HEMATOLOGICAL AND BIOCHEMICAL REFERENCE INTERVALS IN SHETLAND SHEEPDOGS

Short title: Reference Intervals in Shetland Sheepdogs

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Abstract

Background: Several breeds have physiological peculiarities that induce variations in reference intervals compared to the general canine population. Shetland sheepdogs are reported to be more predisposed to different diseases (e.g. hyperlipidemia, gallbladder mucocele, hypothyroidism). Consequently, a breed-specific approach is more often required.

Objectives: The aim of this study is to determine whether reference intervals (RI) of the general canine population may be applied to Shetland Sheepdogs (SS), and to generate breed-specific reference intervals, where appropriate.

Methods: Sixty clinically healthy fasted dogs (36% of the population registered at the Italian Breed association), were examined. Routine hematology and biochemistrywere performed. The transference method was used to compare the results of SS with the RIs of the general canine population. When these RIs were not validated, newRIs were generated according to the guidelines of the American Society of Veterinary Clinical Pathology. Differences associated with sex, age, coat color and use were also investigated.

Results: The transference method validated 30/38 RIs. For 6 of the remaining 8 analytes, the difference with the claimed RIs could depend on pre-analytical or analytical artefacts whereas for glucose and total cholesterol these differences may depend on breed peculiarities. However, in all the dogs the concentration of cholesterol was <500 mg/dL. Relevant differences associated with sex, age, coat color and use were not found.

Conclusions: This study suggests that breed-specific RIs should be used for glucose and cholesterol in SS.

Introduction

Shetland Sheepdog (FCI-Standard N° 88) takes his origins from Great Britain and is included in the Group 1 (Sheepdogs and Cattle dogs) according to the FédérationCynologique Internationale breeds classification.[1] The Shetland Sheepdog can be considered as a companion dog as well as a sheepdog, but an increasing number of these dogs is nowadays excelling in agility competitions. The number of dogs registered at the Italian National Kennel Club (ENCI, Ente Nazionale Cinofilia Italiana) has doubled in the last nine years.[2]

This breed is reported to be predisposed to different diseases, such as primary hyperlipidemia,[3] gallbladder mucocele (GM),[4] hypothyroidism,[5] Multi-Drug Resistance(MDR-1) mutation.[6]

Hyperlipidemia is an increased concentration of lipids (triglycerides, cholesterol, or both) in blood that may be as high as 500 mg/dL.[7] It has been reported or suspected to be more prevalent in certain canine breeds such as Miniature Schnauzer,[8] but, if constitutively present also in Shetland Sheepdogs, may generate problems in theinterpretation of patient's blood tests, when reference intervals (RIs) referred to the general canine population are used. No information is available about the need to establish breed-specific RIs for Shetland Sheepdogs. However, the establishment of these RIs should be the first step to better explain laboratory data from this breed and to better diagnose some diseases reported in Shetland sheepdogs, such as primary hyperlipidemia and GM. In fact, several breeds have physiological peculiarities that induce variations in RIs compared to the general canine population.[9,10] and in these breeds clinical decisions must often be based on the comparison between patient's laboratory results and breed-specific RIs.

The aim of this study is to determine whether RIs referred to the general canine population may be applied to Shetland Sheepdogs from Italy, and to determine breed-specific RIs, when the general RIs were not validated, following the guidelines published by the American Society of Veterinary Clinical Pathology (ASVCP).[11] In addition, we

investigated whether sex, use, age or coat color could influence hematological and biochemical variables in this breed.

Materials and methods

Study design and sample collection

All the dogs included in this study were privately-owned and lived in North of Italy. The study was approved by the Ethical Committee of our Institution (approval number: OBPA 1/2016) and an informed consent was obtained from the owners of the dogs. Blood was collected from the cephalic vein using a 2,5 mL syringe connected to a butterfly needle; One ml of blood has been immediately placed into EDTA tubes with the maximum capacity of 1 mL (Miniplast; LP Italiana S.p.A., Italy) for routine hematology, whereas the remnant blood was transferred into a plain tube (Vacuette; Greiner Bio-One GmbH, Austria) with the maximum capacity of 5 mL. After 10 minutes, plain tubes were centrifuged at 3500 rpm for 5 minutes to obtain serum that was transferred to plain conic tubes (Eppendorf, Germany) and frozen at -20°C. Within a maximum of 6 hours, immediately after arrival in the laboratory, the complete cell blood count (CBC) was performed, and serum was stocked at -20°C for the execution of the biochemical analysis within 1 week. Samples were collected during breeder meetings or before agility competitions from 12 hours-fasted animals, after the collection of history and of clinical information and after a complete physical examination. Agility competitions were performed 5 hours after sampling, to enable to feed the dogs.

