

## Effect of Blood Collection Technique in Mice on Clinical Pathology Parameters

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### ABSTRACT

A study was conducted in normal healthy C57BL/6 mice to determine the effect of method of blood collection on clinical pathology parameters and to provide value ranges for these parameters. Males and females were used and were randomly assigned to treatment groups based upon phlebotomy method. The blood was collected using one of four methods: intracardiac (IC), a single attempt at collection from the caudal vena cava (VC), collection from the caudal vena cava with collection of any extravasated blood from the peritoneum (MC), or retroorbital phlebotomy (RO). Evaluation of blood and serum samples was conducted for a number of serum biochemistries including liver function tests and complete blood count with differentials and platelet counts. Female mice demonstrated higher values for red blood cell number, hemoglobin ( $p < 0.002$ ), hematocrit, alkaline phosphatase, albumin, total protein, and creatinine. Males demonstrated higher values for platelet counts, specific white blood cell numbers (total, neutrophil, lymphocyte, and eosinophil counts), globulin, amylase, and the BUN/creatinine ratio. Overall, the VC method was associated with the least variation in both sexes and appeared slightly better than the IC method for the parameters evaluated. The largest difference between groups was noted for the transaminase levels. While alanine aminotransferase (ALT) values were similar between the IC and VC groups, aspartate aminotransferase (AST) values were associated with less variation for the VC method. Transaminase levels for the MC and RO groups were associated with relatively large ranges and variation. ALT results from the RO method, the only method amenable to repetitive sample collection used in this evaluation, indicate that this is an acceptable method. The results demonstrate the substantial impact that phlebotomy method has on the assay results and that the VC or IC methods provide the most consistent results. The ranges by collection method and sex provided here can be used to select the preferred method of collection when designing a study and for comparison of data obtained with reference ranges. The authors recommend establishment of normal ranges based upon methods employed within an investigator's laboratory.

### OVERVIEW SUMMARY

The effect of phlebotomy method on clinical pathology parameters in C57BL/6 mice was evaluated using one of four methods: intracardiac (IC), a single collection from the caudal vena cava (VC), caudal vena cava with collection of any extravasated blood (MC), or retroorbital (RO). Samples were evaluated for serum biochemistries and complete blood counts. The phlebotomy method had significant impact on the assay results. Overall, the VC

method had the least variation in both sexes and was slightly favorable to the IC method. Transaminase levels were most affected and were significantly greater for MC and RO groups compared to the other two methods. Gender differences were noted for 16 of 25 parameters. The ranges provided by collection method and gender would be valuable for selecting a phlebotomy method or use as normal references ranges. However, establishment of normal ranges by individual investigators is recommended for more accurate evaluations.

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## INTRODUCTION

A NUMBER OF CRITERIA ARE EVALUATED to select the method of blood collection in any given species. Common important criteria include the analyses to be performed, the total volume per sample required, the sampling frequency, and whether the sampling is a terminal or recovery procedure. In smaller animals such as mice, these criteria have more profound consequences due to limitations including their relatively small blood volume and the lack of easily accessible vessels.

The primary purpose of the evaluation of clinical pathology parameters in the context of an experimental design is to determine if there are differences associated with an experimental treatment. To be useful, the values obtained must be accurate, reliable, and consistent in order to draw meaningful and valid conclusions.

Although certain aspects of blood collection are known to produce artifactual effects on values obtained independent of species (Riley, 1992), the blood collection method is frequently not mentioned in the literature. In a summary of clinical pathology parameters obtained in various strains of mice by different groups of researcher, Mohr *et al.* suggested that one of the reasons for the variation noted in values for mice between groups may be due to the collection technique (Loeb *et al.*, 1996).

Evidence that this conclusion is correct at least in rats was shown by Upton and Morgan (1975) in that the values obtained for hemoglobin and hematocrit in the rat were dependent upon the collection site. Archer and Riley (1981) demonstrated that there were differences in blood leukocyte and erythrocyte numbers collected from either the tail vein or jugular vein of unanesthetized rats.

