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Hematological and biochemical reference intervals in Dalmatian pramenka sheep estimated from reduced sample size by bootstrap resampling

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ABSTRACT

Monitoring of animal health status in organic farming relies in part on the availability of exact reference intervals for key hematological and biochemical blood parameters. Once determined, these intervals provide a baseline for interpreting routine laboratory blood tests in order to assess nutritional status, stress or disease. In this study, reference intervals for hematological and blood biochemical parameters were determined for Dalmatian pramenka, an indigenous Croatian sheep breed. A recently developed nonparametric method based on bootstrap resampling was used for calculation of reference intervals, and its performance was compared with that of a conventional nonparametric method. Hematological and blood biochemical profiles from 114 organically reared Dalmatian pramenka sheep were constructed and used for calculation of reference intervals using the new approach. The new method enabled convenient and reliable determination of reference intervals from a relatively small sample size, without the need for transformation or filtering of the raw data. Separate reference ranges for male and female sheep were found to be unnecessary for most of the investigated parameters.

Key words: reference interval, bootstrap, biochemistry, hematology, sheep, pramenka

Introduction

Measurement of key hematological and biochemical values can provide objective information about the condition of an animal at the moment of sampling, revealing its nutritional status, disease conditions or stress it has been subjected to (PEREZ et al., 2003). Reference intervals, usually defined as the values of a given parameter encompassing the central 95% of a healthy population, provide the baseline against which these measurements

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are interpreted. Well-constructed reference intervals are a prerequisite for screening and diagnostic tests aimed at evaluating health and disease in a given population.

Data on the hematological and biochemical parameters of Dalmatian pramenka, the most numerous Croatian indigenous sheep breed, are scarce. Although reference intervals for many species of domestic animals have been published in textbooks (PUGH, 2001), they tend to be general and lack the specificity required for assessment of health in particular breeds under defined conditions. The need for specific reference intervals has been recognized by many authors (BORJESSON et al., 2000; PEREZ et al., 2003), and, for example, a large dataset was collected for determination of reference intervals in Merino sheep (LEPHERD et al., 2009).

The statistical framework for determining percentiles enclosing the central 95% of a population is straightforward, given the normal distribution of the underlying data. Parametric methods achieve good statistical efficiency (narrow confidence intervals) when applied to large sets ($n>120$) of normally distributed data (SOLBERG et al., 2004). However, hematological and biochemical values are rarely normally distributed, and have to be transformed before parametric procedures can be applied. Even if data are successfully transformed into a normally distributed set, application of parametric methods is less statistically efficient, meaning that the confidence intervals are wider. This reduces the most important advantage of parametric methods over their nonparametric counterparts, when data need to be transformed.

For that reason, nonparametric methods are starting to gain prominence in reference interval calculation (HORN and PESCE, 2003). Classical nonparametric methods have the disadvantage of requiring a large number of individuals to achieve the desired robustness and efficiency. To overcome this problem, DIMAURO et al. (2009) have recently developed a nonparametric method for reference interval estimation based on bootstrap resampling (HENDERSON, 2005). This method should be able to produce reliable results in a sample size of 60 healthy subjects, regardless of the probability distribution of data (DIMAURO et al., 2009).

Bootstrapping in the context of statistics implies estimating properties of an estimator (a function of the observed data used to estimate an unknown population parameter) by measuring those properties by resampling from an approximate distribution. In this study, the approximating distribution was represented by a number of resamples, obtained by random sampling with replacement from the original dataset.

The aim of this study was (1) to obtain reference intervals for selected hematological and biochemical parameters of Dalmatian pramenka sheep from reduced sample size using the bootstrap resampling technique and (2) to compare the performance of bootstrapping with that of conventional nonparametric estimation of reference intervals from the same sample.

Materials and methods

Sheep. The investigation was carried out on 114 sheep (33 male and 81 female) aged from four to six months. All animals were clinically healthy. The sheep were fed by free grazing with the addition of hay and grain. The study was conducted in April and June.

Blood sampling and analysis. Blood samples (5 mL each) were taken from the jugular vein into vacuum glass Vactuainer tubes with EDTA anticoagulant for hematological analyses and SST II gel for biochemical analyses.

All hematological analyses were performed within the next 24 hours, and until then the samples were kept at 4 °C. Hematological measurements were done using a Beckman Coulter ACT diff Hematology Analyzer (Beckman-Coulter, USA) equipped with veterinary software. Hematological parameters included total red cell blood count (RBC), total white cell blood count (WBC), hemoglobin (Hgb) and hematocrit (Hct).

