Effect of Daily Serial Phlebotomy in a Rhesus Macaque (Macaca mulatta) Model of Flavivirus Vaccine

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Abstract
Blood collection and analysis in research animals is a necessary component of experimental studies, data analysis, and health monitoring. Current maximum volume recommendations for one time blood collections are dependent on species, weight, and rest period between biosamples. However, volume recommendations for daily phlebotomies are lacking. We examined the effect of daily blood draws in the rhesus macaque over a 15-day period to determine whether serial blood draws under the conditions of a concurrent flavivirus study could be performed safely without impacting their health or well-being. To do this we examined the change in packed cell volume from day 1 to day 15 from a centrifuged tail prick blood sample to determine whether or not the animals became anemic and the degree of anemia. We performed a retrospective comparison of the packed cell volume and complete blood count hematocrit. Finally, we evaluated weight change during the serial blood draws and examined our blood collection methods to identify areas to refine future studies incorporating serial phlebotomy in the experimental design. Although the number of macaques outside of the hematocrit reference range was statistically significant, the anemia was mild. Based on the degree of anemia, absolute change in PCV, clinical signs, and recovery displayed by day 28, we determined that daily blood collection was safe for the macaques' health and welfare while warranting increased monitoring in studies that require daily phlebotomy. In addition, PCV and HCT were significantly different with a positive correlation, a clinically inapparent but statistically significant weight loss was identified, and total blood volume collected was higher than targeted volume.

INTRODUCTION
Blood collection is an integral part of experimental studies involving laboratory animals. Blood analysis allows investigators to examine immunological response, pharmacokinetic activity, biochemical markers, and systemic effects from experimental treatments and challenges. Blood analysis also allows for health monitoring of laboratory animals during the course of experimental trials, routine health screening, and as a tool for assessing sick animals.

Due to the risk of anemia, blood collection creates a welfare and regulatory challenge when investigators require multiple blood draws, large volumes, or a combination of the two over a short period of time.(15) The principle investigator, Attending Veterinarian, and Institutional Animal Care and Use Committee (IACUC) have a responsibility of ensuring that blood collection can be completed safely from the animal while meeting the needs of the study.(1; 2; 13; 21) Current recommendations in literature are based on the weight of the animal and period in between blood draws.(12) Some sources recommend blood draws of no more than 7.5% of the maximum blood volume with a one-week rest period, 10% with a two-week rest period, and 15% with a four-week rest period.(12) Recent studies have demonstrated that even higher amounts can be safely collected weekly in various species.(3; 22; 24) However, the reference sources did not address how much blood can be collected on a daily basis, and published blood volume recommendations for serial phlebotomies were not identified during protocol planning and development.

Studies have been initiated at the Walter Reed Army Institute of Research (WRAIR) that require daily blood draws in the rhesus macaque (Macaca mulatta) over a 15-day period as part of a program to develop a Dengue virus vaccine as well as other flaviviruses. During protocol development, daily blood draw volumes was determined by dividing the maximum volume allowed for a one-time blood draw with a two-week rest period by the number of days requiring serial phlebotomy. The purpose of this project was...
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to evaluate whether such serial blood draws could be performed without compromising the health or well-being of the animals under study. We hypothesized that daily blood collection at a pre-determined volume could be conducted safely with no adverse effect on the well-being of the animals or risk to the animals’ health during the 15-day daily blood draw period of the study. To evaluate the effect of daily phlebotomies on their health, we examined changes in packed cell volume (PCV), total protein (TP), weight, hydration status, and overall presentation and clinical signs. Additionally, we compared PCV values obtained from blood samples collected from tail pricks to automated machine calculated hematocrit (HCT) values obtained from blood samples collected from femoral venipunctures. Finally, we retrospectively examined how blood collection was conducted to see if our procedures could be refined in future studies to further minimize the impact of daily blood collection on the health of the macaques in the colony.

MATERIALS AND METHODS

Animals: 29 adult male and female rhesus macaques (Macaca mulatta; 11 males, age: average, 4.9 years; range, 3.6 to 6.0 years; weight: average 6.8 kg; range, 4.8 to 8.8 kg; 18 females, age: average: 5.0 years; range 3.5 to 6.6 years; weight: average, 5.1 kg, range, 3.9 to 6.7 years) of Indian origin were used to complete this. All procedures were reviewed and approved by the WRAIR Institutional Animal Care and Use Committee (IACUC), and performed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition.