The inclusion criteria for this study were (1) age range: 1-8 years, (2) absence of external clinical signs or evidence of disease, (3) absence of clinical signs in the history.

The exclusion criteria were (1) pregnancy, (2) presence of clinical signs of disease, (3) use of medications except anti-parasitic treatments, (4) sample's alterations that can interfere with the analysis, such as icterus or hemolysis, presence of clots in EDTA

samples. Lipemia in clinically healthy and fasted dogs was not considered "a priori" as an exclusion criterion since one of the aim of the study was to assess the normal lipid concentration in Shetland Sheepdogs, that are known to be potentially hyperlipemic.

Analytical methods

Hematology: the CBC was performed using an automated laser-based hematology analyser (Sysmex XT-2000iV; Sysmex Co., Japan) equipped with a multispecies software for veterinary use. The following variables were measured: hemoglobin concentration (HGB) (g/dL), red blood cells (RBC) count (x 10^6/µL), mean corpuscular volume MCV (fL), white blood cells (WBC) count (x 10^3/µL),
platelets (PLT) (x 10^3/µL) count. Plateletcrit (PCT) (%), hematocrit (HCT) (%), mean corpuscular hemoglobin MCH (pg/cell), and mean corpuscular hemoglobin concentration

(MCHC) (g/dL) were calculated automatically from the impedance counts.[12] Internal quality control and calibration were performed monthly with e-check Xe (Sysmex Co.). External quality control (RIQUAS Monthly hematology, Randox Laboratories, Crumlin, UK) was also run monthly. In addition, manual differential WBC counts were performed on at least 200 WBC on blood smears stained with May–Grünwald-Giemsa by 2 different PhD students, with at least one year of experience in smear evaluation.

During the microscopic analysis of blood smears, all blood cells were examined, and the percentage of nucleated RBCs (nRBCs), the presence of RBC morphologic changes (e.g., anisocytosis or poikilocytosis, Howell-Jolly bodies), quantity and

morphology of PLT (size, shape, granularity, platelet estimate, platelet clumps), WBC morphology (e.g., *toxic neutrophils,* activated lymphocytes or monocytes), if any, were recorded.

Clinical chemistry: biochemistry profiles were performed using an automated chemistry analyzer (Cobas Mira; Roche Diagnostics, Switzerland) and reagents provided by Hagen Diagnostic System (Italy). Internal quality control was performed with 2 levels of human control serum samples (Precinorm U and Precipath U; Real Time Diagnostic System, Italy) before each assay, and calibration was performed with human based calibrators (Calibrator; Real Time Diagnostic System). External quality control (RIQAS Monthly clinical chemistry, Randox Laboratories) was also run monthly. The serum concentration or activity of the following analytes was measured: glucose (GOD-POD method), urea (urease method), creatinine (Jaffé method), GGT (γ-glutamyltransferase, kinetic IFCC method), total protein (biuret method), ALP (alkaline phosphatase, kinetic IFCC method), ALT (alanine aminotransferase, kinetic IFCC method), calcium (ortho-cresophtalein method), inorganic phosphate (molibdate method), triglycerides (GOD-PAP method), and cholesterol (CHOD-POD method), sodium, potassium and chloride (ion selective electrodes). Information on the coefficient of variations of the hematological and biochemical analytes included in this study are reported in the supplemental table S1.

