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Hematological profile of healthy Thoroughbred foals from birth to one month of age in Trinidad, West Indies

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OBJECTIVE
This study aimed to develop a sound database for the hematological reference intervals of thoroughbred foals in Trinidad, West Indies from birth to 1 month of age.

ANIMALS
89 foals.

METHODS
Whole blood samples were taken from 89 foals throughout Trinidad at approximately 1 day, 1 week, and 1 month of age. These foals were examined to be classified as healthy or free from disease. Complete blood count (CBC), microscopic analysis of blood smears, and conventional PCR for *Theileria equi* and *Babesia caballi* were performed.

RESULTS
Of the 89 foals, 67 were deemed healthy and suitable for establishing reference intervals. Foals in this study had lower mean hemoglobin and hematocrit values for all 3 times of sampling when compared to their North American counterparts. Age had a significant effect on hemoglobin, hematocrit, white blood cell (WBC), neutrophil, and platelet counts of the foals from birth to 1 month of age.

CLINICAL RELEVANCE
Variations in reference intervals can occur due to differences in demographic, physiological, and environmental factors such as age, gender, breed, and geographical location. Given the changes in the hematological values over time, this study provides clinicians with valuable information that can be used to monitor the health status of newborn foals and detect disease conditions.

Keywords: complete blood count, horse, neonates, reference intervals, Trinidad

Reference intervals represent the results expected in 95% of a population of healthy animals and is a primary tool used by veterinary practitioners in the diagnosis, prognosis, management, and treatment of cases.[1] Once signs of ill health are observed, 2 of the common diagnostic tests chosen to evaluate the animal are a complete blood count (CBC) and a serum biochemistry profile.[1]

According to Latimer et al (2011), hematological reference intervals differ for horses in tropical compared to temperate climates. Other than geographical location, variations in these parameters can occur due to factors such as age, breed, gender, and physiological status.[2] Hematological reference intervals should be established for each country. Similarly, variations due to breed have been observed; for example, small breed dogs such as Poodles have higher hematocrit reference intervals compared to large breed dogs.[3]

Some of the disease conditions of newborn foals that can alter the hemogram include neonatal isoerythrolysis, septicemia, equine piroplasmosis, and diarrhea.[4,5] When these are known, a more complete evaluation can be made to diagnose and assess foals evaluated during these early periods resulting in a better outcome or prognosis.[6]

The American Society of Veterinary Clinical Pathology (ASVCP) guidelines recommend that appropriate reference intervals should be used, as interpreting clinical data with inappropriate reference

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intervals can lead to both misdiagnosis and improper treatment of a patient.[7] Factors that preclude using previously published data as reference intervals are based on these data being derived from an inadequate number of healthy subjects, low frequency of sampling as well as a short sampling period.

Trinidad, the southernmost island of the West Indies is located at 10.69° N, 61.22° W. It has a tropical climate with a population of thoroughbred horses that are mainly used for racing. Trinidad is also endemic for equine piroplasmosis that is caused by *Theileria equi* and *Babesia caballi* and can be transplacentally transmitted.[5,8] At present, the hematological reference intervals for Thoroughbred foals have not been established in this region. Therefore, the reference intervals that are currently being used by veterinarians in this country may be inappropriate and misleading. This study aims to establish reference intervals for Thoroughbred foals in Trinidad that can then be applied to foals raised under similar climatic and geographical locations.

Methods

Sample and data collection

This study was approved by the Ethics Committee of the Faculty of Medical Sciences, the University of the West Indies, St Augustine, Trinidad, and Tobago. This study was a prospective cohort study with an estimated sample size of 100 foals based on an a priori estimate of 100 foals being born annually in Trinidad. One hundred and eleven pregnant mares from 10 farms were monitored until parturition for resorptions, abortions, or stillbirths. Data on the foals and factors associated with their births were recorded. A physical examination of the foals in this study was performed as well as a distance examination to observe the behavior of the foal. The information recorded included the body condition score, activity level, hydration status, if the foal suckled, if the mare had a difficult birth, characteristics of the suckling reflex, ability to stand, color of the mucous membrane, and the presence of any clinical abnormality such as diarrhea or musculoskeletal conditions. If these were abnormal or the foal exhibited clinical signs or symptoms associated with any disease conditions, they were excluded and not used in the generation of reference intervals. Blood samples were taken aseptically from the jugular vein of the foals within the first 36 hours of life and at approximately 7 and 30 days after birth. Four milliliters of blood was placed in K^+ EDTA tubes (Vacuette tube, Greiner Bio-One North America Incorporated) for hematology, transported to the laboratory on ice packs, and stored at 4 °C until analyzed.