Animals were housed in visual and auditory contact with conspecifics and pair-housed when possible. Animals were provided with biscuits (Lab Diet 5038 Monkey Diet) twice per day with water provided ad libitum through lixits. Rooms were maintained at temperatures and humidity level IAW the Guide and AWA regulations. Florescent lighting was provided on a 12h:12h light/dark cycle (light period 0600-1800 daily). Scheduled environmental enrichment was provided through food enrichment, toys and manipulanda placed in the cage, room-based auditory and visual enrichment, and play caging provided on a weekly basis. All animals tested negative for simian retrovirus, simian t-cell leukemia virus, simian immunodeficiency virus, and Macacine herpesvirus 1 on annual serologic and PCR laboratory tests, and were negative for tuberculosis based on semiannual skin testing. Complete blood count (CBC) (Seimens Advia 120) and serum chemistry (Sysmex XT-2000iV) analysis was performed on all macaques within two weeks of the beginning of the 15-day blood draw for groups 1-4, and on day 1 for groups 5-6.

Experimental groups: This study was conducted in conjunction with an approved experimental flavivirus vaccine study that was already underway. 30 animals were divided into six groups of five macaques each. Four groups (#1-4) received experimental inactivated flavivirus vaccines as part of the parent study 150 days prior to the 15-day serial blood collection period followed by subcutaneous injection with challenge virus on day 1. Flavivirus challenges in the rhesus macaque are believed to be subclinical, resulting in viremia without signs of disease.(10; 11; 16; 28; 30) Macaques in group 5 were not vaccinated or challenged serving as a control for the effect of vaccination and viral challenge. One macaque assigned to group 5 was removed due to diagnosis of nonregenerative microcytic hemolytic anemia prior to the initiation of daily blood draws. Therefore group 5 only had four members. Group 6 served as a control group for the effect of daily blood draws. This group did not receive experimental vaccinations, viral challenge, or undergo daily blood draws, but were sedated and examined daily.

Daily health assessment. Prior to out-of-cage procedures animals were sedated with ketamine/acepromazine at a dose of approximately 5 mg/kg and 0.5 mg/kg respectfully. After sedation animals were removed from their cages, and their weight, body temperature, pulse, and respiration rates were recorded. Skin turgor response was evaluated as a measure of hydration status, with a prolonged skin-tent considered as an indication of dehydration. Mucous membrane color was evaluated as an assessment of anemic state, with pink or light pink considered normal, and pale pink or white considered abnormal. Capillary refill time (CRT) was determined by pressing the gums with a cotton-tipped applicators and observing the amount of time required to return to the original color. CRT provides an assessment of peripheral perfusion and is considered normal when the refill time is < 2 seconds. Dehydration can also cause a delayed refill time. CRT in an anemic patient is normal unless hypoperfusion is also present, but can be difficult to assess due to lack of color contrast.(20)

Serial blood collection. On days 1-15, macaques in groups...
1-5 underwent daily phlebotomies, with approximately 1.5-2.0 mL blood drawn from the femoral vein using a 23-gauge needle into a 3.5 mL serum separator tube (SST) (BD Vacutainer). These biosamples were provided to the primary study investigator for analysis of the animals’ immune responses and virus titers. Blood collection was repeated on day 28 for all groups. The volume of blood collected from each animal was estimated to the nearest 0.1 ml by comparing the level of blood in the collection tube to an indexed serum separator tube marked at 0.5 mL intervals.

**Packed cell volume and hematocrit defined:** In general, PCV and HCT refer to the fraction of whole blood volume that contains red blood cells. The terms are often used interchangeably. PCV, also referred to as spun HCT, is the percentage of the total blood volume occupied by red cell mass after centrifugation. HCT is most often calculated by an automated analyzer and not directly measured. It is determined by multiplying the red cell count by the mean cell volume. For the purpose of this paper, PCV is defined as the percent of red blood cells in the blood sample obtained from a tail prick, collected with a micro-hematocrit capillary tube, centrifuged, and determined by comparison to a scaled hematocrit chart (Critocaps, Oxford Labware). HCT is defined as the percent of red blood cells in the blood sample obtained by venipuncture, collected in a 2.0 mL purple-top EDTA blood collection tube (BD Vacutainer), and processed using an automated CBC analyzer.