While there have been reports of clinical pathology ranges (Frith *et al.*, 1980; Harrison *et al.*, 1978; Loeb *et al.*, 1996; Wolford *et al.*, 1986), to our knowledge, there has been no controlled assessment of the collection site implications on a broad panel of clinical pathology parameters including complete blood counts and serum biochemistries in mice. In our previous experience, the method of blood collection in mice resulted in variances that encumbered interpretation of the results. We therefore set out to determine the cause of variance and to determine the influence, if any, that the collection technique had on the results in mice. Any noted differences between collection methods would be critical to compare data when different methods are used, i.e., survival versus nonsurvival. Determination of the cause of variability and identification of a preferred bleeding site if the variability was due to collection site were needed.

Due to the lack of published literature relating to the method of blood collection and also a lack of reported normal ranges by technique in the C57BL/6 mouse, we conducted a study to compare the hematology and serum chemistry results obtained using four common methods of blood collection.

## MATERIALS AND METHODS

A total of 60 male and 60 female C57BL/6 mice were obtained from Taconic. The study was conducted according to the Guide for the Care and Use of Laboratory Animals and was approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Mice were allowed to acclimate for 63 days to allow sufficient time for normalization of blood values after any shipment stress. At the time of blood collection, the animals were between 13 and 15 weeks of age. Females weighed between 20.2 and 26.7 g (average = 22.2 g) while males weighed between 22.3 and 32.0 g (average = 28.5 g). Each sex was separately and randomly divided into four groups based upon the method of blood collection: intracardiac (IC), single stick caudal vena cava (VC), caudal vena cava for maximum blood collection (MC), and retroorbital (RO). The difference between the VC and MC methods is that the vena cava was phlebotomized only once for the VC group, whereas the MC group received a caudal vena cava stick and all blood spilling into the abdomen was also collected. A 1-ml tuberculin-type syringe with a 25-gauge 5/8-inch needle (Exel, Los Angeles, CA) was used for all collections except the RO group. IC collection was performed via percutaneous access using a caudal approach through the diaphragm. The VC and MC collections were obtained after opening the abdomen for direct visualization of the caudal vena cava. For the RO group, an untreated capillary tube (Fisher, St. Louis, MO) was used to access the retroorbital sinus, with blood being dripped into the respective tubes. For the phlebotomy, all animals were given intramuscular ketamine hydrochloride (Ketaset®, Fort Dodge, IA) and xylazine hydrochloride (Anased®, Lloyd Labs, Shenandoah, IA) at 120 and 10 mg/kg, respectively. All animals were unfasted and samples were collected in the afternoon.

Blood for hematology was collected into Microtainer Brand Tubes with EDTA (Becton-Dickinson, Franklin, NJ) as an anticoagulant. Blood for serum biochemistry analysis was collected into preservative-free Microtainer Brand Serum Separator Tubes (Becton-Dickinson, Franklin, NJ). For all collection techniques, the stopper from the tube was removed and blood was deposited either directly from the syringe (IC, VC, MC) after removal of the needle or directly by dripping into the tube (RO). The total volume for hematology was 250  $\mu$ l and the remaining volume was used for serum biochemistries. The blood for serum biochemistry evaluation was allowed to clot at room temperature and was centrifuged for 10 min using an Adams Compact II centrifuge (Becton-Dickinson, Franklin, NJ), and the serum was separated for shipment. Blood and serum were sent to the contract laboratory for analysis on ice packs via express delivery. All samples were processed in the same manner and were shipped within the same boxes in order to prevent postcollection artifact.

The analysis of the samples was conducted by a contract laboratory (Laboratory Corporation of America, Research Triangle Park, NC). The hematological analysis included a complete blood count with differential and platelet count. Within the limitation of available serum volume, a panel of serum biochemistries was conducted and included alanine aminotransferase (ALT), albumin, aldolase (ALD), alkaline phosphatase (alk phos), amylase, aspartate aminotransferase (AST), bilirubin (total), blood urea nitrogen (BUN), BUN/creatinine ratio, chloride, creatine kinase (CK), creatinine, globulin (calculated), lipase, and total protein. The globulin was calculated by subtracting the albumin value from the value for the total protein.