Blood in the serum tubes was allowed to clot for at least 30 min prior to centrifugation. Serum samples were kept frozen at -20 °C until biochemical analyses were performed. Biochemical parameters were determined by a SABA (AMS, Rome, Italy) analyzer according to the manufacturer's protocol. Reagents were supplied by Herbos dijagnostika d.o.o., Sisak, Croatia. Parameters for biochemistry panel included creatinine (CRE), glucose (GLU), total bilirubin (BIT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), urea (BUN), total protein (TP), albumin (ALB), cholesterol (CHO) and globulin (GLO).

Determination of reference intervals. Classical 95% reference intervals for all hematological and biochemical parameters were calculated using the nonparametric quantile estimator described by HARRELL and DAVIS (1982). This is one of the most efficient nonparametric methods for small samples, as recommended by HARRIS and BOYD (1995). 90% confidence intervals of each reference interval were determined by the jackknife method (EFRON, 1982).

Determination of reference intervals (95%) by bootstrap resampling was performed using the method of DIMAURO et al. (2009), which is suitable for reduced sample sizes. Briefly, the standard error of the mean was represented by the bootstrap standard error (standard deviation of the bootstrap distribution). The bootstrap standard deviation was calculated from the standard error and subsequently used for parametric estimation of reference intervals and their 90% confidence intervals. The root mean square error of the mean was used for the calculation of bias, which represented a measure of accuracy provided by the bootstrap method. For each biochemical and hematological parameter, 10,000 bootstrap resamples were generated.

Partitioning into subgroups. In order to assess whether division of the experimental group into subgroups according to sex would be appropriate, the method described in detail by LAHTI et al. (2002) was used. Briefly, the calculation was based on determining

the percentages of the subgroup distribution outside the common reference limits. If this difference, expressed as the *z* statistic, and adjusted for sample size, exceeded a certain limit, the groups were considered good candidates for subdivision. The value of this limit was set at five, as recommended by HARRIS and BOYD (1995).

Other statistical tests. Normal distribution of the data for each parameter, as well as the distribution of bootstrapped means, was assessed by the Anderson-Darling test. Correlation between data skewness and the overlap was tested using the Pearson's r.

Overlap between reference intervals determined by different methods was calculated as the ratio of the overlapping subrange to the combined range. Overlapping subrange was the interval contained in both ranges, while the combined range represented the interval between the lowest and the highest endpoint of the both ranges taken together. Overlap was therefore represented by a real number between 0 and 1.

Computer programs. The R language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria) was used for all calculations, bootstrap resampling and preparation of figures. Support for Anderson-Darling test was provided by the package "nortest", for Harrell-Davis quantile estimator by the package "Hmisc" and for the calculation of skewness by the package "moments".

Results

Hematological and biochemical profiles obtained from 114 animals are summarized in Table 1. Only seven out of the seventeen parameters tested could be considered normally distributed according to the results of the Anderson-Darling test (Table 1, Fig. 1. A and B). Mean values of the datasets generated by resampling were normally distributed, although they were mostly drawn from non-normally distributed data (Fig. 1.C). This demonstrates the effectiveness of bootstrap resampling. The calculated bias of the bootstrap method was consistently much smaller than 0.25 SE_{boot} for all parameters (data not shown), which assures that the bootstrap technique provides accurate results.

Comparison between the reference intervals obtained by bootstrapping and those calculated by the selected classical nonparametric method reveals a close match for parameters with data points symmetrically distributed around the mean (Fig. 1.A). Discrepancies between the two methods were pronounced only when the data were heavy-tailed (e.g. Fig. 1.B). This is evidenced by the significant negative correlation between the overlap of the two methods and skewness of the underlying data ($r=-0.84$, $P<0.01$).

Subdivision of the experimental group and calculation of separate reference intervals for male and female animals was not necessary for most parameters. Partitioning would only be justified for platelets ($z^* = 6.59$), cholesterol ($z^* = 7.04$) and γ-glutamyl transferase ($z^* = 5.49$). Due to the small size of the subgroups, separate reference intervals for these three parameters were not calculated.

Table 1. Reference ranges of selected hematological and biochemical parameters in sheep of the Dalmatian pramenka breed, as determined by the bootstrap method (DIMAURO et al., 2009).