CBC analysis

CBCs were performed using the IDEXX VetAutoread™ Hematology Analyzer (IDEXX Laboratories Incorporated). This machine was validated by IDEXX, and quality controls were performed once every 2 months. Wright Giemsa-stained thin blood smears were examined microscopically at 1,000X, especially for the presence of the intraerythrocytic inclusions of the equine piroplasms (*T equi* and *B caballi*). A white blood cell (WBC) differential count was performed manually according to standard procedure.[9] Blood smears were also evaluated for platelet clumps. Samples containing platelet clumps were excluded from establishing reference intervals for platelet numbers. Microhematocrit tubes (Chase Scientific Glass Incorporated) containing whole blood were centrifuged using the microhematocrit centrifuge (Hematokrit 210 Andreas Hettich GMbH & Co) to evaluate plasma protein and fibrinogen levels. Plasma protein and fibrinogen levels were determined using a refractometer (Cambridge Instruments Incorporated) and the heat precipitation method, respectively, according to standard procedures.[10]

DNA extraction and PCR

DNA was extracted from 100 µL of EDTA blood using the Qiagen DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. Conventional PCR (cPCR) for *T equi* and *B caballi* were conducted as described previously.[11,12] The 18S rRNA gene was amplified to detect *T equi* using the forward and reverse primers BEC-UF2 (5′-TCGAAGACGATCAGATACCGTCG) and Equi-R (5′-TGCCTAAACTTCCTTCCTTGCGAT) (Sigma Aldrich Inc), respectively, which yielded a product of approximately 392 base pairs.[11] The positive control was a horse with clinical theileriosis that was confirmed by nucleotide sequencing and DNA extracted from PCR grade water was used as a negative control.

Five microliters of extracted DNA in elution buffer was amplified in a 25 µL reaction volume. The reaction conditions consisted of 25 pmol of forward and reverse primer, 2.5 µL GC Enhancer, 12.5 µL AmpliTaq Gold 360 Master Mix (Applied Biosystems, Life Technologies), and 4.5 µL PCR grade water (Sigma-Aldrich Inc). The PCR was performed in a Techne Flexigene Thermal Cycler (Techne) using the following cycling parameters: initial denaturation step at 96 °C for 10 minutes, followed by 40 cycles of denaturation at 96 °C for 60 seconds, annealing at 54.2 °C for 60 seconds, and extension at 72 °C for 60 seconds. There was a final extension step at 72 °C for 10 minutes.[11] Samples were held at 4 °C at the end of the process.

The PCR products were subjected to electrophoresis in 2.0% agarose (Sigma-Aldrich Inc) in 1% TBE buffer (Sigma-Aldrich Inc) prestained with ethidium bromide to visualize under ultraviolet (UV) light.

The 18S rRNA gene was again amplified to detect *B caballi* using the universal forward primer BEC-UF2 (5′-TCGAAGACGATCAGATACCGTCG) and a reverse primer Cab-R (5′-CTCGTTCATGATTTAGAATTGCT) (Sigma-Aldrich Inc). The PCR reaction yielded a product of approximately 480 base pairs.[12] The positive control DNA was obtained from a horse previously diagnosed with *B caballi* infection that was confirmed by sequencing. DNA extracted from PCR-grade water was used as a negative control. The reaction conditions were the same as previously described for *T equi* except that the annealing temperature used for *B caballi* was 51.3 °C for 60 seconds.

Foals that were cPCR positive for *T equi* or *B caballi* or had suspected intraerythrocytic piroplasms on microscopic examination of their blood smears were excluded from the study. If a foal developed a medical condition during the first month, the CBC values were not used to establish reference intervals.

Statistical analysis

The American Society of Veterinary Clinical Pathologists (ASCVP) reference interval guidelines were utilized for data analysis to generate the reference intervals for the different age groups of the Thoroughbred foals.[7] Scatterplots were generated for the evaluation of the parameter distributions and detection of possible outliers. No observations were removed from the analysis because the authors understand that since healthiness was a selection criterion and there were no unexpected observations for healthy animals, there is no need to exclude observations through algorithms. As the number of healthy subjects ranged from 40 to 120, reference intervals were generated using nonparametric methods. Ninety-five percent of reference intervals were calculated by bootstrapping 5,000 times. Ninety percent confidence limits were calculated for each reference interval of the different hematological parameters.[7] The analysis was conducted in R using the "referenceIntervals" and "tidyverse" packages.[13,14]

A mixed model repeated measures linear regression analysis was conducted on each blood parameter measured repeatedly over time. A random effect for foals was included in the analysis. The intraclass correlation coefficient (ICC) was calculated to determine the degree of relatedness among repeated measures. The ICC can range from 0 to 1, where an ICC value close to 0 implies that the variance within the cluster is greater than the variance between clusters. The log likelihood ratio test of the constant-only model vs the full model was used to determine if to include the random effect parameter, foal into the model. Gender was also introduced as a covariate in the model to determine if there was a significant difference in the values obtained for male and female foals in this study. Statistical significance was set at $P < .05$.