Statistical evaluation was performed by the Biostatistics and Data Analysis Core of the Department of Biostatistics and Epidemiology at the University of Pennsylvania. Analysis of variance (ANOVA) was fitted using blood collection technique and

gender as classification variables using PROC GLM in SAS (SAS Institute, 1990, 1996). The interaction of these effects was also examined, as values may have been impacted by potential differences in collection technique due to body size differences between the genders. A 0.01 level of significance was used for all statistical evaluations.

## RESULTS

The hematology and biochemistry results are provided in Tables 1 and 2. The tables provide ranges (minimum and maximum values), means, and standard deviations broken down by collection method and gender.

### Gender

Gender differences were apparent for 16 of the 25 parameters evaluated, with  $p$  values of  $<0.0001$ , except as indicated. Female mice demonstrated higher values for red blood cell number, hemoglobin ( $p \leq 0.002$ ), hematocrit, alkaline phosphatase, albumin, total protein, and creatinine. Males demonstrated higher values for platelet counts, specific white blood cell numbers (total, neutrophil, lymphocyte, and eosinophil counts), globulin, amylase, and the BUN/creatinine ratio.

### Hematology

Slight anisocytosis and polychromatophilia were noted in all animals in this study. Additionally, Howell-Jolly bodies were noted in 18 of the 120 animals (15%), with no correlation to gender or method of blood collection. Smudge cells occurred in two of the MC animals.

The red blood cell count, hemoglobin, and hematocrit demonstrated statistically significant effects due to the collection method ( $p < 0.0001$ ) when comparing methods with each other. These differences were due to the higher values obtained within the RO group and the lower values in the IC group. One substantial outlier was noted for platelet counts within the male RO group, with a confirmed value greater than  $1000 \times 10^5/\text{mm}^3$ .

A statistically significant difference was noted between the methods of collection for both the total white blood cell counts and lymphocytes counts ( $p = 0.0001$ ). The difference was due to the IC group having lower values than the other methods. The MC group was statistically different than the VC group for these same values, with the ranges and standard deviation being somewhat larger for the MC group. The values for basophils and bands were always zero for all collection methods.

### Serum chemistries

AST demonstrated statistically significant differences between all collection methods (Fig. 1A). The VC method values were the lowest and the least variable in comparison to other methods. While the range of values for the IC method was lower as compared to the MC and RO methods, it was significantly higher than the VC method. The range for the VC method was 30–57 IU/dl in the VC method. The MC and RO groups demonstrated very large ranges. The ranges for the MC females were 21–1430 IU/dl and the ranges for the RO males were 50–627 IU/dl. Unlike the results for AST, the IC and VC techniques provided similar results ( $p = 0.5877$ ) for ALT. The val-

ues for the MC and RO groups were significantly greater in comparison to either of the VC or IC groups (Fig. 1B).  $\gamma$ -Glutamyltranspeptidase (GGT) was not evaluated in this study, as our previous experience in mice has demonstrated very low values (generally 0.0 to 0.1 IU/dl) and no demonstrable correlation between the assay results and corresponding histopathology of the liver.

The IC method gave higher values for BUN as compared to the MC and RO groups. The VC gave the greatest variability, but was not significantly different than the MC group ( $p = 0.0972$ ). The retroorbital group was statistically lower than the IC and VC groups ( $p = 0.0001$  and  $0.0012$ , respectively), but was similar to the MC groups. In general, the BUN values were highest for the IC group.

The alkaline phosphatase for the IC and RO groups yielded similar results ( $p = 0.5455$ ) as did the VC and MC groups ( $p = 0.8412$ ), with slightly lower values for the vena cava collection methods. While the VC and MC groups revealed similar values for albumin and total protein, the RO group had higher values and was statistically different than the other groups ( $p = 0.0001$ ) with the IC groups having intermediary values. The values for globulin were not substantially different between groups. Creatine kinase values were lower and more consistent for the IC and VC methods. The MC method clearly gave the highest values, with the RO method demonstrating intermediary values.

While amylase results were similar between groups, substantial differences were noted between groups ( $p = 0.0001$ ) for lipase. For this parameter, the VC and RO groups had maximum values of 12, whereas the MC and IC had maximum values of 123 and 1348, respectively. Aldolase values were different between groups, with the least variability noted in the VC group. Aldolase values were similar for the VC and IC methods, with the highest values noted in the MC groups. Total bilirubin could not be evaluated due to lack of sufficient volume to consistently analyze between groups.

