Hematological and Biochemical Reference Intervals of the Visayan Warty Pig in Negros Occidental, Philippines

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INTRODUCTION

The Philippines is considered to be one of the world's biodiversity hotspots (1). Possessing extensive biodiversity and endemism, it houses over two-thirds of the Earth's species (2). However, excessive hunting and habitat loss have brought about a staggering decline in the population of endemic wildlife (3, 4). While the country is among the few mega-biodiverse countries in the world, it is also one of the top global conservation priority areas with at least 700 threatened species (5). If left unchecked, these species may go extinct, adversely affecting the world's ecosystem.

The Visayan warty pig (*Sus cebifrons negrinus*) is recognized as one of four endemic *Sus* (pig) species in the Philippines (6). Known for the distinct mohawk-like hair on their heads and the large canines that protrude from the mouths of the males, this forest-dwelling pig is considered to be one of the rarest wild species of pigs in the world, only naturally found across the Negros-Panay region of the country (6). This species was listed as a *critically endangered* species in 2008 and continues to be considered as one by the International Union for Conservation of Nature (IUCN) (7). Species identified to be *critically endangered* experience population declines of 80% to 90% over the last decade and are at an extreme risk of becoming extinct (8). Factors such as habitat loss and hunting for trade and consumption (7) are believed to have contributed to the sharp decline in their population. Primary forest cover has been diminished to only 5.4% and 2.1% in Panay and Negros Island, respectively (9). Pathogens and biological factors such as the African swine fever (ASF) (10), classical swine fever (CSF) (11), and genetic contamination through hybridization (12) also pose a threat to the survival of the population. Despite *in situ* conservation action and efforts, their population continues to decline at an alarming rate (7). Repopulating the species has also proven to be a challenge because they produce only three to five piglets a litter. At present, the exact number of these pigs in the wild remains unknown, but they are extinct in at least 98% of their primary habitat. As of 2021, there are roughly 70 captive pigs reported across the conservation centers in Negros Occidental, Philippines.

Ecological functions rely on different species' habitats and their relationships with other ecological components. The extinction of a species brings cascading effects to the environment, disrupting many interactions, eventually shifting the balance within the whole ecosystem (13, 14). For instance, flowering plants in rainforest ecosystems are known to rely on animals for pollination, so the loss of these pollinators could lead to a proportional decline in plant reproduction (9). Studies suggest that biodiversity loss is linked to an increased risk of human exposure to zoonotic pathogens (15), lower fishery catches and greater incidences of stock collapse (14), reductions in crop yield, and a decline in future drug development potential (16). The Visayan warty pig contributes to biodiversity and to the maintenance of ecological cycles. They are known to forage for tubers, roots, fruits, and earthworms, thereby aiding in the dispersal of various plant seeds (17). The preservation of this species is, therefore, deemed essential because their absence would impede nutrient transport and plant reproduction within the forest areas of Negros Island and Panay, and in turn, affect the overall wellness of the ecosystem.

One of the conservation programs that aid in the protection of Visayan warty pigs is the Talarak Foundation, Inc. (TFI). Efforts include the assessment and monitoring of the health status of threatened species by looking into their hematological and biochemical profiles to maintain a healthy captive population. TFI reported that there are no set reference intervals for blood analyses within the Visayan Warty pig population housed by the foundation. Values are interpreted using an overview of the general blood baseline values of the adult Visayan warty pigs from the textbook *Fowler's Zoo and Wild Animal Medicine* (18).

Measurements from laboratory tests are interpreted with the use of a set of values defined as the typical results in a healthy reference population (19). Termed the *reference interval* (RI), it is an interval between and including two values, the upper and lower reference limits (20).

Establishing reference intervals requires careful analysis and selection of reference individuals. Guidelines set by the International Federation of Clinical Chemistry (IFCC) and the Clinical and Laboratory Standards Institute (CLSI) provide references for properly measuring these variables (20). Values are determined based on a well-characterized healthy population, considering factors such as age, sex, breed, and diet (21, 22).

Several studies have confirmed that age affects reference intervals. Variations between age groups were reported to be influenced by changes in growth and development among individuals (23, 24). Moreover, physiological factors can differ due to milk-feeding and weaning (25). Furthermore, juvenile animals tend to be more prone to illness. Thus, it is crucial for regular monitoring of serum biochemical analytes (25). This calls for the determination of hematological and biochemical reference intervals for juvenile Visayan warty pigs, as adult values are not applicable for the assessment and monitoring of the health status of these juveniles.

