Research Report

An equation to determine the percentage of whole blood contamination in stallion semen, based on packed cell volume*

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Abstract

The purpose of this experiment was to determine a method to estimate the percentage of whole blood contamination in stallion semen. Experiment 1 used a mathematical simulation to predict packed cell volume (PCV) of hemospermic semen, based on whole blood PCV. A regression line was plotted for each simulation and the equation of the line was calculated that produced the following equation:

Percent whole blood contamination in semen = $[PCV_{semen} + (PCV_{blood}/100)] \div (PCV_{blood}/100)$

The equation was then used to predict the percentage of whole blood contamination in semen and compared to the actual percentage. Experiment 2 tested the equation using blood-semen mixtures at 5, 10, 20, 40 and 50% dilutions. Experiment 3 expanded the dilution range used in Experiment 2 to span from 0-100% at 20% increments. PCV of blood and semen dilutions from Experiments 2 and 3 were measured. The mean difference between the actual percentage of whole blood contamination and predicted was −0.59 ± 3.53 in Experiment 2, and the test for zero bias was not significant and there was no significant correlation in the independence of bias. In Experiment 3, mean difference between the actual percent of whole blood contamination and predicted was −2.73 ± 0.87 and the test for zero bias and correlation in the independence of bias were significant. These data suggested that the amount of whole blood contamination in semen can be estimated based on stallion's PCVs blood and semen sample.

Keywords: Stallion, hemospermia, equation, prediction

Introduction

Hemospermia has been associated with reduced fertility in stallions.[1,2] Mares inseminated with raw semen containing 20% whole blood had a 7.7% (1/13) per cycle pregnancy rate.[2] Mares inseminated with semen containing 5% whole blood contamination had similar fertility to 0% contamination whereas none of the mares bred with semen containing 50% whole blood contamination became pregnant.[1] Red blood cells have an impact on semen and fertility; however, when 20% of serum was added to semen, it did not interfere with fertility.[2]

Immediate dilution with milk-based extender in at least a 2:1 (volume/volume) extender: semen ratio can rescue the fertility and sperm kinetics of semen containing high amount of blood when using fresh semen for breeding.[3] Effects of red blood cells on fertility in semen subjected to cooling or cryopreservation remain unknown; however, increasing blood contamination reduced sperm viability after 24 hours of cooled storage in a dose-dependent manner.[4] This suggested that cooled storage of hemospermia samples could reduce fertility. The exact threshold at which blood contamination impacts fertility is unknown and the mechanism remains to be elucidated.

In this study, packed cell volume of semen (PCV_{semen}) refers to the total sedimented cellular fraction obtained following centrifugation of raw semen in a microhematocrit tube.

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Following centrifugation, erythrocytes within blood-contaminated semen form a visually distinct packed layer separate from the sperm sediment, as observed in prior unpublished observations. Because the erythrocyte layer can be estimated independently of the sperm pellet, the proportion of whole blood within a semen sample can be calculated based on the PCV of the donor's blood. Estimating the amount of blood in semen may aid in determining the threshold at which blood contamination reduces fertility and support decision-making regarding whether semen should be used for breeding, cooled transport, or further processing. The purpose of this experiment was to determine a method to estimate the percentage of whole blood contamination in semen.

Materials and methods

Ethical approval was granted by the Oklahoma State University Institutional Animal Care and Use Committee under protocol number IACUC-21-04-STW.

Experiment 1

A mathematical simulation was used to predict the PCV of hemospermic semen, based on the PCV of whole blood. For the simulation, the PCV of blood was assumed to be either 30, 35, or 40% and the range of blood and semen dilutions from 0-100% was modeled by multiplying the percent dilution of the blood-semen combination by the blood PCV to give a predicted PCV for the semen. A regression line was plotted for each simulation, and the equation of the line calculated. By examining the similarities between regression line equations, an equation was developed to predict the percentage of whole blood contamination based on blood and semen PCV.