Finally, body weight was evaluated for evidence of other interactions and as a measure to qualitatively assess the randomization. There was no statistical difference between groups ( $p = 0.93$ ). The males overall were heavier than the females ( $p = 0.0001$ ).

## DISCUSSION

As defined by Riley (1992), preanalytical variation is the change in the concentration of analytes (serum levels of biochemicals or cell parameters) in samples introduced before the samples are analyzed. There are a number of factors originating from a broad range of events that may influence the assay results. The variations caused by these factors negatively impact the predictive and evaluative value of the assay results due to the introduction of artifacts. Known sources of artifacts have been reviewed by Riley (1992). These alterations can be divided into actions performed prior to sample collection, during the sampling process, or during the period following sampling until the actual analysis. Additional variation may occur during the analysis process. All of these variables impact the assay results obtained. This report concentrates on the effect of the sampling process, specifically the collection method.

One of the more common major target organ for toxicity,

TABLE 1. SUMMARY DATA FROM FEMALE MICE BY THE COLLECTION METHOD<sup>a</sup>

Parameter	Units	p value	Intracardiac (IC)				Vena cava (VC)				Maximum collection vena cava (MC)				Retroorbital (RO)			
			Mean	Min	Max	SD	Mean	Min	Max	SD	Mean	Min	Max	SD	Mean	Min	Max	SD
RBC	10 <sup>6</sup> /mm <sup>3</sup>	0.0001 <sup>b</sup>	9.42	8.98	9.84	0.245	9.81	9.05	10.7	0.529	9.80	9.04	10.2	0.343	10.4	9.94	11.0	0.325
HGB	g/dl	0.002 <sup>b</sup>	13.9	13.5	14.4	0.273	14.6	13.9	15.6	0.639	14.4	13.1	15.1	0.546	15	8.8	15.8	1.8
HCT	%	0.0001 <sup>b</sup>	45.9	44.1	49.1	1.59	48.4	42.9	52.6	3.70	48.3	45.6	50.6	1.37	50.5	48.4	53.0	1.60
Platelet	10 <sup>5</sup> /mm <sup>3</sup>	0.0001 <sup>c</sup>	8.9	6.4	11.3	1.3	10.8	9.3	14.2	1.8	10	8.4	12.2	1.1	10	8.2	14.2	1.7
MCV	U <sup>3</sup>		48.9	47	51	1.23	49	46	52	1.9	49	48	51	0.78	49	48	50	0.63
MCH	UUG		14.8	14.2	15.2	0.294	14.9	14.6	15.6	0.300	14.7	14.4	15.3	0.237	14.3	8.0	15.2	1.9
MCHC	%		30.3	28.7	31.3	0.857	30.3	28.3	32.3	1.28	29.8	28.8	30.8	0.560	29.3	16.6	31.2	3.85
WBC	10 <sup>3</sup> /mm <sup>3</sup>	0.0001 <sup>c</sup>	2.8	1.6	4.2	0.81	3.4	1.3	5.5	1.6	5.9	2.6	9.7	2.0	4.6	2.2	8.5	1.5
PMN	%	0.0001 <sup>c</sup>	7	2	15	4	7	2	16	3	7	1	12	3	5	1	9	2
Lymphocyte	%	0.0001 <sup>c</sup>	87	79	93	4.6	88	76	94	4	90	83	96	4.2	92	88	97	2.5
Monocyte	%		5	2	9	2	4	1	9	2	3	1	7	2	2	1	5	1
Eosinophil	%	0.0001 <sup>c</sup>	2	0	6	2	0.9	0	2	0.8	0.4	0	2	0.7	0.3	0	1	0.5
AST	IU/liter		91	45	176	33	59	35	80	15	311	21	1430	380	209	50	555	171
ALT	IU/liter	0.5954	40	20	88	18	33	24	42	6.0	206	30	1085	278	59	30	135	34
Alk phos	IU/liter	0.0001 <sup>b</sup>	120	88	145	17	100	66	126	19	105	87	120	8.6	125	105	175	19.3
Albumin	g/dl	0.0001 <sup>b</sup>	3.6	3.2	4.0	0.20	3.5	3.0	4.0	0.33	3.5	3.3	4.5	0.34	3.9	3.5	5.0	0.48
Globulin	g/dl	0.0001 <sup>c</sup>	1.1	0.8	1.5	0.2	0.8	0.5	1.2	0.3	0.9	0.5	1.2	0.2	1.0	0.5	1.2	0.2
Total bilirubin	U/liter	0.0006 <sup>b</sup>	0.2	0.2	0.3	0.05	0.6	0.6	0.6	0.0	0.3	0.2	0.5	0.08	0.3	0.3	0.3	0.0
Amylase	U/liter	0.0001 <sup>c</sup>	2308	1664	7850	1538	1851	1554	2184	169.7	1861	1653	2472	197	2008	1650	3140	449.9
Aldolase	U/liter		22.0	3.6	45.6	10.1	35.3	22.4	49.2	7.41	125	50	450	111	62.7	17.5	150	41.0
Protein	g/dl	0.0001 <sup>b</sup>	4.7	4.0	5.2	0.29	4.4	4.0	5.0	0.37	4.4	4.2	5.0	0.27	4.9	4.0	6.0	0.57
BUN	mg/dl		31	27	35	2.5	29	21	40	5.8	26	21	32	3.2	25	20	35	4.5
Creatinine	mg/dl	0.0001 <sup>b</sup>	0.8	0.5	1.6	0.3	1	0.7	1.6	0.3	0.8	0.6	1.5	0.3	0.9	0.6	1.5	0.3
BUN/creatinine	—	0.0001 <sup>c</sup>	40	20	70	11	29	20	50	8.8	35	20	50	9.9	30	16	45	8.0
CK	U/liter		187	63	445	109	536	133	1432	403	5593	603	28575	7537	2002	141	6675	2140
Weight	g	0.0001 <sup>c</sup>	22.52	20.59	24.95	1.470	22.03	20.90	23.71	0.9653	21.75	20.24	23.82	0.9651	22.31	20.52	26.66	1.653
Lipase	U/liter		96	0.0	1348	346	0.0	0.0	0.0	0.0	32	0.0	123	30	0.0	0.0	0.0	0.0