MATERIALS AND METHODS

Animals

Blood sampling of all juvenile Visayan warty pigs were performed in the mornings of the months of June to October of 2021 in the Negros Forest Park and TFI-Kabankalan, which are both located in Negros Occidental, Philippines. The subject pigs were two years old or younger, weaned, unvaccinated, had not received blood transfusion, and had not been previously diagnosed with disease. Their diets also consisted of the same food, such as pig pellets, bananas, and sweet potatoes. A total of 30 piglets were selected

for sampling. Prior to blood extraction, physical examinations, including those of the pig's head, eyes, snout, ears, mouth, skin, abdomen, hooves, feet, and rear end were performed by the licensed in-house veterinarian of Negros Forest Park to ensure that the subjects were healthy. All subjects did not display signs of illness and were deemed healthy.

Hematological and Biochemical Variables

Once the pig was determined to be fit for blood collection, blood was drawn from the cephalic vein, while it was restrained by the snout. Fresh whole blood was extracted from the subjects by the same veterinarian and was placed in a 10 mL tube containing ethylenediaminetetraacetic acid (EDTA). Then, the blood was subjected to hematological analysis using the MYTHIC^{TM} 18 Vet Hematology Analyser, and biochemical analysis using the MNCHIP^{TM} Pointcare® V2 Chemistry Analyzer in the Santoceldes Animal Hospital in Bacolod City, Philippines. The hematological components, in alphabetical order, were granulocyte (GRA), hematocrit (HCT), hemoglobin (HGB), lymphocyte (LYM), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean platelet volume (MPV), monocyte (MON), platelet (PLT), platelet distribution width (PDW), procalcitonin (PCT), red blood cell (RBC), red cell distribution width (RDW), and white blood cell (WBC). On the other hand, the biochemical components, in alphabetical order, were alanine aminotransferase (ALT), albumin (ALB), ALB/GLO, alkaline phosphatase (ALP), amylase (AMY), blood urea nitrogen (BUN), BUN/CRE, calcium (Ca^{2+}), cholesterol (CHOL), creatinine (CRE), creatine kinase (CK), globulin (GLO), glucose (GLU), phosphorus (P), total bilirubin (TBIL), and total protein (TP).

Data Analysis

The Tukey test was used to remove data that behaved as outliers. An outlier is defined as a data point that lies outside the distribution of the dataset. As recommended by the guidelines of the American Society of Veterinary Clinical Pathology (ASVCP) (26), suspected or near-outliers were retained, whereas far-outliers were removed.

Before the RI can be estimated, the data must be normally distributed, so the Shapiro–Wilk test was used to assess the distribution of variables. Normally distributed data were encoded in Microsoft® Excel® 2016, where they were explored and where descriptive statistics, including the sample size, mean, standard deviation, and minimum and maximum value were calculated for each hematological and biochemical variable. By estimating the Mean and the standard deviation (SD) from a normally distributed population, the RIs were defined, through a parametric method, to begin at $(\text{Mean} - 2 \times \text{SD})$ and end at $(\text{Mean} + 2 \times \text{SD})$. This method was recommended by the ASVCP, especially for critically endangered species, with samples $20 \leq n \leq 40$. Data that did not exhibit Gaussian (i.e., normal) distribution were subjected to a log transformation (LT)(27, 28) to bring the distribution closer to normal behavior. If the LT failed, a Yeo-Johnson transformation (YJ)(29) was performed. The distribution was then reassessed before defining the RI.

LIMITATION

While the complete dataset had 30 juvenile pigs, hematological and serum biochemical RIs were based on different sample sizes due to difficulties encountered during blood collection. Hematological analysis covered 30 samples, but only 28 samples were subjected to biochemical analysis due to challenges involving blood clotting. We acknowledge that the total sample size does not meet the IFCC- and ASVCP-recommended minimum sample size of 120 healthy individuals. This limitation is attributed to the fact that

the species is critically endangered and its population is small. While it was ensured that all subjects were weaned, had the same diet, and were considered healthy, it should be noted that food proportions were left uncontrolled and may have had an effect on the overall physiological status of the subjects. Moreover, reference intervals were distinguished by age alone; sex was not considered because there were significantly more male juvenile pigs (80%) in the population and the sample size was too small. This factor may have also influenced the RIs calculated. In addition, while there were few similarities between the RIs of the juvenile and adult pigs, there was insufficient information in the related literature to evaluate inferential statistics, and therefore, statistical comparison could not be performed.

REFERENCE INTERVAL

This study defined a total of 31 hematological and serum biochemical RIs. 30 juvenile pigs were used as samples to calculate the hematological RIs, while only 28 of these 30 pigs were used to calculate the biochemical RIs.

The default units of the automatic analyzers described in the MATERIALS AND METHODS section were used to report our results. Hematological (**Table 1**) and biochemical (**Table 2**) RIs were presented in two separate tables. Each table presents the variables and their units, the sample sizes *n*, the descriptive statistics (Mean, SD, minimum value [Min], maximum value [Max]), the lower and upper limits of the RI, the number of outliers that were eliminated based on the Tukey test, and the transformations (Trans) used, if any. The arithmetic mean was used for data exhibiting Gaussian distribution and for those transformed by YJ, while the geometric mean was used for those that were transformed by LT. The SDs of the transformed distributions were also reported for data that required transformation.