Results

In this study population, 89 of the 111 mares gave birth to live foals. There was an abortion rate of 19.8% as 22 of the mares lost their fetuses between the 2nd and 11th month of pregnancy (median 4th month of pregnancy). The majority of losses occurred during the 3rd month of pregnancy as 5 (4.5%) mares had fetal losses during this period. The mares in this study were housed individually in stalls with their foals and allowed on pasture once daily.

Of the 89 foals, 67 were classified as healthy as well as negative for *T equi* and *B caballi* by cPCR **(Figure 1)** and were used to establish hematological reference intervals for foals between the ages of 0–2 days. Of the 67 foals, 37 were male and 30 were female. The 22 foals that were classified as not healthy consisted of 7 foals (32%) that had failure of passive transfer due to a weak sucking reflex or the mother did not allow the foal to suckle, 5 foals (23%) that developed diarrhea, 5 foals (23%) which showed symptoms associated with colic, 4 foals (18%) with conformational problems, and 1 foal (4.5%) that had icteric mucous membranes and symptoms associated with equine piroplasmosis. Eight of these foals (36%) developed complications within the first month of life died or were euthanized. Foals that had complications were excluded from this study. For the establishment of hematological reference intervals for foals 5–10 days old, blood samples of 66 foals (37 males, 29 females) were used as the blood sample of 1 of the foals was clotted. Blood samples of 61 healthy foals were used to establish hematological reference intervals for foals 20–32 days of age (34 males, 27 males). Reference intervals for platelets were established using platelet values from 59, 55, and 48 foals for the different age groups of 0–2, 5–10, and 20–32 days old, respectively. The hematological reference intervals obtained for the foals in this study are displayed **(Table 1)**.

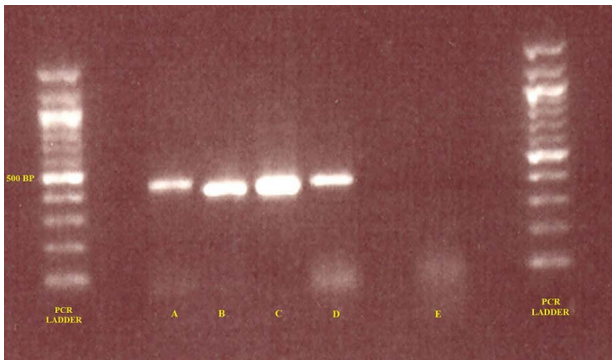

Figure 1—Image of an agarose gel showing the conventional PCR results for *Babesia caballi* and *Theileria equi* of some of the foals in this study. PCR 100 bp DNA ladder. A–E, samples, sample A: *B caballi* positive control, sample B: *T equi* positive control, sample C: *T equi* positive sample, sample D: *B caballi* positive sample, E: negative control.

Hemoglobin ($P = .0076$), hematocrit ($P = .01$), WBC count ($P = .027$), absolute neutrophil count ($P = .034$), and platelets ($P = .0003$) were significant when introduced in a mixed model repeated measure linear regression analysis. Gender did not have a significant effect on any of the hematological parameters of the foals in this study when introduced as a covariate in the model. In this study, there were progressive decreases in the hemoglobin, hematocrit, WBC, and absolute neutrophil counts of the foals from birth to 1 month of age. There was also a decrease in the mean platelet levels at 5–10 days of age followed by an increase at 20–32 days old. The ICC is a measure of the relatedness of clustered data. It accounts for this by comparing the variance within clusters with the variance between clusters. For example, the ICC value for hematocrit was 0.180 **(Table 2)** which means that the within measures of the foals for the different age groups was 18.0% similar and the between foal measure was 82.0% similar

Table 1—Hematological reference intervals established for healthy thoroughbred foals from birth to 1 month of age in Trinidad.