<sup>a</sup>Note that the p value is shown for those parameters for which a significant difference was noted between sexes.

<sup>b</sup>The values for this parameter were higher in the females.

<sup>c</sup>The values for this parameter were higher in the males.

TABLE 2. SUMMARY DATA FROM MALE MICE BY THE COLLECTION METHOD<sup>a</sup>

Parameter	Units	p value	Intracardiac (IC)				Vena cava (VC)				Maximum collection vena cava (MC)				Retroorbital (RO)			
			Mean	Min	Max	SD	Mean	Min	Max	SD	Mean	Min	Max	SD	Mean	Min	Max	SD
RBC	10 <sup>6</sup> /mm <sup>3</sup>	0.0001 <sup>b</sup>	9.23	8.44	10.2	0.487	9.07	7.77	9.77	0.490	9.38	8.94	10.1	0.327	10	9.10	10.9	0.497
HGB	g/dl	0.002 <sup>b</sup>	13.6	12.6	14.6	0.591	13.4	12.0	14.5	0.616	13.8	13.4	14.6	0.337	14.7	13.7	15.6	0.612
HCT	%	0.0001 <sup>b</sup>	46.0	41.9	49.9	2.14	44.9	39.8	48.6	2.09	46	43.4	50.3	1.88	48.7	44.6	53.4	2.40
Platelet	10 <sup>5</sup> /mm <sup>3</sup>	0.0001 <sup>c</sup>	12.3	9.8	13.9	1.0	13.1	9.9	18.4	1.88	12	4.9	14.9	2.9	104	10.8	1001	298
MCV	U <sup>3</sup>		50	48	52	1.0	50	49	51	0.64	49	48	50	0.85	49	48	50	0.70
MCH	UUG		14.7	14.3	15.3	0.230	14.8	14.5	15.4	0.226	14.8	14.4	15.3	0.245	14.8	14.3	15.5	0.339
MCHC	%	0.0001 <sup>c</sup>	29.5	28.6	30.8	0.546	29.9	29.2	30.6	0.372	30.1	29.0	31.7	0.683	30.3	29.2	32.1	0.765
WBC	10 <sup>3</sup> /mm <sup>3</sup>	0.0001 <sup>c</sup>	5.4	2.3	7.7	1.5	5.8	4.4	7.2	0.81	7.5	1.8	10	2.2	6.6	4.5	9.8	1.8
PMN	%	0.0001 <sup>c</sup>	12	2	34	10	14	2.0	30	7.9	19	3	53	13	20	3	66	20
Lymphocyte	%	0.0001 <sup>c</sup>	84	59	97	11	81	60	98	8.7	78	46	94	13	76	32	92	20
Monocyte	%		3	0	6	2	4	0	7	2	2	1	4	1	3	0	6	2
Eosinophil	%	0.0001 <sup>c</sup>	1	0	2	0.8	1	0	3	1	1	0	2	1	1	0	3	1
AST	IU/liter		82	51	232	45	47	30	57	8.9	130	92	206	31	255	63	627	160
ALT	IU/liter	0.5954	38	30	51	6.3	39	28	51	7.9	60	40	93	15	77	33	171	36
Alk phos	IU/liter	0.0001 <sup>b</sup>	79	58	96	9.0	72	40	90	13	68	54	80	6.9	72	45	87	12
Albumin	g/dl	0.0001 <sup>b</sup>	3.0	2.5	3.6	0.29	2.7	2.4	3.0	0.23	2.9	2.4	3.3	0.24	3.2	2.7	4.3	0.41
Globulin	g/dl	0.0001 <sup>c</sup>	1.3	1.2	1.6	0.18	1.2	0.8	1.5	0.25	1.3	1.2	1.6	0.14	1.2	0.2	1.5	0.37