Of the 31 hematological and biochemical variables, 21 were compared with RIs reported in (18). Units of the adult RIs that differed from the default units reported by our instruments were converted for uniformity. No statistical methods were applied for comparison. Overall, results further support the importance of determining age-specific RIs as findings indicate (by visual inspection) differences between the calculated values for juvenile Visayan warty pigs and the established adult values from related literature (18).

Table 1 presents the 15 hematological RIs calculated from 30 juvenile pigs. The untransformed values of LYM, MON, and GRA (referred to as the differential count) sum up to the value of WBC. GRA includes basophils, eosinophils, and neutrophils.

Two variables did not behave normally, so transformations were performed. The SDs show that the majority of variables were highly scattered. Notably, PLT showed a very dispersed range of values, which was reflected by its wide RI. This can also be said of HCT, MCV, and PDW.

It can also be observed in **Table 1** that there were differences between the values of juvenile and adult pigs for the variables LYM, RBC, HGB, HCT, and PLT, which are higher in juveniles than in adults. These can be attributed to the fact that younger pigs are still developing, so their higher lymphocyte counts may be due to their immature immune systems establishing defense against pathogens (30). Furthermore, it was observed that the value of LYM was higher when compared with the values of MON and GRA because by nature, LYM is the second most common white blood cell after neutrophils. Heightened levels of MON and GRA are indicative of inflammation or infection (31).

PCT is produced in response to the presence of inflammatory or infectious agents. A minimum value of zero in PCT is not considered unusual in mammals, and such a measurement is usually indicative of the absence of systemic inflammation (32).

Table 1.The calculated hematological RIs for the juvenile Visayan warty pig.

Variable	Unit	n	Descriptive Statistics				RI		Outliers	Trans	Adult RI reported by (18)
			Mean	SD	Min	Max	Lower limit	Upper limit			
WBC	$10^3/\mu L$	30	9.92	2.63	4.50	16.1	4.77	15.0	0	None	5.9-23.3
LYM	$10^3/\mu L$	30	6.48	2.23	2.50	13.4	2.11	10.8	0	None	1.328-7.52
MON	$10^3/\mu L$	30	0.290	0.0634	0.200	0.006	0.170	0.410	0	YJ	0.112-1.066
GRA	$10^3/\mu L$	30	2.87	1.15	1.40	6.40	1.43	5.79	0	LT	
RBC	$10^6/\mu L$	30	10.3	0.912	8.17	12.1	8.50	12.1	0	None	4.8-9.7
HGB	g/dL	30	15.7	2.31	9.50	21.4	11.2	20.2	0	None	9.9-15.9
HCT	%	30	56.1	7.39	36.2	74.1	41.6	70.6	0	None	30.9-53.2
MCV	m^3	30	54.5	5.74	42.8	65.6	43.3	65.7	0	None	25.9-69.6
MCH	pg	30	15.2	1.62	11.6	17.9	12.1	18.4	0	None	14.2-22.3
MCHC	g/dL	30	28.0	1.26	25.7	30.2	25.5	30.5	0	None	0.6-33.2
RDW	%	30	19.2	0.91	17.2	20.9	17.4	20.9	0	None	
PLT	$10^3/\mu L$	30	373	173	26.0	746	34.0	712	0	None	0.194-296
MPV	μm^3	30	8.69	0.996	6.90	11.3	6.74	10.7	0	None	
PCT	%	30	0.316	0.151	0	0.690	0.0200	0.610	0	None	
PDW	%	30	44.8	7.31	26.7	55.5	30.5	59.1	0	None	

* SD = Standard deviation; Min = Minimum; Max = Maximum; Trans = Transformation

* WBC = white blood cells; LYM = lymphocytes; MON = monocytes; GRA = granulocytes; RBC = red blood cells; HGB = hemoglobin; HCT = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; RDW = red cell distribution width; PLT = platelets; MPV = mean platelet volume; PCT = procalcitonin; PDW = platelet distribution width.

* YJ = Yeo-Johnson Transformation; LT = Log Transformation

For biochemical RIs, 16 variables were calculated. Six variables that were not normally distributed were transformed.

In **Table 2**, juvenile RIs were marked by higher TP, CA, GLU, P, CHOL, ALP, BUN, and CRE when compared with adult RIs. Differences may be attributed to the overall physiological conditions within developing individuals. For instance,the dissimilarity in ALP levels may be brought about by high bone formation activity among juveniles (33).