Hematological parameter		0–2 days Average = 0.83 days	5–10 days Average = 7.54 days	20–32 days Average = 26 days
Hemoglobin (g/L)	N	66	66	56
	Reference interval	95–166	78–150	85–142
	90% CI for lower limit	86–97	62–89	81–86
	90% CI for upper limit	164–172	148–157	141–151
Hematocrit (L/L)	N	67	66	56
	Reference interval	0.32–0.48	0.22–0.41	0.22–0.40
	90% CI for lower limit	0.26–0.34	0.20–0.26	0.19–0.22
	90% CI for upper limit	0.45–0.52	0.40–0.42	0.40–0.43
MCHC (g/L)	N	65	66	56
	Reference interval	289–378	331–390	284–396
	90% CI for lower limit	239–289	316–331	226–319
	90% CI for upper limit	376–385	384–400	395–404
Platelets (X 10^9/L)	N	59	55	48
	Reference interval	43–423	72–457	106–718
	90% CI for lower limit	22–45	10–103	85–109
	90% CI for upper limit	409–458	431–518	666–974
Plasma protein (g/L)	N	23	22	15
	Reference interval	57–76	54–75	54–74
	90% CI for lower limit	54–57	50–54	49–54
	90% CI for upper limit	76–80	75–79	74–80
Fibrinogen (g/L)	N	21	16	15
	Reference interval	1–8	1–7	1–10
	90% CI for lower limit	NA	NA	NA
	90% CI for upper limit	8–10	7–10	10–13
WBC count (X 10^9/L)	N	67	66	55
	Reference interval	6.4–21.4	6.3–22.4	5.3–21.9
	90% CI for lower limit	5.3–7.1	5.5–6.9	3.8–6.0
	90% CI for upper limit	19.3–24.7	21.6–24.9	21.2–25.5
Segmented neutrophils (X 10^9/L)	N	67	66	55
	Reference interval	3.88–17.69	3.35–18.29	2.23–17.13
	90% CI for lower limit	2.88–4.81	2.22–4.65	1.17–2.68
	90% CI for upper limit	16.59–19.63	16.15–21.64	16.86–19.44
Band neutrophils (X 10^9/L)	N	67	67	58
	Reference interval	0.00–0.46	0.00–0.30	0.00–0.00
	90% CI for lower limit	0.00–0.00	0.00–0.00	0.00–0.00
	90% CI for upper limit	0.39–0.82	0.14–0.53	0.00–0.00
Lymphocytes (X 10^9/L)	N	67	66	55
	Reference interval	0.92–4.64	1.04–5.04	1.15–5.84
	90% CI for lower limit	0.66–1.04	0.81–1.28	0.47–1.56
	90% CI for upper limit	4.24–5.44	3.92–5.80	5.12–7.23
Eosinophils (X 10^9/L)	N	67	66	58
	Reference interval	0–0.10	0.00–0.24	0.00–0.73
	90% CI for lower limit	0.00–0.00	0.00–0.00	0.00–0.00
	90% CI for upper limit	0.10–0.17	0.20–0.28	0.61–1.10
Monocytes (X 10^9/L)	N	67	66	58
	Reference interval	0.00–1.27	0.00–1.14	0.00–1.35
	90% CI for lower limit	0.00–0.00	0.00–0.00	0.00–0.00
	90% CI for upper limit	1.24–1.91	0.70–1.39	1.26–1.72
Basophils (X 10^9/L)	N	67	66	60
	Reference interval	0.00–0.21	0.00–0.43	0.00–0.20
	90% CI for lower limit	0.00–0.00	0.00–0.00	0.00–0.00
	90% CI for upper limit	0.14–0.39	0.43–0.66	0.20–0.27
Leukocytes (%)				
Segmented neutrophils (%)	N	67	67	61
	Reference interval	52–91	45–89	36–84
	90% CI for lower limit	44–59	34–59	26–38
	90% CI for upper limit	91–94	87–95	84–89
Band neutrophils (%)	N	67	67	59
	Reference interval	0–3.2	0.0–2.3	0.0–0.5
	90% CI for lower limit	0.0–0.0	0.0–0.0	0.0–0.0
	90% CI for upper limit	0.4–5.4	1.6–4.3	0.0–1.0

Table 1 (*continued*)

Hematological parameter		0–2 days Average = 0.83 days	5–10 days Average = 7.54 days	20–32 days Average = 26 days
Lymphocytes (%)	N	67	67	61
	Reference interval	8–44	6–45	10–59
	90% CI for lower limit	6–8	0–6	6–10
	90% CI for upper limit	32–53	43–52	53–68
Eosinophils (%)	N	67	66	60
	Reference interval	0–1	0–2.0	0–5.5
	90% CI for lower limit	0–0	0–0	0–0
	90% CI for upper limit	1–2	2–3	5–8
Monocytes (%)	N	67	66	61
	Reference interval	0–8	0–14	0–10
	90% CI for lower limit	0–0	0–0	0–0
	90% CI for upper limit	8–11	4–21	10–12
Basophils (%)	N	67	66	60
	Reference interval	0–2	0–3	0–1
	90% CI for lower limit	0–0	0–0	0–0
	90% CI for upper limit	2–4	3–4	NA–NA

MCHC = Mean corpuscular hemoglobin concentration. NA = Not available.