Total bilirubin	U/liter	0.0006 <sup>b</sup>	0.2	0.2	0.2	0.0	0.2	0.2	0.2	0.04	0.2	0.2	0.3	0.0	0.4	0.2	0.6	0.1
Amylase	U/liter	0.0001 <sup>c</sup>	2351	1992	2756	205.4	2263	1827	2645	228.2	2238	1984	2442	136.7	2314	2080	2516	145.5
Aldolase	U/liter		23.1	16.8	44.2	6.37	24.6	17.5	40.8	6.16	98.6	67.6	148	25.4	73.3	23.8	117	26.7
Protein	g/dl	0.0001 <sup>b</sup>	4.3	4.0	4.8	0.25	3.9	3.2	4.5	0.35	4.2	3.8	4.5	0.21	4	4	5	0.3
BUN	mg/dl		31	24	36	2.8	29	24	40	4.9	28	24	33	2.8	26	21	30	3.5
Creatinine	mg/dl	0.0001 <sup>b</sup>	0.8	0.6	1.0	0.1	0.6	0.4	1.0	0.2	0.6	0.4	0.9	0.1	0.7	0.1	1.0	0.2
BUN/creatinine	—	0.0001 <sup>c</sup>	40	30	60	8.6	50	30	80	17	47	30	70	11	55	30	280	64
CK	U/liter		187	52	606	141	282	68.0	680	201	3915	891	12420	2896	2114	168	5271	1571
Lipase	U/liter		8.8	0.0	26	10	0.9	0.0	12	3.3	31	0.0	80	27	1.5	0.0	12	3.9
Weight	g	0.0001 <sup>c</sup>	28.20	22.29	31.81	2.544	28.29	23.38	32.01	2.309	29.06	25.95	31.35	1.567	28.53	24.84	30.40	1.742

<sup>a</sup>Note that the p value is shown for those parameters for which a significant difference was noted between sexes.

<sup>b</sup>The values for this parameter were higher in the females.

<sup>c</sup>The values for this parameter were higher in the males.

particularly in adenoviral gene transfer studies, is the liver. These studies are designed for a number of pragmatic and welfare issues to utilize the minimum numbers of animals while obtaining the maximum amount of information through multiple evaluation methods. Evaluations of clinical pathology and gene expression often compete for a limited blood volume. Because of this competition for blood volume, the maximum vena cava collection was historically used in our studies. Difficulty in statistical interpretation of the results, particularly in determining a no-observed adverse effect level (NOAEL) for toxicity studies based upon serum transaminase levels, occurred due to often wide interanimal variability. As other potential causes including underlying nonclinical disease were excluded, the method of collection employed was the most likely cause. This study was conducted because of the need to determine the cause of the variability and to select the preferred method.

