute and chronically infected rhesus macaques using a branched DNA (bDNA) signal amplification assay.
12. Lu X, Kiyono H, Lu D, et al. Targeted lymph-node immunization with whole inactivated simian immunodeficiency virus (SIV) or envelope and core subunit antigen vaccines does not reliably protect rhesus macaques from vaginal challenge with SIVmac251. *AIDS*. 1998; 12:1–10. [PubMed: 9456249]

13. Knapp LA, Lehmann E, Piekarczyk MS, Urvater JA, Watkins DI. A high frequency of Mamu-A*01 in the rhesus macaque detected by polymerase chain reaction with sequence-specific primers and direct sequencing. *Tissue Antigens*. 1997; 50:657–661. [PubMed: 9458122]
14. Evans DT, Knapp LA, Jing PC, et al. Rapid and slow progressors differ by a single MHC class I haplotype in a family of MHC-defined rhesus macaques infected with SIV. *Immunol Lett*. 1999; 66:53–59. [PubMed: 10203034]
15. Bowling AT, Eggleston-Stott ML, Byrns G, et al. Validation of microsatellite markers for routine horse parentage testing. *Anim Genet*. 1997; 28:247–252. [PubMed: 9345720]
16. Chan AWS, Dominko T, Lutjens CM, et al. Clonal propagation of primate offspring by embryo splitting. *Science*. 2000; 287:317–319. [PubMed: 10634789]
17. Penedo, MC.; Cordova, KI.; Ward, T., et al. Animal Genetics Symposium. Minneapolis, Minnesota: 2000. A parentage test for rhesus macaques using human primers for microsatellite markers.
18. Penedo, MC.; Bertolini, L.; Marthas, ML.; Bowling, AT. Microsatellites for the MHC region of rhesus macaques. Human and Animal Immunogenetics: Comparative Evolution of the Mammalian MHC Conference; University of Manchester, Manchester, England. September 8–10 2000;
19. Aspin AA, Welch BL. Tables for use in comparisons whose accuracy involves two variances, separately estimated. *Biometrika*. 1949; 36:290–296. [PubMed: 15409356]
20. Trickett WH, Welch BL, James GS. Further critical values for the two-means problem. *Biometrika*. 1956; 43:203–205.
21. Zar, JH. Biostatistical Analysis. 2nd. Prentice-Hall; Englewood Cliffs, New Jersey: 1984.
22. Morin PA, Kanthaswamy S, Smith DG. Simple sequence repeat (SSR) polymorphisms for colony management and population genetics in rhesus macaques (*Macaca mulatta*). *Am J Primatol*. 1997; 42:199–213. [PubMed: 9209585]
23. Miller C, Alexander N, Sutjipto S, et al. Effect of virus dose and Nonoxynol-9 on the genital transmission of SIV in rhesus macaques. *J Med Primatol*. 1990; 19:401–409. [PubMed: 2172544]
24. Miller C, Alexander N, Gettie A, Hendrickx A, Marx P. The effect of contraceptives containing nonoxynol-9 on the genital transmission of simian immunodeficiency virus in rhesus macaques. *Fertil Steril*. 1992; 57:1126–1128. [PubMed: 1315297]
25. Greenier JL, Miller CJ, Lu D, et al. Route of simian immunodeficiency virus inoculation determines the complexity but not the identity of viral variant populations that infect rhesus macaques. *J Virol*. 2001; 75:3753–3765. [PubMed: 11264364]
26. Daniel MD, Letvin NL, Sehgal PK, et al. Long-term persistent infection of macaque monkeys with the simian immunodeficiency virus. *J Gen Virol*. 1987; 68:3183–3189. [PubMed: 2826656]
27. Lewis M, Bellah S, McKinnon K, et al. Titration and characterization of two rhesus-derived SIVmac challenge stocks. *AIDS Res Hum Retroviruses*. 1994; 10:213–292. [PubMed: 8198874]
28. Lu Y, Pauza CD, Lü X, Montefiori DC, Miller CJ. Rhesus macaques that become systemically infected with pathogenic SHIV 89.6-PD after intravenous, rectal, or vaginal inoculation and fail to make an antiviral antibody response rapidly develop AIDS. *J AIDS Hum Retrovirol*. 1998; 19:6–18.
29. Kimata JT, Kuller L, Anderson DB, Dailey P, Overbaugh J. Emerging cytopathic and antigenic simian immunodeficiency virus variants influence AIDS progression. *Nat Med*. 1999; 5:535–541. [PubMed: 10229230]