Statistical analysis

The transference method[13] was used to compare the results of 20 Shetland Sheepdogs, randomly selected from the dataset, with the RIs of the general canine population in use in our laboratory. If less than 10% of values were outside the claimed reference interval, this latter RI was validated, while if more than 25% of values were outside the claimed

reference interval, this latter was rejected. When the proportion of values outside the claimed RI was within 10% and 25%, additional 20 results were randomly selected and re-examined, using the 10% threshold to validate or reject the claimed RI. When the claimed RIs were rejected, new reference intervals were calculated according to the ASVCP guidelines.[11] Based on data distribution, a non-parametric method or the Robust method were used to define the reference intervals with 90% confidence interval.

The RIs were determined using an Excel (Excel; Microsoft Corp., USA) spreadsheet with the Reference Value Advisor (version 2.0) set of macroinstructions.[14] The software performs computations according to the IFCC-CLSI recommendations[13] as

suggested by ASVCP guidelines.[11] Descriptive statistic, test of normality according to Anderson–Darling with histograms and Q-Q plots, and Box–Cox transformation were calculated. To find outliers, we used both the Dixon–Reed and the Tukey's test. Following

the ASVCP guidelines[11], outliers considered as "suspected" by the software were retained. Conversely, far outliers were removed from the analysis.

Differences of all hematological and biochemical results recorded in dogs of different sex (excluding neutered females and the castrated male, that were too few to allow a reliable statistical comparison), and use (companion vs agility dogs) were investigated with a U Mann-Whitney U test, using an Microsoft Excel with the Analyse-it set of

macroinstructions (Analyse-it software, version 2.21; Analyse-it Software Ltd, UK), whereas the possible differences associated with the coat colour (sable, bi-Black, tri-colour and blue merle) were assessed using a non-parametric ANOVA test (analysis of variance) for independent samples (Kruskal-Wallis test) followed by a Bonferroni test in the case of significant differences. The possible age-related differences were investigated by linear regression using the Reference Value Advisor (version 2.0) set of macroinstructions mentioned above.

Results

Samples from 60 clinically healthy and fasted Shetland Sheepdogs (24 males, 1 neutered male, 31 females and 3 spayed females) with a median age of 3,5 years (age range: 1-8 years), were examined. This caseload represents the 36% of the population registered at the National Breed association.[2]

All the dogs fulfilled the inclusion criteria described above. No sample was hemolytic, icteric or lipemic. Hematologic RIs were determined for 59 samples (one sample in EDTA was clotted) whereas sodium, potassium and chloride were only evaluated in 40 dogs due to the insufficient volume of the collected serum. Others biochemical analyses were performed in all the 60 Shetland Sheepdogs.

Comparison with claimed RIs and establishment of new RIs

The percentage of observations falling outside the RIs in use in our laboratory is reported in Table 1. Based on these results, the transference method validated 15/16 hematological and 7/14 biochemical RIs. For 8 analytes, the claimed RIs were rejected and new RIs were created (Table 2). Histograms displaying the distribution of data for these 8 analytes are reported in the Supplementary Figure 1.

Partitioning based on use, sex, age and color of the coat

A significant difference (P<0.01) between males (47,3 U/L +/- 27,26) and females (27,5 U/L +/- 8,94) concerning the activity of ALT was found (Figure 1). No other differences related to gender, use or color of the coat were found.

A weak correlation (Figure 2) was found between serum cholesterol concentration and age (-0.026 – p=0.045).

Discussion

This study suggests that for the large majority of biochemical and hematological analytes, the RIs derived from the general canine population may be used also in Shetland Sheepdogs. The transference method rejected only 8 RIs. However, as recommended by the guidelines for establishing RIs[11] the opportunity to apply these newly generated RIs may depend also on non-statistical factors. From this perspective, we think that for 6 out of 8 analytes (platelets, sodium, chloride, phosphate, triglycerides and total protein), pre-analytical or analytical factors may explain the rejection of claimed RIs using the transference method, or the analysis of the newly established reference intervals may demonstrate that, despite the results of the transference method, the use of the pre-existing RIs from the general canine population may be still valid. More specifically, the analysis of the distribution of data evidences that the minimum value recorded for sodium (and consequently the lower reference limit of the RI), and the maximum values recorded for both the sodium and chloride (and consequently the upper and lower reference limits of the RIs) are respectively so low and high that sampled animals should have clinical signs consistent with electrolyte or acid-base disturbances and possibly changes also in the concentration of potassium,[15,16] but this was not the case in our sampled population. Therefore, the most likely interpretation is that results of sodium and chloride