As the results show, the method of collection impacts both the actual values obtained for an assay and the range and standard deviation of the values. Overall the preferred method, based upon low variability, is the VC followed closely by the IC method. Further, both the RO and MC techniques were associated with considerable variability and relatively high values for various parameters.

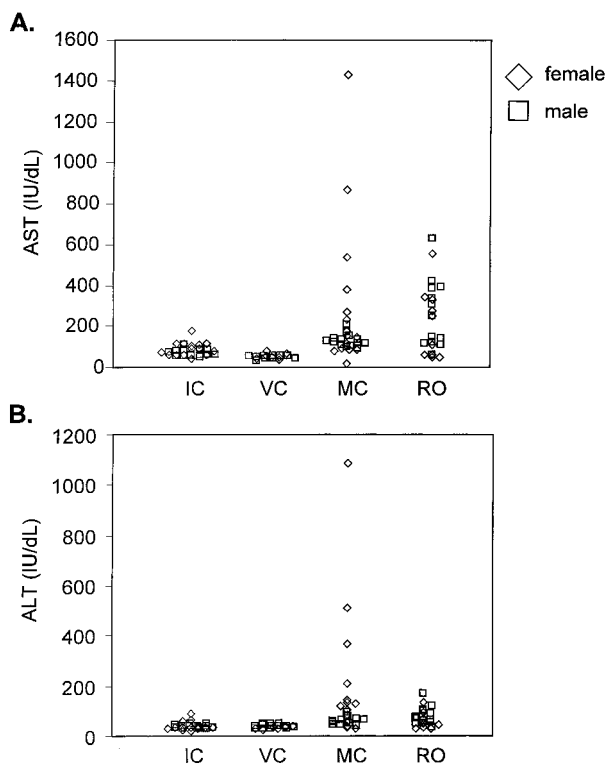
It is particularly important to use either the VC or IC method whenever serum transaminase levels are the primary marker, such as in studies involving hepatotropic vectors. The most critical impact of the phlebotomy method was upon these levels. The results indicated that the VC method yields the lowest and most consistent results, particularly with AST. Importantly, the MC method was associated with substantial impact on the range of values and standard deviation. The primary impact on AST values using this method appears to have been on females. As compared to the MC groups, the variation in RO groups was more consistent throughout the range in both sexes. AST was more affected by the RO method, and this alteration likely is due to trauma either to the periocular tissues or to the blood cells. This finding is consistent with that from our previous study in which evaluation of the toxicity was obscured by the wide value range not allowing for discernment effects. Based upon the results of this study, we have changed our preferred method to the VC method.

The VC method is perhaps easier to learn and to perform due to the direct visualization of the vessel, with the most significant disadvantage being the collapsibility of the vessel requiring gentle aspiration. However, such gentle aspiration is perhaps one of the primary contributors to the tight standard deviation ranges, as increased negative pressure is associated with more hemolysis (Riley, 1992).

The IC method is also a preferred method. As it is a blind stick, training and experience may be necessary to perform it well. The heart is less collapsible than the vena cava, but rapid aspiration may also lead to collapse of the cardiac chamber. It is a less time-consuming method of collection, as it does not require the dissection required for the VC method or involve relatively slower blood flow as in the RO method.

The primary advantage of the RO method is that it is a recovery procedure and therefore clinical pathology effects can be monitored over time in the same animals. Although widely used as a preferred technique for the National Toxicology Program (Thompson, 1992), the RO method was associated with substantial limitations in the present study. However, carbon dioxide was used in that study and therefore there may be anesthesia-related effects. As an example, the AST levels ranged from 63 to 627 (standard deviation = 160.5) in males and 50 to 555 (SD = 171.3) in females in these untreated animals by this route. Such large ranges and deviations make evaluation of this parameter using this method quite problematic. Further, the retroorbital method in this study was also associated with the highest values for the serum transaminases, creatine kinase, alkaline phosphatase, aldolase, and albumin. This study suggests that the method may have practical utility if the primary clinical pathology indicators have not demonstrated intolerable effects. Although the ALT value range was larger for the RO group than the VC or IC groups, as a recovery method it may be useful for assessment of potential hepatotoxicity of a gene transfer agent over time within the same animal. The RO method may also be acceptable to obtain samples for gene expression prior to terminal phlebotomy, if sufficient time has been given for resolution of effects due to blood withdrawal and healing of the injury to the RO plexus.