Fig. 1. Comparison of *in vitro* titration of SIVmac251-5/98 stock in primary PBMCs from Indian and Chinese origin rhesus monkeys. Note that the range of p27 antigen levels in PBMCs from all of the animals is similar.

Fig. 2.
SIV RNA levels in plasma of rhesus macaques of Chinese and Indian origin.

Fig. 3.

(A) Levels of SIV RNA in plasma 2 and 6 weeks after IVAG inoculation for macaques of Chinese origin (filled symbols) and Indian origin (open symbols). The means (indicated by arrows) for Chinese and Indian origin animals are significantly different at 6 weeks postinfection, but not at 2 weeks postinfection (see Table 3). (B) Decrease in SIV RNA plasma levels from 2 to 6 weeks after IVAG inoculation with SIV for macaques of Chinese origin (filled symbols) and Indian origin (open symbols). The means (indicated by arrows) for Chinese and Indian origin animals are significantly different (see Table 3).

Table 1
Experimental Design, Virus Isolation from PBMCs, and Plasma Antibody Responses of Indian and Chinese Origin Rhesus Macaques IVAG Inoculated with SIVmac251

Ancestral country	Animal no.	Age ^b (years)	No. of inoculations ^c	Virus isolation results (weeks pi)							Anti-SIV Ab titer (reciprocal) ^d	
				2	4	6	8	10	6 weeks pi	8 weeks pi		
India	27762 ^d	5.7	1	-	-	-	ND	ND	Neg	Neg		
	28209 ^d	4.9	1	-	-	-	ND	ND	Neg	Neg		
	28336	4.8	1	+	+	+	+	ND	200	200		
	28349	4.8	1	-	+	+	+	ND	32,000	400,000		
	28418	4.8	1	-	+	+	+	ND	32,000	3,200		
	28549	4.5	1	+	+	+	+	ND	16,000	800,000		
	27307	6.2	1	+	+	+	+	+	32,000	800,000		
	28209 ^d	4.9	1	+	+	+	+	+	32,000	400,000		
	29058 ^d	3.9	1	-	-	-	-	-	Neg	Neg		
	31167	13.1	1	+	+	+	+	+	16,000	320,000		
	31168	16.0	1	-	+	+	+	+	Neg ^e	Neg ^e		
	22162	16.1	2	+	+	+	ND	+	3,200	NA		
	22237	13.2	2	+	+	+	ND	+	16,000	NA		
	22990	12.4	2	+	+	+	ND	+	32,000	NA		
	23657	12.3	2	+	+	+	ND	+	64,000	NA		
	23737	12.0	2	+	+	+	ND	+	200,000	NA		
China	24185	11.3	2	+	+	+	ND	+	80,000	NA		
	27762 ^d	5.7	2	+	+	+	ND	+	200	NA		
	29058 ^d	3.9	2	+	+	+	ND	+	200	NA		
	31403	4.6	1	+	+	+	+	ND	100,000	400,000		
	31404	4.8	1	-	-	-	-	ND	Neg			
	31424	4.8	1	+	+	+	+	ND	100,000	400,000		
	31426	5.5	1	-	-	-	-	ND	Neg	Neg		
	31427	4.8	1	-	-	-	-	ND	Neg	Neg		
	31428	4.8	1	+	+	+	+	ND	16,000	160,000		