**Packed cell volume and total protein measurement and evaluation.** Because the blood volume collected was based on the maximum recommended blood volumes and dedicated for the Dengue study, it was necessary to limit additional blood collection to samples that could be obtained from a skin prick. On days 1, 15, and 28, tail tips of macaques in groups 1-5 were pricked with lancets (Microlance, 5 mm) at the site of the coccygeal vein. A small amount of blood was collected in two glass sodium heparinized micro-hematocrit capillary tubes (Chase Instruments) per macaque. To ensure uniformity in collection methods, one person was responsible for blood collection. The micro-hematocrit tubes were centrifuged (TRIAC Centrifuge, Clay Adams) according to manufacturer’s directions. Packed cell volume (PCV) was measured by using a scaled hematocrit chart. To the authors’ knowledge, there is no published reference interval for macaques for PCV values obtained from a tail or skin prick. Therefore the HCT reference range was used for the assessment of anemia. After measuring PCV, a small amount of serum was placed on a refractometer to determine the total protein (TP) value. The average value of the two PCV and TP readings were used for statistical analysis.

**Complete blood count and serum chemistry.** As part of the flavivirus vaccine study, baseline CBC and serum chemistry values were obtained for groups 1-4 within two weeks prior to the start of the 15-day daily phlebotomy period. CBC and chemistry values for groups 5-6 were obtained on day 1 of the 15-day period. CBC and chemistry values were repeated for all groups on day 28. Due to the volume of blood collected for the Dengue study, samples were not available to determine CBC and chemistry values on day 15 for groups 1-5.

**Statistical analysis.** Baseline (day 1) differences among groups were tested using one-way analysis of variance. Changes in PCV, TP and weight from day 1 to day 15 were analyzed using a linear regression model with the change to day 15 value as the dependent variable and the following three independent variables: day 1 value, experimental group and whether the group was a phlebotomy group. If the experimental group was significant, Tukey’s test to determine pairwise differences was to be performed. The proportion of macaques with PCV values falling outside of the normal HCT range was analyzed using test of proportions with Yates’ continuity correction. The p-values for PCV (day 1) vs. HCT (baseline), PCV (day 28) vs. HCT (day 28), and for impact of blood volume collected on change in PCV were calculated using paired t-tests (for differences between measures) and Pearson’s correlation coefficient (for association between measures).

All statistical analyses were performed as two-tailed tests with a p-value less than 0.05 considered significant. Except for the adjusted p-values from the Tukey test which controls the experimental error rate, no adjustments for multiple comparisons were planned.

**RESULTS**

**Clinical assessment.** Baseline CBC and serum chemistry values were unremarkable for the 29 macaques in the study. No differences on baseline values were detected among groups. On days 1-15 and 28, all macaques had body temperatures, pulse rates, respiration rates, mucous membrane color, skin turgor, and capillary refill times that were within established reference ranges. No vomiting or diarrhea was observed. Eating and drinking was reported as normal by the animal caretakers.
The PCV difference from day 1 to day 15 between animals receiving phlebotomies (groups 1-5) and the non-bled control animals (group 6) was significantly different. The mean and 95% confidence interval of the difference between the phlebotomy group and the control without phlebotomy was -5.3% (-9.28% to -1.27%; p-value <0.0001). The average PCV difference in groups 1-5 was -5.9% (range: -17.0 to 0.5%). The average PCV difference in group 6 was -0.6% (range: -6.5 to 5.5%) (Table 1).

The proportion of macaques with PCV values falling out of the HCT reference range (34.8-55.2%) was significantly different at day 1 vs. day 15. No animals were above the reference range at either time point. One macaque was below the reference range on day 1, and nine macaques were below the reference range on day 15, i.e., eight more macaques fell below the normal to low HCT threshold of 34.8 on day 15 than on day 1 (9/29=31.0%, up from 1/29=3.4%; p=0.0150). None of these were in the non-bled control group. (Table 1)

Table 1

<table>
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<th>Hematocrit (HCT) %</th>
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There were no significant differences comparing total protein (TP) measured at day 1 vs. day 15 between individuals (mean [SD] change of 0.0 [0.45], p=0.7285).
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**Figure 3**
CBC HCT % (Day 28) vs. PCV tail prick (Day 28) %

**Blood volume collected.** The average blood volume collected per individual over the 15-day serial blood draw in groups 1-5 was 36.0 mL (range: 31.8-38.1 mL).