Experiment 2

Actual percent of whole blood contamination in semen to predicted percent of whole blood contamination in semen based on PCV of blood and hemospermia sample was compared. Semen was collected from 1 stallion and blood was drawn from the jugular vein of 10 donor horses into 10-ml EDTA tubes using a vacutainer needle. Blood was loaded into microcapillary tubes and centrifuged (10,030 x g for 5 minutes) and PCV was determined using hematocrit reader chart. Blood from each donor was added to semen at 5, 10, 20, 40 and 50% (volume/volume) to create 0.5 ml aliquots. Raw semen and blood-semen dilutions were loaded into microcapillary tubes, centrifuged, and measured as previously described. This experiment was performed in 2 replicates with different micropipettes used for each replicate. Replicate 1 utilized an air displacement style (Eppendorf, Enfield, CT, USA) pipettes for all dilutions whereas replicate 2 utilized positive displacement style (Mettler-Toledo Rainin, Oakland, CA, USA) pipettes for all dilutions. The equation produced from Experiment 1 was used to predict the percentage of whole blood contamination in semen, based on the PCV of whole blood and blood-semen combination.

Experiment 3

Semen was collected from 2 stallions and blood was drawn from the jugular vein of the same 2 stallions and 1 mare through a vacutainer needle into 10 ml EDTA tubes. Raw semen and blood were loaded into microcapillary tubes, centrifuged (10,030 x g for 5 minutes), and PCV was read using a hematocrit reader. This confirmed that the raw semen samples had no blood and established baseline PCV values. Artificially induced hemospermic dilutions were created by mixing blood (from mare and same stallion or other stallion) with semen at 0, 20, 40, 60, 80 and 100% dilutions (volume/volume) to create 1 ml aliquots using a 100 µl positive displacement pipet (Mettler-Toledo Rainin). Dilutions were loaded into microcapillary tubes, centrifuged, and measured as previously described.

Data analysis

For Experiment 2, replicates 1, 2 and the combined data, SAS (version 9.4) SGPlot procedure was used to generate Bland-Altman plots of the difference and mean of the actual percentage of whole blood contamination and predicted percent of whole blood contamination. Student's *t*-test was performed for zero bias using actual and predicted values and independence of bias was tested using the Corr procedure using the bias (difference between actual and predicted) and magnitude (average of actual and predicted). Outliers were removed (n = 2) from the combined dataset (Replicates 1 and 2) when > 3 standard deviations and the data were reanalyzed.

For Experiment 3, logistic regression was performed to evaluate whether there was a difference between blood donor sources (mare, same or other stallion). Because there was no significant effect of donor type on the outcome, data from all blood sources were combined without removing any outliers for subsequent analyses using a Bland-Altman plot, as previously described.

Results

In Experiment 1, we derived the following equation:

$$\text{Percent whole blood contamination in semen} = [PCV_{semen} + (PCV_{blood}/100)] \div (PCV_{blood}/100)$$

For Experiment 2, semen had no evidence of hemospermia based on the color after collection and centrifugation of raw semen microcapillary tubes for either replicate.

Bland-Altman plot for Replicates 1 and 2 are displayed in Figures 1A and 1B, respectively. For Replicate 1, the mean difference between the actual percent of whole blood contamination and predicted was –2.2440 ± 6.3808. The test for zero bias (actual versus predicted values) had a weak tendency toward significance (p = 0.09) and there was correlation (p = 0.01) in the independence of bias. For Replicate 2, the mean difference between the actual percent of whole blood contamination and predicted was –0.3600 ± 2.9022. The test for zero bias (actual versus predicted values) was not different (p = 0.54) and there was a correlation (p = 0.03) in the independence of bias.

Because there was not a significant difference in either replicate between the actual versus predicted values for Replicates 1 and 2, the data were combined for analysis. Bland-Altman plot for the combined data is displayed in Figure 2. The mean difference between the actual percentage of whole blood