Ancestral country	Animal no.	Age ^b (years)	No. of inoculations ^c	Virus isolation results (weeks pi)					Anti-SIV Ab titer (reciprocal) ^d	
				2	4	6	8	10	6 weeks pi	8 weeks pi
	31405	4.4	2	+	+	+	–	+	100,000	400,000
	31406	5.4	2	+	+	+	–	+	200,000	400,000
	31409	5.5	2	+	+	+	+	+	64,000	800,000
	31410	4.6	2	+	+	+	+	+	16,000	32,000
	31415	5.3	2	+	+	+	ND	ND	Neg ^e	NA ^e
	31433	4.8	2	–	–	–	–	–	Neg	Neg
	31436	4.9	2	+	+	+	+	+	64,000	160,000
	31437	5.3	2	+	+	+	+	+	100,000	800,000

Abbreviations: NA, Sample was not available; Neg, no SIV-specific antibodies were detected; ND, not determined; pi, postinfection.

^aAntibody end-point dilution titers; the titer shown is the reciprocal of the highest serum dilution that was positive.

^bAge of animals in years at the time of the first IVAG inoculation.

^cNumber of IVAG inoculations with SIVmac251-5/98.

^dAfter these animals failed to become infected following the first SIV inoculation, they were reinoculated.

^eAlthough SIV infected, these animals failed to make an anti-SIV antibody response and they rapidly progressed to terminal AIDS by 6 (31415) or 12 weeks pi (31168).

Table 2

Repeated Measures Analysis of Variance to Test for Significant Effects of Geographic Origin, Number of IVAG SIV Inoculations, and Time after Inoculation on SIV RNA Levels in Plasma of SIV-Infected Rhesus Macaques

Factor ^a	F value	Degrees of freedom ^b	Probability of a larger F value
Ancestral country	10.15	1	0.004 ^c
Number of IVAG SIV inoculations	0.01	1	0.937
Weeks pi ^d	31.45	2	<0.0001 ^c
Ancestral country × weeks pi ^e	1.40	2	0.258
IVAG inoculations × weeks pi ^f	0.12	2	0.890

^aAnalyses were performed on the log₁₀ of the number of copies of SIV RNA per milliliter of plasma.

^bDegrees of freedom for F value.

^c*p* 0.050 was considered statistically significant.

^dComparison of the temporal trend in SIV plasma RNA levels among SIV-infected animals of Indian and Chinese origin. Data for weeks 2, 4, and 6 postinfection were analyzed; missing data for some animals precluded analyses of all times postinfection.

^eComparison of the temporal trend in SIV plasma RNA levels between animals of different geographic origin.

^fComparison of the temporal trend in SIV plasma RNA levels between animals that received different numbers of IVAG SIV inoculations.

Table 3

Unpaired *t* Test Comparisons of SIV RNA Levels in Plasma in Infected Rhesus Macaques of Chinese and Indian Origin at Specific Times after IVAG Inoculation

Time after IVAG inoculation	Chinese origin mean ^a ± SEM ^b (<i>n</i> ^c)	Indian origin mean ± SEM (<i>n</i>)	Mean difference ^d	Probability of a larger <i>t</i> value
2 weeks pi	7.2 ± 0.3 (10)	7.7 ± 0.1 (16)	0.5	0.094
6 weeks pi	5.0 ± 0.2 (9)	6.3 ± 0.2 (16)	1.3 ^e	<0.001 ^e
2–6 weeks pi ^f	2.1 ± 0.2 (9)	1.4 ± 0.2 (16)	−0.7 ^e	0.020 ^e

^aAnalyses were performed on the log₁₀ of the number of copies of SIV RNA per milliliter of plasma; mean values shown are log (copies of SIV RNA per milliliter of plasma).

^bStandard error of the mean.

^c*n* = number of animals from which data were analyzed.

^d(Mean value for Indian origin animals) minus (mean value for Chinese origin animals).