Parameter (unit)	Mean ± SE	Bootstrap RI		Harrell-Davis RI		G_1	A-D
		Lower bound (CI_{90})	Upper bound (CI_{90})	Lower bound (CI_{90})	Upper bound (CI_{90})		
Leukocytes (10^9/L)	18 ± 0.8	0 (0-2)	36 (33-38)	8.5 (7.9-9.2)	44 (35-53)	1.669	No
Erythrocytes (10^{12}/L	10 ± 0.1	7 (7-8)	13 (13-14)	6.7 (6.1-7.4)	13 (12-13)	-0.567	No
Hemoglobin (g/L)	102 ± 1.2	77 (74-81)	127 (124-131)	75 (70-80)	124 (119-128)	-0.321	Yes
Hematocrit (%)	31.73 ± 0.37	23.7 (22.6-24.8)	39.7 (38.6-40.8)	23.6 (22.8-24.4)	39.3 (37.9-40.7)	-0.084	Yes
MCV (fL)	31 ± 0.3	25 (24-26)	38 (37-39)	27 (27-28)	42 (40-44)	1.861	No
MCH (pg)	10.1 ± 0.1	8.4 (8.2-8.7)	11.7 (11.5-12)	9.2 (9.16-9.23)	13 (12-14)	2.049	No
MCHC (g/L)	324 ± 1.8	286 (281-291)	361 (356-366)	290 (284-296)	371 (359-383)	0.531	No
Platelets (10^9/L)	524 ± 21.5	70 (9-131)	978 (917-1039)	126 (93-160)	999 (925-1074)	0.183	Yes
AST (U/L)	127 ± 2.2	79 (73-86)	174 (168-181)	85 (81-88)	180 (166-194)	0.693	Yes
γ-GT (U/L)	59 ± 1.3	31 (27-35)	87 (84-91)	27 (21-34)	91 (78-103)	0.214	No
Bilirubin - total (μM)	7 ± 0.3	0.5 (0-1.4)	14 (13-15)	4.8 (4.7-4.9)	17 (12-22)	5.492	No
Glucose (mM)	4.8 ± 0.10	2.7 (2.5-3.0)	6.9 (6.6-7.2)	3.3 (3.0-3.6)	8.2 (6.5-9.9)	2.498	No
Cholesterol (mM)	1.7 ± 0.04	0.8 (0.7-0.9)	2.5 (2.4-2.6)	1.0 (0.9-1.0)	2.6 (2.4-2.9)	0.756	Yes
Blood urea N (mM)	4.5 ± 0.22	0.0 (0.0-0.5)	9.1 (8.5-9.8)	2.7 (2.5-2.8)	12.0 (9.7-14.2)	2.384	No
Creatinine (μM)	108 ± 0.8	91 (89-93)	125 (123-127)	91 (87-95)	124 (123-126)	-0.001	Yes
Total protein (g/L)	77 ± 0.6	65 (63-67)	89 (87-90)	66 (65-67)	89 (88-90)	0.137	Yes
Albumin (g/L)	38 ± 0.3	31 (30-32)	44 (43-45)	31 (30-33)	44 (42-46)	-0.168	No

Results obtained by the classical nonparametric Harrell-Davis quantile estimator are shown for comparison. G_1 represents the skewness of the sample, and the A-D column represents the normal distribution of the sample as determined by the Anderson-Darling test. SE, standard error; RI, reference interval; CI_{90}, 90% confidence interval MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; AST, aspartate transaminase; γ-GT, γ-glutamyl transferase.

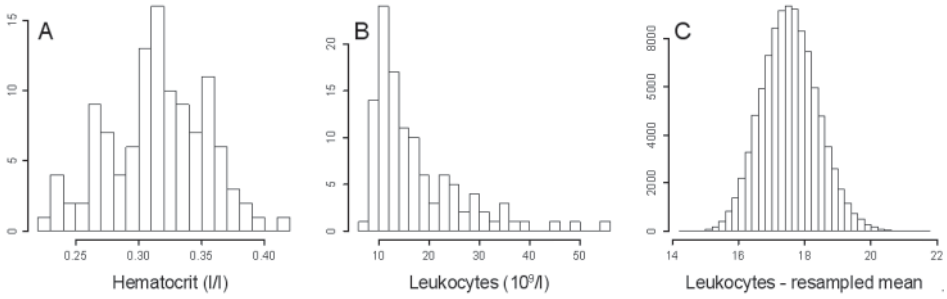

Fig. 1. Histogram showing probability distribution of a normally distributed parameter (hematocrit, A), a parameter from a highly skewed distribution (leukocytes, B), and the resampled (bootstrapped) mean constructed from the skewed distribution (C). As expected according to the central limit theorem, the resampled mean shows the typical Gaussian shape, and normal distribution of the bootstrapped statistic was confirmed by the Anderson-Darling test.