The gender of the animal had a substantial impact on the obtained values for 17 of 26 parameters, and therefore use of separate normal ranges based upon gender are important to increase the sensitivity of observing treatment-related effects in this



**FIG. 1.** Comparison of transaminase levels by method. (A) The aspartate aminotransferase (AST) by group. The VC method had minimal variability in comparison to other methods. The MC method was associated with the largest range of values, particularly due to outliers in the females. The RO method had a more even distribution within a broad range of values. (B) The values for alanine aminotransferase (ALT) demonstrating results similar to AST. Note that while the ALT range is substantially less for the RO method, it is significantly greater than either the VC or IC methods.

strain of mouse. Frith *et al.* (1980) reported similar but fewer gender differences, namely for hemoglobin, phosphorus, glucose, AST, total protein, and alkaline phosphatase. Female mice have higher hemoglobin and hematocrit values than males, which is opposite that found in humans.

The "gender" effects noted are likely due to collection-dependent effects in addition to those that are due to true physiological differences between the genders. As the females weighed significantly less than the males, available blood volume was less. Because of this, collection of a suitable sample volume is relatively more difficult in females than in males. Artifactual elevations due to relatively more damage to blood cells in addition to abdominal fluid contamination of the blood likely occurred to a greater extent in females. Suggestive evidence for this is that the standard deviation in males for AST was only approximately 35-fold higher for the MC as compared to the VC method, whereas the difference in females was approximately 25-fold. The variability in the MC method in females was likely due to the fact that collection of the requisite blood volume was easier than in males than females. The collection of an equal volume of blood was more difficult and likely artifactually increased certain parameters, e.g., serum transaminases (see Fig. 1).

Effects related to collection technique may have been heavily influenced by the ease of blood collection. The differences between the results of the VC and IC groups, for which collection involved minimal manipulation, and those of the MC and RO groups, which required substantial manipulation, suggest that this additional disturbance may be the cause of these variations. This is especially true for the VC versus MC, as the differences between the two are associated with relatively protracted collection and potential abdominal fluid contributing to the analytes for the MC method. Alterations in the RO method may have been related to potential true variations in blood components, trauma, and damage to the cellular components.

Within gene transfer studies, evaluation of serum levels of surrogate markers, such as erythropoietin, is often used to confirm gene expression. Unfortunately, other methods (IC and VC) provide less blood relative to the MC method and therefore additional animals may be required for evaluation of gene expression via serum markers, as applicable. If collection of samples for both clinical pathology and other such assays is necessary, an alternative would be to use one of the preferred methods followed by maximum collection via the caudal vena cava, which may provide the ability to achieve both goals. However, it would be necessary to confirm that the collection technique does not also intolerably affect the results of these secondary assays.

In conclusion, the site of blood collection plays a substantial role in both the values and variations in clinical pathology results. The single-stick caudal vena cava method and intracardiac method are preferred for consistency as determined by lower standard deviations and tighter ranges. The technique-dependent effects must be considered when selecting the method of collection, and the data relative to the collection method provided here would facilitate this selection. Further, it is critical to evaluate clinical pathology data in light of potential artifacts related to collection technique. This is particularly true for comparison with "historical data" or between published reports for which techniques between studies may have varied. Similar to use of historical histopathological control data, valid statistical analysis requires comparison of "like" with "like" (Roe, 1994). The data

provided here will assist in these comparisons, as well as selection of the preferred method of collection for a particular study design. While reference to published reference ranges is useful, the authors recommend valid control data and reference ranges be developed when more precise data evaluation is necessary. As technique-dependent differences occur, the sample collection technique should be fully described whenever clinical pathology results are reported or interpreted.

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