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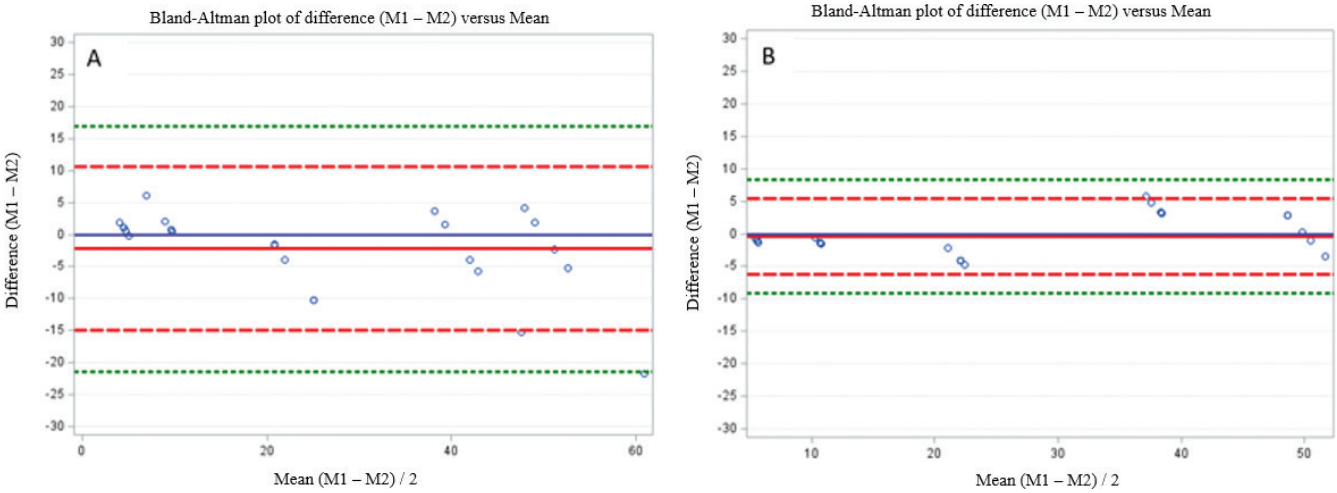

Figure 1. Bland-Altman plot of the difference (actual percent of whole blood contamination minus predicted amount of whole blood contamination) to the mean [(actual percent of whole blood contamination plus predicted)/2] for Experiment 2, replicate 1 (A) and replicate 2 (B). (A), the solid blue line represents actual percent of whole blood contamination minus predicted amount of whole blood contamination = 0, The solid red line represents the mean difference (actual percent of whole blood contamination minus predicted amount of whole blood contamination = -2.244), the dashed red lines represent 2 standard deviation limits (12.76), and the dashed green lines represent 3 standard deviation limits (19.14). (B), the solid blue line represents actual percent of whole blood contamination minus predicted amount of whole blood contamination = 0, The solid red line represents the mean difference (actual percent of whole blood contamination minus predicted amount of whole blood contamination = -0.360), the dashed red lines represent 2 standard deviation limits (5.80), and the dashed green lines represent 3 standard deviation limits (8.71)

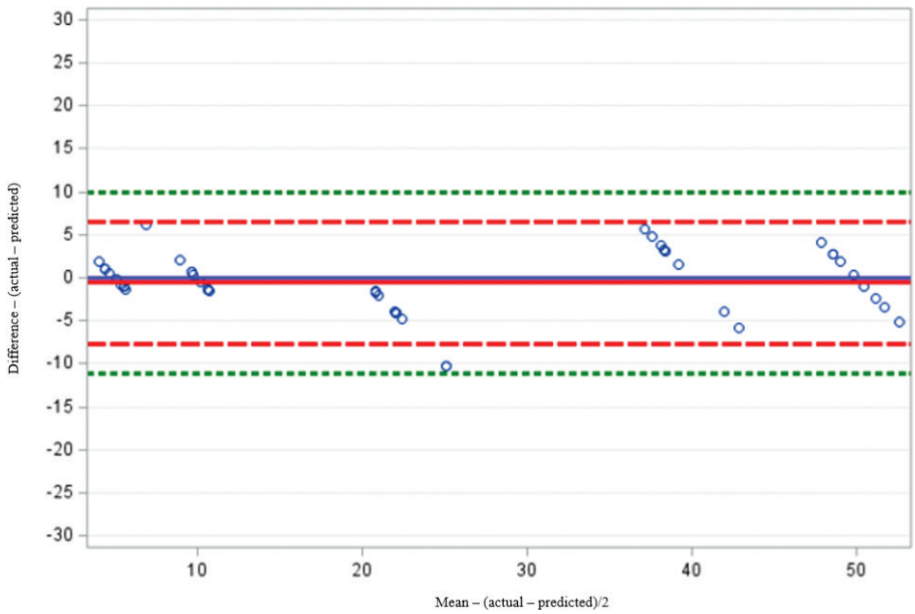

Figure 2. Bland-Altman plot of the difference (actual percent of whole blood contamination minus predicted amount of whole blood contamination) to the mean [(actual percent of whole blood contamination plus predicted)/2] for the combined data for Experiment 2 for replicates 1 and 2. The solid blue line represents actual percent of whole blood contamination minus predicted amount of whole blood contamination = 0, The solid red line represents the mean difference (actual percent of whole blood contamination minus predicted amount of whole blood contamination = –0.59), the dashed red lines represent 2 standard deviation limits (7.06), and the dashed green lines represent 3 standard deviation limits (10.59)

contamination and predicted was –0.59 ± 3.53. The test for zero bias (actual versus predicted values) was not significant ($p = 0.26$) and there was no correlation ($p = 0.80$) in the independence of bias.