**Body weight.** 28 of 29 macaques demonstrated a weight loss over the 15-day period. The average weight change among all 29 macaques was -0.29 kg (range: -0.52 to 0.05 kg, p=0.0128). The average percent of weight change from the day 1 weight was -5.0% (range: -9.3% to 0.9%). Weight changed from a mean (SD) of 5.7 (1.34) to 5.4 (1.27) kg. There was no difference among groups. (Fig. 4)

**Figure 4**
Body weight (kg) on Day 1 vs. Day 15

**DISCUSSION**
The decrease in PCV from day 1 to day 15 for macaques that were bled daily can be attributed to the daily blood draws, with 30% of the population having 15-day PCV values below the reference range. There were no reports or indications of decreased activity level. Clinical presentation, physical exam, and mucous membrane color was normal for all patients during pre-blood draw evaluation. By day 28, 26/29 macaques had PCV values within the HCT reference range, with the remaining 3 within 2% or less of the lower limit. HCT values for 23/29 macaques were within the reference range, with 5/6 of those outside the reference range within 2% or less.

Dehydration-masked anemia, defined as artificially high PCV/HCT values due to hemoconcentration, was considered. Animals with dehydration would be expected to have and increased TP. Instead, there was no significant difference between day 1 and day 15, supporting the assessment of normal hydration status. Physical signs did not indicate notable dehydration, with skin turgor and CRT normal, and no individual presentation of sunken eyes or dry tongues. Based on PCV values within or mildly below the reference range, the absence of clinical signs of anemia on presentation, and the general improvement of HCT and PCV in the population by day 28, we concluded that daily blood draws, under the conditions of the study, were safe for the macaques with no adverse effects or health risks.

It was noted that the PCV was significantly different from the HCT, although there was a positive correlation between these two measurements. The positive correlation between the day 28 PCV and day 28 HCT was stronger compared to day 1 PCV and baseline HCT. This is not surprising as the baseline CBC and chemistry biosamples were not collected on the same day as the day 1 PCV for group 1-4. In general, PCV for venous samples and HCT values of laboratory animals should be within 3% of each other.(26) Other studies have found skin prick PCV values in animals and humans vary compared to machine-determined HCT values.(7; 14) In general, the potential for error can be greater when calculating HCT with an automated analyzer compared to the PCV method in species other than the dog due to their smaller erythrocytes if the machine is not calibrated to the animal of interest.(8) The analyzer used at our institution allows the user to enter the type of animal (i.e., “monkey”), but not necessarily the specific species. In addition, PCV values can vary based on the collection site.(4; 6; 7) With this in mind, the authors feel that the macaque PCV values obtained from a tail prick are a reliable indicator of an anemic state. In the absence of a PCV reference range for a blood sample obtained from a tail or skin prick, the HCT reference range should be used as a guideline and not an absolute when diagnosing anemia in a macaque. Clinical presentation, a complete physical exam,
and the effect of anesthetic drugs should be considered as well.(17; 27; 29)

Although the 5% average weight loss was not apparent or clinically significant on daily physical exams, it was statistically significant. It is also notable that all but one macaque lost weight over the 15-day period. One possible cause is a reduction in caloric intake. Due to the requirement for daily blood draws, morning feedings were withheld to reduce the risk of vomiting and aspiration while under sedation. Although macaques were provided caloric supplementation through treats, fruits, and extra biscuits in addition to their normal afternoon feedings, the additional supplementation did not prevent weight loss. Weight loss attributable to the residual effects of repeated sedation with ketamine/acepromazine has been documented in other studies. (11) Stress from daily sedations and manipulations likely contributed to the weight loss as well. Previous studies reported that African green monkeys (Cercopithecus aethiops) that did not receive daily ketamine injections, but observed other animals being sedated, showed reduced feed consumption.(25)

Another notable finding was that the actual blood volume collected was higher than the intended volume. The targeted blood volume per day was 1.5–2.0 mL per animal. With a 15-day period of daily phlebotomies, the expected range of total blood volume collected would be 22.5-30.0 mL. The actual total volume collected averaged was 36.0 mL (range: 31.8–38.1 mL) indicating a consistent overfilling of SST tubes during blood collection. One possible explanation is that there was a subconscious effort to ensure that 1.5–2.0 mL of whole blood was the minimal amount obtained for the primary vaccine study. With samples being drawn daily, it would not be possible to obtain a second sample on the same day should the first sample provide an insufficient volume of serum for analysis. Since the daily serum sample collected from 1.5-2.0 mL of whole blood would be relatively small, an under filled SST tube could provide a sample that was too small to be analyzed, resulting in lost data. Under these conditions, it is reasonable that technicians could unintentionally slightly overfill the tubes to ensure that a sufficient volume was collected.