^e*p* 0.050 was considered statistically significant.

^fThe decrease between SIV plasma RNA levels at week 2 and week 6 postinfection for individual animals was analyzed; missing data for 6 weeks postinfection precluded one Chinese origin animal (31415) from analysis.

Genotypes and Microsatellite Genotypes of Indian and Chinese Origin Rhesus Macaques IVAG Inoculated with SIVmac251

Table 4

Country	Animal no.	Rhesus MHC alleles		Microsatellite locus genotype ^d												
		Mamu A *01	Mamu B*01	D7S794 ^b	D17S804	D7S513	D3S1768	D13S765	D11S925	D6S291	D6S276	D6S1691	D10S1412	D18S72	D16S403	D8S1106
	27762	–	–	124/128 ^b	118	189/207	209/221	216/224	308	204	225/235	197	157/163	308/330	168	164/172
	28209	+	–	120/128	118	197/211	193/229	208/236	308/330	200/214	233/235	197/203	163	306/330	168	144/152
	28336	–	+	108/124	114/118	195/207	205/225	224/228	312/330	204	221/235	197/213	160	306/328	156/158	152/156
	28349	+	+	108	118	187/205	217/221	232/236	310/330	206	225	213/221	157/163	306/308	164/168	160/164
	28418	+	–	120/124	118	189/203	193/217	224/228	308	204	227/235	197	157/163	306	156/174	140/144
	28549	–	–	124	118	187/219	205/229	212/224	308/312	206/214	233	199	157	308	164	144/152
	27307	–	+	120/124	118	195/207	213	224/232	312/330	204/206	215/227	177/197	157/163	310/324	152/168	160
	29058	–	+	128	118	197/203	225	208/224	312/338	206/214	225/231	197/209	157	306	164/174	144/152
	31167	–	–	128	118	187/189	201/213	224/236	312/342	206/214	225/235	197	157/163	306/308	164/168	144/160
	31168	–	+	120/128	118	191/203	213/221	216/232	308/338	206/208	221/235	197/215	157/166	306/308	156/158	148/168
	22162	–	–	108/124	118	203/207	205/221	208/232	308/338	206/214	205/221	197/203	157/160	306/314	164/170	144/172
	22237	–	–	108/128	118	195/207	201/205	212/220	312/330	204	227/235	195/197	157/160	302/328	144/164	132/144
	22990	–	–	128	118	187/207	201/225	224/228	330	206/212	225/233	191/197	157/163	308/330	152/164	144/168
	23657	–	+	128/136	118	187/207	225/229	224	338	206/214	233	199/211	157	306/308	164/168	148
	23737	–	+	124/128	118	219/233	201/225	232/260	308/338	204/206	235/237	195/201	157/160	306/308	156/168	152/156
	24185	–	–	108/128	118	205/211	205/217	228/260	308/312	204	221/225	205/213	157/163	306/328	164	144/160
		3/16	7/16													

AIDS Res Hum Retroviruses. Author manuscript; available in PMC 2012

No. of alleles	5	2	11	9	9	6	6	9	14	4	8	8	10
Most common allele ^d (freq.)	128 (13/32) 0.41	118 (31/32) 0.97	207 (7/32) 0.22	225 (6/32) 0.13	224 (10/32) 0.31	308 (10/32) 0.31	204 (12/32) 0.38	235 (8/32) 0.25	197 (14/32) 0.44	157 (17/32) 0.53	306 (13/32) 0.41	164 (11/32) 0.34	144 (9/32) 0.28
Freq. het. ^{s,e}	11/16 0.69	1/16 0.06	16/16 1.00	14/16 0.88	15/16 0.94	12/16 0.75	10/16 0.63	13/16 0.81	12/16 0.75	11/16 0.69	13/16 0.81	12/16 0.75	14/16 0.88