Raw semen had no evidence of whole blood contamination, based on color after collection and centrifugation of raw semen in microcapillary tubes for both stallion samples (Experiment 3); Bland-Altman plot is

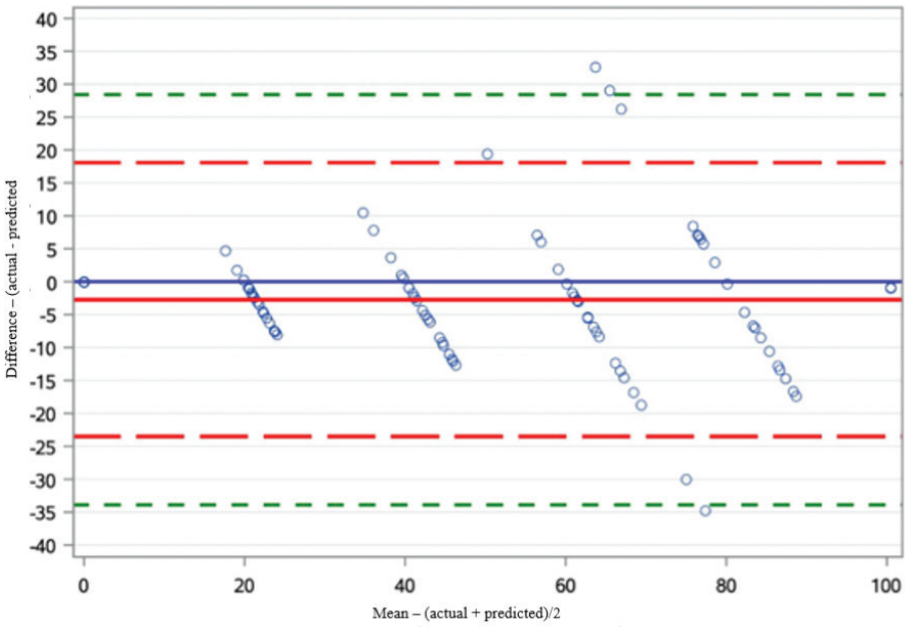

Figure 3. Experiment 3's Bland-Altman plot of difference (actual percent of whole blood contamination minus predicted amount of whole blood contamination) to the mean ([actual percent of whole blood contamination plus predicted]/2]) for the combined data of self, other stallion, and mare. The solid blue line represents actual percent of whole blood contamination minus predicted amount of whole blood contamination = 0, The solid red line represents the mean difference (actual percent of whole blood contamination minus predicted amount of whole blood contamination = −2.73), the dashed red lines represent 2 standard deviation limits (24.79), and the dashed green lines represent 3 standard deviation limits (34.05)