The blood collection technique and requirement to estimate when 1.5-2.0 mL was collected could have contributed to the decrease in PCV between day 1 and day 15. The smallest volume SST tubes available for the parent study were 3.5 mL. This required technicians to estimate when the appropriate amount was collected and then remove the tube and needle. Blood collection from the femoral vein requires that the SST tube be inverted. Due to the separating gel at the bottom of an upright SST tube, and visual occlusion of collected blood due to the tube cap of an inverted SST tube, the biosample volume would appear less during collection when compared to an upright tube of the same volume (Fig. 5). The inverted SST blood collection tube appears to have 0.4 mL less volume when compared to an upright tube. In both cases, although a small amount of excess blood drawn would be inconsequential for a one-time sample, there would be an additive effect on the macaque due to the total blood volume removed over the 15-day period.

Figure 5
Comparison of and inverted 3.5 mL SST blood collection tubes. (A) Both tubes filled with 2.0 mL dyed water. (B) Both tubes filled to the same level. The inverted tube on the right required an additional 0.4 mL of water (C) Both tubes upright, with the inverted tube in (B) on the right.

The consistent overfill during blood collection could be refined in several ways. The most appropriate change would be to use collection tubes that would only collect the intended volume of blood. At the time of blood collection only 3.5 mL SST tubes were commercially available. Although 2.5 mL SST tubes are produced, according to the manufacturer’s website this size is only available in Europe, and would still require incomplete filling to obtain a 1.5-2.0 mL sample.(5) Partially filling tubes was considered a reasonable adaption to the resources on hand that would allow collection of a usable sample size for the parent study while protecting the well-being and health of the macaques. The targeted blood volume determined during pre-protocol planning was based on the individual with the lowest body weight, adding an additional margin of safety for the other 28 macaques. Additional tail prick PCV measurements were performed during the 15-day blood draw collection to
provide additional monitoring of anemia; these values were not included in data analysis. In this case, one study adaptation would be to change the blood collection site. The femoral vein is the most common site for blood collection in nonhuman primates. Other sites for collection include the cephalic, saphenous, and coccygeal vein. Blood could be collected at peripheral veins using a syringe to draw the exact amount before filling the SST collection tube. The drawback to this technique would be an increased risk of personnel needle-stick injuries during collection, as well as the potential difficulty re-accessing peripheral veins towards the end of the 15-day period due to cumulative daily vessel trauma. Another refinement would be to conduct training with the technicians on estimating when the correct blood volume has been collected. The instructions for estimating the target blood volume during collection included demonstrating the approximate fill level of 2.0 mL of blood in an upright SST tube. Instruction with an inverted SST tube would have provided a more realistic example of estimating 2.0 mL at the moment of collection.

The excessive blood volume collection and weight loss were unexpected findings from this study and likely would have been unnoticed had it not been for the statistical analysis of the 15-day change in weight and documentation of the actual blood volume collected. These findings illustrate the benefits of protocol monitoring concurrent with experimental procedures and data collection. Protocol review, approval, and monitoring are a legal, administrative, and ethical requirement covered by many laws and regulations. However, peer-reviewed articles that address findings discovered during animal manipulation and data collection may be underrepresented in the scientific community, especially when unrelated to the actual study being performed. Animal research and welfare would benefit from investigators publishing findings discovered incidentally or during oversight of animal procedures. It allows other institutions to improve their animal care and use programs, and meets the intent of “refinement” as described by Russell and Burch. Professions outside of research with animals have successfully embraced identifying and communicating potential hazards and corrective actions as a method to prevent more serious events. In this study, analysis of data not required for the parent flavivirus study identified two areas that could be refined for future studies. As a result, increased nutritional support to prevent weight loss, technician training on estimating the targeted blood volume, and PCV monitoring to detect early, subclinical anemia have been implemented in a similar study with daily blood-draws that is ongoing at the authors’ institute at the time of this article’s submission.

Based on these results, under the conditions of the study, we concluded that the 15-day daily blood draw was safe for the macaques with no adverse effects or health risks, and that future studies warrant increased monitoring of the animal’s well-being during periods of serial blood collection. Although 30% of the macaques were below the HCT reference range by day 15, the anemia was mild, clinical presentations were absent of signs of anemia, and showed a return to or towards normal values by day 28. In addition, this study identified how biosample collection technique and animal husbandry can be refined in similar studies and serves as an example of how evaluation of experimental procedures leads to improved research and animal care.


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