GLU and CRE are found to be correlated to adiposity and muscle growth which may contribute to the high variances of the two variables observed in the results (24). CK is a muscle enzyme influenced by inflammation, trauma, or disease. This may have been affected by increased movement which was observed as the subjects were being restrained during blood collection (33). Certain subjects exerted more physical effort during restraint than others. This may be the reason for the high variability observed in CK.

The study presents RIs essential for analyzing juvenile Visayan warty pig hematological and serum biochemical profiles. It may aid in the practice of veterinary medicine and serve as a basis for interpreting

juvenile Visayan warty pig blood measurements in other veterinary clinics. Moreover, this study can provide more information on the species, aid in related future studies, and potentially inform conservation efforts. The data may also be used for comparison with other studies related to the establishment of RIs. Our findings show that the RIs of adult and juvenile pigs differ, but research on the underlying physiological processes may be necessary to achieve a better understanding of the various phenomena surrounding the results.

Table 2. The calculated biochemical RIs for the juvenile Visayan warty pig.

			Descriptive Statistics				RI				
Variable	Unit	n	Mean	SD	Min	Max	Lower limit	Upper limit	Outliers	Trans	Adult RI reported by (18)
ALB	g/L	28	43.3	5.86	26.6	53.5	31.6	55.0	0	None	40.0-57.0
GLO	g/L	28	27.8	10.1	12.0	53.9	7.57	48.0	0	None	
A/G		28	1.66	0.192	0.500	3.70	0.700	3.94	0	LT	
TP	g/L	28	71.1	10.0	53.1	87.0	51.0	91.1	0	None	47.0-72.0
CA	mmol/L	28	2.39	0.324	1.69	2.96	1.74	3.03	0	None	0.42-0.57
GLU	mmol/L	28	9.13	2.83	5.82	16.1	5.27	15.8	0	LT	3.5-8.55
P	mmol/L	27	2.30	1.68	1.23	10.2	1.05	3.51	2	LT	0.19-0.47
AMY	U/L	28	653	142	292	928	368	937	0	None	778-1994
CHOL	mmol/L	26	3.52	0.930	2.22	6.28	2.20	4.67	1	None	0.89-4.94
ALT	U/L	28	77.0	25.0	37.0	144	27.1	127	0	None	41.0-189
TBIL	µmol/L	28	3.94	2.22	2.05	11.9	1.69	9.17	0	LT	
ALP	U/L	28	119	110	21.0	459	30.6	460	0	LT	20.0-299
BUN	mmol/L	28	4.14	1.29	1.97	8.11	1.57	6.72	0	None	0.44-1.25
CRE	µmol/L	28	102	33.3	49.0	175	35.6	169	0	None	79.58-141.47
BUN/CRE		28	10.9	4.09	4.00	20.0	2.70	19.1	0	None	
CK	U/L	28	850	706	366	2800	253	2860	0	LT	

* SD = Standard deviation; Min = Minimum; Max = Maximum; Trans = Transformation

* ALB = albumin; GLO = globulin; A/G = albumin/globulin; TP = total protein; CA = calcium; GLU = glucose; P = phosphorus; AMY = amylase; CHOL = cholesterol; ALT = alanine aminotransferase; TBIL = total bilirubin; ALP = alkaline phosphatase; BUN = blood urea nitrogen; CRE = creatinine; BUN/CRE = blood urea nitrogen/creatinine; CK = creatinine kinase.

* LT = Log Transformation

QUALITY CONTROL

Blood collection and blood analysis were performed by a licensed veterinarian and medical technologist. Disposable materials used for blood collection were newly purchased, while blood analyzers met the required standards, were calibrated, and were up-to-date. Standard values were set before running the hematological analyzer for each test, accompanied by automatic cleaning (34). The biochemical analyzer was WiFi-enabled, and updates were run automatically. Reagents were verified by scanning the QR codes on their containers before analysis. The filters were cleansed daily. Our study protocol dictated that if the machines or the samples did not fulfill requirements, the analyses would not be performed.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICAL CONSIDERATION

The handling of animals was conducted by a veterinarian licensed in accordance with Philippine Republic Act No. 9268: AN ACT TO REGULATE THE PRACTICE OF VETERINARY MEDICINE IN THE PHILIPPINES, REPEALING FOR THE PURPOSE REPUBLIC ACT NO. 382 AND FOR OTHER PURPOSES, and was conducted in strict accordance with Philippine Republic Act No. 8485: AN ACT TO PROMOTE ANIMAL WELFARE IN THE PHILIPPINES, OTHERWISE KNOWN AS ”THE ANIMAL WELFARE ACT OF 1998”.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions in conceiving and designing the study, and in writing the manuscript. MA carried out the blood collection procedure while JA, FE, JN processed the blood samples in the laboratory and analyzed the data. MT advised and assisted with the writing of the study, and critically revised the manuscript. All authors read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

Datasets analyzed and presented in this study are available through IEEE dataport (35).

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