were probably affected by analytical artefact. This hypothesis is supported by the fact that sodium and chloride were proportionally increased or decreased compared with the claimed RIs only in a minority of cases. External and internal quality control runs did not reveal analytical problems of the instrument or the method. Therefore, abnormal results may depend on sample collection, on sample pre-processing or on the presence of interfering substances in the tubes or in serum that affected the measurement of these two electrolytes. Despite the nature of this artefact cannot be determined in this study, it may be advisable to continue to use the RIs of the general canine population until new studies specifically focused on these electrolytes in Shetland Sheepdogs will be run. Similarly, the lower reference limit for platelets is too low to not be associated with clinical signs. However, falsely low platelet count may frequently occur in dogs, due to the dificulties of automated instruments to correctly differentiate platelets from erythrocytes[17] or to platelet clumping.[18] Low platelet counts, usually associated with increased mean platelet volume (macrothrombocytopenia) is reported in the Cavalier King Charles Spaniel.[19] However, the MPV of Shetland Sheepdog was not increased compared with the general canine population and the observation of blood smear did not reveal the presence of macroplatelets. Conversely, platelet clumps were found in only one sample, and the platelet estimate was adequate in all the samples. This suggests that platelet counts may have suffered from an analytical artefact that lowered the actual number of platelet present on the samples(pseudothrombocytopenia), as often occurs with automate counts based on the impedance method.[20] Therefore, the lower limit of platelet count should be cautiously interpreted and it may be advisable to continue to use the claimed RIs, with the recommendation to pay particular attention on the presence of platelet clumps on smears. The distribution of data regarding total proteins and phosphate did not evidence any possible pre-analytical or analytical artefacts and therefore either the transference method or the newly established RIs may be theoretically acceptable. However, either the lower or the upper reference limit of both these analytes are so similar to those of the general canine population that it may be superfluous to use a different RI and therefore it would be more practical to use the RI referred to the general canine population. As a

support to this hypothesis, for both the analytes, the difference between the claimed and the newly established RIs corresponds to the intrinsic variability of the method (see supplemental table S1). Finally, the high proportion of samples on which the concentration of triglycerides was higher than the claimed RIs likely depends on the inadequacy of the latter. The RI in use in our laboratory for triglycerides (38 mg/dL) was adopted from Kaneko[21] using the method of transference of reference intervals described above, but other textbooks or articles report that the upper reference limit for this analyte may be as high as 200 mg/dL.[22] From this standpoint, the maximum value and the upper reference limit recorded in this study (86 and 79.7 mg/dL, respectively) may be considered as a normal values and, therefore, the use of RIs from the general population reported in recent articles may be used also in Shetland Sheepdogs.

Conversely, breed-specific RIs should be used for glucose and total cholesterol in Shetland Sheepdogs to reduce the misinterpretation of laboratory results. In these cases, in fact, the presence of pre-analytical or analytical artefacts is unlikely. Theoretically, high glucose concentration may depend on stress, possibly associated with sampling or travelling to breed meetings, presumably from epinephrine[23] or cortisol[24] secretion. However, no other signs of stress (e.g. lymphopenia) were found and the dogs included in this study were accustomed to travelling and to competition. Therefore, it may be assumed that the concentration of glucose in this breed is constitutively slightly higher than in other dogs. Also the RIs of cholesterol was higher than in the general canine population. However, all the dogs presented cholesterol levels lower than 500 mg/dL, which is considered as a clinically relevant value.[7] However, the RI for serum cholesterol (and the confidence interval of its upper limit) were very wide due to the presence of a single dog with a concentration of serum cholesterol very close to 500 mg/dL and to additional 10 dogs with values ranging from 300 to 400 mg/dL. A complete abdominal ultrasound screening was not performed in this dog, and therefore the possible presence of occult diseases typical of this breed (e.g. gallbladder mucocele) cannot be ruled out. However, ultimately, our results were different to those of Sato and others[3] that in 64 Shetland Sheepdog found a mean plasma cholesterol level of 335 +/-