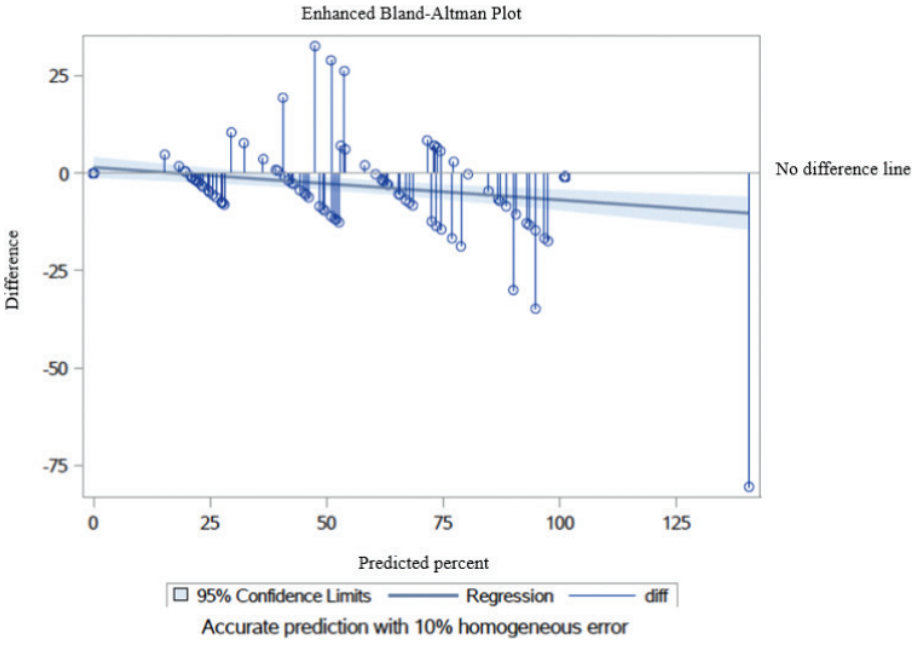

Figure 4. Experiment 3's enhanced Bland-Altman plot illustrating the agreement between actual and predicted percent whole blood contamination. Most data points fall below the zero-difference line, particularly at higher mean values, indicating a consistent underprediction (fixed bias). The spread of differences increases with the mean, and the fitted regression line demonstrates a slight negative slope, consistent with a significant proportional bias (r = –0.17, p = 0.039)

displayed (Figure 3). The test for zero bias (actual versus predicted values) was different (p = 0.002); mean difference was –2.73 ± 0.87. There was a difference (p = 0.039) in correlation (r = –0.17) in the independence of bias. It was noted that variation increased with the percent blood dilution (Figure 4).

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Discussion

This is the first report of a method to determine the amount of hemospermia in semen. This is a practical method that utilizes common equipment in clinical practice and requires little time. Most practices have the equipment to measure PCV and can easily obtain a blood sample to measure PCV. This provides an easy and practical method to determine the level of whole blood contamination in semen without the need for specialized equipment.

It is likely this will work in other species (e.g. cases of hemospermia in dogs) since there is nothing unique to the stallion in this calculation. It is based on the equation: $concentration_1 \times volume_1 = concentration_2 \times volume_2$. Thus, by knowing the PCV of blood and the PCV of semen the percent of whole blood contamination in semen can be calculated. This premise should hold true regardless of species.

Although the difference of the actual and predicted values in Replicate 1 was not significant, there was more variation than in Replicate 2. This can be attributed to the type of pipet used.[5] Air pipettes have greater error compared to positive displacement pipettes.[6] Although, this was not tested in this experiment, the greater difference between the actual and predicted values in Replicate 1 than in Replicate 2 supported the idea of positive displacement pipettes as being more accurate and should be used when accuracy is paramount.

In Experiment 3, paired *t*-test demonstrated a fixed bias ($p = 0.002$) between actual and predicted values (mean difference = -2.73 ± 0.87), which indicated that predictions systematically underestimated actual values. This fixed bias is visually supported in the enhanced Bland-Altman plot (Figure 4), where most points fall below the zero-difference line, particularly at higher values, indicating a consistent underprediction trend. This may be related to the preparation of dilutions in Experiment 3 that required multiple transfers using a positive displacement pipette, due to limitations in available pipette volume. Consequently, several pipetting steps were required to achieve the target dilutions for each sample. An increase in variance was observed across the 20-80% dilution range that became more pronounced with additional transfer steps. Thus, cumulative pipetting errors may have contributed to the observed increase in variability at higher dilution levels. Additionally, increased pipetting may have introduced mechanical stress and turbulence, which could explain the hemolysis noted in 40, 60 and 80% dilutions. Hemolysis caused by the increased number of pipetting may be artificially reducing the actual percent contamination. In samples where hemolysis was present, the prediction equation deviated by up to 10%, suggesting reduced accuracy compared to nonhemolyzed samples. Although less accurate, the impact of larger single-step (to minimize hemolysis) transfer using an air-displacement (instead of positive-displacement as in the current study) pipette can be investigated.

These data suggested that the amount of whole blood contamination in semen can be estimated based on the PCV of the stallion's blood and PCV of semen. Combining fertility records with estimate of the degree of hemospermia in semen will allow researchers and practitioners to determine what level of hemospermia reduces fertility.

Authors' contribution statement and agreement

SL: Experiment 3 data collection and manuscript preparation; **RH:** Experimental design and editing; **CT:** Experimental design and editing and DK: Experimental design, Experiment 1 and 2 data collection, data analysis and manuscript preparation. Authors have read and approved final submission.

Conflict of interest

None to report.

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