31403	–	–	–	124/128 ^b	106/114	189/207	213/221	220/232	330/332	204/206	211/215	197/217	154/157	316/320	166	144/152
31404	–	–	–	112/124	108/118	207/219	221/225	220/236	344/350	204/206	211/227	197/211	160	312/314	158/186	160
31424	ND	ND	ND	124	118	177/207	185/189	224/228	308/312	202/218	223/237	211/217	157/160	312/326	140/152	144/148
31426	–	–	–	128	114	198/297	205/217	220/228	328/332	204/206	225	209/211	157/163	316/332	142/156	156/160

AIDS Res Hum Retroviruses. Author manuscript; available in PMC 2012 July 20.

Country	Animal no.	Rhesus MHC alleles		Microsatellite locus genotype ^d														
		Mamu A *01	Mamu B*01	D7S794 ^b	D17S804	D7S513	D3S1768	D13S765	D11S925	D6S291	D6S276	D6S1691	D10S1412	D18S72	D16S403	D8S1106		
	31427	ND	ND	128	120/126	199/217	197	228/264	328/330	204	231	177/203	157	312/316	156/160	148/160		
	31428	ND	ND	108/128	118/122	205/207	209	224/272	328/330	208/222	223/227	177/213	157	308	152/166	148/160		
	31405	-	-	124	114/122	195/209	213/221	220/232	330/332	204/206	211/215	197/217	154/157	316/320	166	144/152		
	31406	-	+	124	114/122	193/199	185/213	220/224	328/332	208/216	211/213	195/213	160	312/320	150/172	160		
	31409	-	-	124/128	114	191/207	201/217	220/224	308/310	204/206	211	201/205	154/163	306	158/160	144/156		
	31410	-	-	108/128	114/120	199/211	185/229	232/236	320/324	204	223/229	203/205	154/160	314	142/158	152/156		
	31415	-	-	108/128	114/118	193/197	193/213	200/224	322/330	202/214	211	207	157/160	318/324	142/166	144/160		
	31433	-	-	124/132	112/122	199/209	205/221	264/268	312/348	202/204	211	207/209	157/172	312/336	158/162	140/160		
	31436	-	-	128	108/118	205	185/201	224/228	330/332	200/202	211	193/199	157	310/322	144/166	144		
	31437	-	-	124	116	189/193	209/217	228	312/348	200/204	223	203/211	154/157	312/318	146/162	148/152		
		0/10		1/10														
No. of alleles				6	9	13	12	9	12	9	9	13	5	12	13	6		
Most common allele (freq.)				124 (12/28) 0.43	114 (9/28) 0.32	207 (6/28) 0.21	185, 213, 221 (4/28) 0.14	220, 224, 228 (6/28) 0.21	330 (6/28) 0.21	204 (11/28) 0.39	211 (12/28) 0.43	211 (4/28) 0.14	157 (13/28) 0.46	312 (6/28) 0.21	166 (7/28) 0.25	160 (9/28) 0.32		
Freq. het.s ^e				7/14 0.50	10/14 0.71	13/14 0.93	11/14 0.79	13/14 0.93	14/14 1.00	12/14 0.86	7/14 0.50	13/14 0.93	9/14 0.64	11/14 0.79	12/14 0.86	11/14 0.79		

D, Not done.

h heterologous human primers.

esignated as for human microsatellite marker locus; alleles at each locus are designated by a number indicating mobility of PCR product on a polyacrylamide gel; two numbers separated by a slash (/) indicates a heterozygote; a single number indicates only one most likely a homozygote; alternatively, a null allele could also be present.

es observed in all animals from one ancestral country.

s) that are most frequently detected in animals from one ancestral country. The number of alleles over the total number of alleles (2 × number of individuals for diploids) is given in parentheses. Animals for which a single allele was detected were inferred to be , thus, were assumed to carry two copies of the same allele.

terozygous animals (i.e., two different alleles detected) at each locus defined as: the number of animals heterozygous at a locus over the total number of animals.