312 mg/dL but in the cited study the presence of diseases than can lead to hypercholesterolemia (e.g. hypothyroidism, hyperadrenocorticism) was not excluded. According to Graham et al.,[5] the prevalence of hypothyroidism in Shetland Sheepdog is 14% in a population of 5765 dogs of this breed. However, our study did not confirm the primary hypertriglyceridemia reported by Aguirre et al.[4] or the presence of primary hyperlipidemia that was demonstrated in other studies such as those of Sato et al.[3] mentioned above or of Mori et al.[25] that found high concentrations of both triglycerides and total cholesterol but in samples obtained from non-fasted dogs on which the higher serum concentration of both the analytes may also depend on feeding. However, a trend to hypercholesterolemia was found in many of the in outwardly clinically healthy Shetland Sheepdogs included in the current study. Therefore, further studies are needed to investigate if primary hyperlipidemia may occur in this breed. This may be interesting since primary hyperlipidemia has been reported as a possible predisposing factor for gallbladder disorders, that are common in this breed.[4]

According to our results, cholesterol concentration appeared to decrease with the increasing age. This result appears to be not justified by biological causes; moreover, the correlation is very weak. In any case, this result contrasts with what reported by Mori et al.[25] that found a positive correlation between the severity of hyperlipidemia and aging in Shetland Sheepdogs.

No other age related differences were found, likely because very young or old dogs, that may usually have evident differences regarding hematological or biochemical analytes compared with adult dogs, were not included in this study.

As regards gender related activities, Mori et al.[25] found that hypertriglyceridemia was more frequent in females whereas in male prevails the hypercholesterolemia. However, the result of the current study did not confirm this finding. Conversely, the presence of significant gender-related differences of ALT activity recorded in the current study was not reported in previous studies. Mean and median values were within the RI of the general canine population in both groups; clinical signs potentially associated with hepatocellular

damage (e.g. e.g. apathy, inappetence, jaundice, vomiting, acholic feces, weight loss, polyuria/polydipsia, distended abdomen)[26] were never detected in this study even if sometimes it is not possible to rule out an hepatocellular injury without further diagnostic procedures (ultrasound examination and liver biopsy). Therefore, in the absence of an evident explanation about its possible pathogenesis, this finding could be considered as non-biologically relevant.

In conclusion, this study suggests that breed-specific RIs may be used for glucose and cholesterol in Shetland Sheepdogs to reduce the misinterpretation of laboratory results. This confirms that the recommendation to generate, for some analyte, breed-specific reference intervals, as suggested recently either in dogs[27,28] or in cats,[29,30] may be valid also in this breed.

However, the current study demonstrated some additional peculiarities, such as a significant difference between male and female for ALT activity. Conversely, this study didn't confirm the presence of primary hyperlipidemia in Shetland Sheepdogs, although it may be advisable, in routine practice, to monitor the dogs with cholesterol values close to upper limit the newly generated RIs, to exclude the possible presence of occult or upcoming gallbladder disease.

These conclusions could be a starting point to further investigations about this breed and the possible predisposition of Shetland Sheepdogs to different diseases.

Acknowledgements

Conflict of interest

The authors do not have conflicts of interest potentially interfering with the results of this study.

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Table 1. Canine RIs in use in our laboratory and results of the transference method: the percentage of 20 or 40 (when the first 20 values were included in the claimed RIs) randomly selected observations falling outside the claimed RI is reported.

Analyte	% Of results falling outside the claimed RIs (1st and 2nd analysis)	Claimed RIs in use at our laboratory
Glucose	**30%**	80.0-100.0 mg/dL
Urea	0%	20.0-60.0 mg/dL
Creatinine	0%	<1.5 mg/dL
Sodium	**35%**	141-152 mmol/L
Potassium	5%	3.7-5.8 mmol/L
Calcium	5%	8.0-12.0 mg/dL
Phosphate	**30%**	3.5-6.2 mg/dL
Chloride	**50%**	105.0-115.0 mmol/L
Cholesterol	**45%**	135.0-270.0 mg/dL
Triglycerides	**55%**	<38.0 mg/dL
ALT	10%	<60.0 U/L
ALP	10%	<180.0 U/L
GGT	0%	<14.0 U/L
Total Protein	**20% (30%)**	5.4-7.5 g/dL
RBC	10%	5.7-8.8 x10^6/µL
HGB	5%	12.9-18.4 g/dL
HCT	10%	37.0-57.0 %
Platelets	**20% (30%)**	200.0-500.0 x10^3/µL
MCHC	10%	310.0-360.0 g/L
MCH	0%	19.5-24.2 pg
MCV	10%	60.0-77.0 fL
WBC	10%	6.0-19.5 x 10^3/µL
Neutrophil	5%	3.0-11.5 x10^3/µL
Eosinophil	10%	0.1-1.2 x10^3/µL
Lymphocyte	5%	1.0-4.8 x10^3/µL
Monocyte	5%	0.1-1.5 x10^3/µL