Table 2—Hematological parameters that were significant with age of the foal and their ICC values.

Parameters	*P* value	ICC value	SE	95% CI
WBC count	.027	0.147	0.081	0.046–0.380
NQ value	.034	0.139	0.081	0.041–0.377
Hemoglobin	.0076	0.201	0.087	0.079–0.422
Hematocrit	.01	0.180	0.087	0.064–0.412
Platelets	.0003	0.294	0.088	0.153–0.490

ICC = Intraclass correlation.

within the different age groups. However, the ICC values for these hematological parameters that were significant were less than 0.3. This indicates that most of the variation can be explained within the age group compared to the variation between the different age groups in this study.

Discussion

There is limited data available from other studies to compare population statistics as these studies sampled foals at different frequencies resulting in different age groups compared to this study. Strong inferences could not be made when comparing the results of this study to those of other studies due to variation in times of sampling of foals, parameters evaluated as well as the unavailability of raw data from other studies. Most studies reported the mean ± SD, but this study provides additional information such as the 95% confidence intervals and 90% confidence intervals for the upper and lower reference limits.

There were noticeable differences in the mean values of some of the hematological parameters such as mean corpuscular hemoglobin concentration (MCHC), total WBC, and neutrophil counts of the foals in this study for the 3 times of sampling that were greater than their North American counterparts.[15] The mean and 95% confidence interval for the WBC counts of the foals for the 3 times of sampling were 11.9 (11.1–12.8), 10.4 (9.6–11.3), and 9.9, (8.9–11.0) X 10^9/L, respectively. Comparison with other similar studies revealed that Thoroughbred foals in this study had higher mean WBC counts but lower mean hematocrit values than foals in Brazil and Turkey for the 3 times of sampling.[16,17]

The trend observed for the mean hemoglobin and hematocrit values of the foals in this study from birth to 1 month of age was not in agreement with the trends reported in other studies. There was a progressive decrease in the hemoglobin and hematocrit levels of the foals for the 3 times of sampling in this study; however, other studies reported increases in these parameters from 1 week to 1 month of age.[15,16] The decreases in the hemoglobin and hematocrit levels of the foals can be attributed to fluid shifts after the absorption of immunoglobulins, possible decrease RBC survival time, decrease iron delivery to the bone marrow, decrease stimulus for erythropoietin production, and enhanced oxygen delivery to tissues due to lower 2, 3 diphosphoglycerate concentrations.[15,17]

Lymphocytes and neutrophils are the predominant WBCs of the foal, contributing to more than 90% of the total WBC count.[15] The trend observed in this study was in agreement with Uluisik et al (2013) who observed a significant decrease in the WBC counts of thoroughbred foals from 1 to 3 days of age. The authors of that study attributed the high neutrophil count of the foal at birth due to cortisol. The neutrophil count then decreased to mean adult values by 4 to 6 months of age.[17] It is important to note that in a study conducted by Brommer et al (2001) on 43 Dutch warm-blooded foals from birth to 5 months of age in the Netherlands, an increase in the WBC, neutrophil, and lymphocyte counts was observed.[18] However, the frequency of sampling in the study by Brommer et al (2001) was on a monthly basis. Thus any fluctuations in hematological parameters occurring during the first month of life would remain undetected in that study.

The platelet counts of foals in this study remained relatively constant from birth to 1 month of age with minor fluctuations in the number of platelets for the different time periods. This agreed with a study conducted

in the US that reported that platelet numbers remained constant throughout the first year of life.[15]

This study is important as it created a valuable guide for equine practitioners in tropical countries on the hematological reference intervals of thoroughbred foals from birth to 1 month of age. Age had a significant effect on the hemoglobin, hematocrit, WBC, absolute neutrophil, and platelet counts of the foals; however, most of the variation was within the group compared to between age groups. In this study, there were decreases in the hemoglobin and hematocrit levels for the 3 times of sampling as compared to the other studies. Additionally, the WBC, absolute neutrophil, and lymphocyte counts in this study were discordant with the findings from Brommer et al (2001) but were similar to the findings of Uluisik et al (2013). Establishing hematological parameters is an ongoing process and data should be collected over different time periods to detect trends that can reflect on the health or disease status of the foals.

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