RDW	0%	11.9-18.5 fL
PCT	10%	0.2-0.4%
MPV	20% (5%)	8.0-13.0 fL
PDW	15% (5%)	8.0-18.0 fL

Data in bold indicates the analytes for which the claimed RIs were rejected since the percentage of observations falling outside the claimed RI was >25% at first analysis or >10% in two consecutive analyses of the dataset.

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Table 2. Newly established RIs of the analytes for which the claimed RIs were rejected.

Analyte	N	Mean	Median	SD	Min	Max	Out	RI	90% CI (LRL)	90% CI (URL)	Dist.	Meth
Glucose(mg/dL)	60	109.9	108.5	16.0	82.0	151.0	1 (S)	83.1 - 150.0	82.0 - 85.6	137.0- 151.0	G	NP
Sodium(mmol/L)	40	145.8	145.0	7.0	130.0	161.0	1 (S)	130- 160.9	130.0- 133.0	154.0- 161.0	G	NP
Phosphate(mg/ dL)	59	4.0	4.1	0.5	3.0	5.7	1 (R)	3.1- 5.4	3.1- 3.4	4.8- 5.7	G	NP

Analyte	N	Mean	Median	SD	Min	Max	Out	RI	LRL	URL	Dist	Meth
							.1 (S)					
Chloride(mmol/L)	36	113.8	115.0	6.3	95.0	125.0	3 (S)	102.3-128.7	98.2-106.4	124.6-129.1	NG	Rob
Total Cholesterol(mg/dL)	60	241.2	233.5	74.1	112.0	468.0	1 (S)	116.7-420.8	112.0-130.9	360.4-468.0	G	NP
Triglycerides(mg/dL)	60	43.3	40.0	12.8	28	86.0	3 (S)	28.0-79.7	28.0-29.0	69.0-86.0	NG	NP
Total protein(g/dL)	59	6.1	5.9	0.8	5.0	8.0	1 (R).2 (S)	5.1-8.0	5.0-5.2	7.6-8.0	NG	NP
Platelets (x 10³/μL)	58	276.1	275.5	91.1	13.0	503.0	2 (S)	57.7-487.8	13-146.1	428.3-503.0	G	NP

N = number of valid observations; SD = standard deviation, Out = outliers; RI = reference interval; LRL = lower reference limit; URL = upper reference limit; Dist = distribution; Meth = method; (S) = number of suspected outliers; (R) = number of far outliers that were identified and removed; G = gaussian; NG = non Gaussian; NP = non parametric; Rob = robust method

Figure captions

Figure 1. Difference between males (M) and females (F) concerning the activity of serum ALT. The boxes indicate the I–II interquartile range (IQR), the horizontal line indicates the median values, whiskers extend to further observation within quartile I minus 1.5 × IQR or to further observation within quartile III plus 1.5 × IQR. '+' indicates near outliers (i.e. values exceeding quartiles I or III minus or plus 1.5 × IQR); the asterisks indicate far outliers (i.e. values exceeding quartiles I or III minus or plus 1.5 × IQR).

Figure 2. Scatter plot representing the negative correlation between cholesterol concentration (Tot Cho) and age. A weak correlation was found (-0.026 – p=0.045).

Supplemental figure S1. Distribution of 8 hematological and biochemical values recorded in our study and that need new RIs in Shetland sheepdog. For each analyte, the observed and fitted distribution are reported (blue bars and pink line, respectively). The blue vertical lines indicate the upper and lwer limit of the reference intervals. The 90% CI of both the reference limits are indicated by the dotted lines.