Discussion

Reference intervals for key hematological and biochemical parameters were determined using a new approach recently developed by DIMAURO et al. (2009), which enables estimation of reference intervals from a reduced sample size (down to 60 individuals) using bootstrap resampling as a single method. The advanced nonparametric method based on the quantile estimator described by HARRELL and DAVIS (1982), which performs reasonably well even with small samples, was used for comparison. The results obtained by both methods yielded similar results for parameters symmetrically distributed around the mean, regardless of their underlying distribution. However, skewed data gave different results due to the symmetry inherent to this particular bootstrap approach. The effect was quantified by a simple calculation of the overlap, which was highly correlated with skewness. This is a strong indication that the lack of symmetry of the underlying data causes discrepancies between the two methods.

Although both methods for calculation of reference intervals performed similarly, the bootstrap approach has been specifically developed for simplicity and performance with reduced sample sizes (DIMAURO et al., 2009). However, for samples closer to 150 individuals, the described nonparametric quantile estimator might be superior. This is

particularly true for highly skewed data, which are treated by the bootstrap method as if they were symmetrical, leading to inaccuracy in reference interval estimation. When confronted with skewed data and small sample size simultaneously, a good choice might be the robust method of HORN et al. (1998).

Subdivision of the sample into groups according to sex and estimation of separate reference intervals for male and female animals might be physiologically justified (BORJESSON et al., 2000; PEREZ et al., 2003). Indeed, a simple *t*-test yields a statistically significant difference between the mean values of the male and female subgroups for almost all parameters (data not shown). However, the t-test is a poor criterion for partitioning because it does not provide information on the magnitude of the difference, which would be necessary to establish physiological relevance. Further, by sufficiently increasing the sample size, even the smallest difference would eventually become significant. For these reasons, the method outlined by HARRIS and BOYD (1990), which involves the ratio between subgroup standard deviations and uses a normal deviate test for comparison of group means, was chosen as an objective test for partitioning. Using this criterion warranted partitioning according to only 3 out of the 17 tested parameters. Based on this finding, reference intervals were determined for the combined group comprising both males and females. For an in-depth discussion of partitioning criteria, see LAHTI et al. (2004).

A direct comparison with reference intervals published in other studies would require access to the original datasets and additional information on the exact analytical methodologies used to obtain them. Therefore, it is much more challenging to compare reference intervals than to simply correlate means. Although difficult to compare confidently, it is obvious that the results are largely similar to other published reports (PUGH, 2001; KRAJNICAKOVA et al., 1997), especially when considering means. This holds true for a similar study by LEPHERD et al. (2009) conducted on a large number of Australian Merino sheep. The mean values for most parameters in pramenka sheep closely matched those found in literature, but the whole reference range was consistently shifted towards the lower values, with the exception of leukocyte count, AST activity and concentrations of total bilirubine and urea. This shift was also observed in reference intervals obtained by the Harrell-Davis method (HARRELL and DAVIS, 1982), so it cannot be explained as an artifact of the bootstrap procedure.

Conclusions

Reference intervals for key hematological and biochemical parameters in Dalmatian pramenka sheep were determined. They represent a starting point for further investigation of this group of sheep and also provide a useful tool for monitoring of health status, particularly in organic farming.

The reference intervals were calculated from a reduced sample size using the bootstrap approach. This method compares favorably to established classical methods, with the advantage of being reliable even for reduced sample sizes. However, for highly skewed data, a robust estimator might be more appropriate.

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SAŽETAK

Praćenje zdravstvenog statusa u ekološkom uzgoju dijelom se temelji na raspoloživosti preciznih referentnih raspona za ključne hematološke i biokemijske pokazatelje. Jednom utvrđeni, ovi rasponi služe kao polazište za interpretaciju rutinskih laboratorijskih pretraga krvi, dajući informacije o hranidbenom statusu životinje te izloženosti stresu ili bolesti. Ovim istraživanjem utvrđeni su referentni rasponi za ključne hematološke i biokemijske pokazatelje ovaca izvorne hrvatske pasmine dalmatinska pramenka. Pri tome je korištena razmjerno nova neparametrijska metoda temeljena na ponovljenom uzorkovanju s ponavljanjem (bootstrap), a njeni rezultati su uspoređeni s rezultatima odabrane konvencionalne neparametrijske metode. Hematološki i biokemijski profili 114 ekološki uzgajanih ovaca pasmine dalmatinska pramenka poslužili su za računanje referentnih raspona novom metodom, koja se pokazala jednostavnom i pouzdanom čak i na malom uzorku, bez potrebe za transformacijom ili filtriranjem izvornih podataka. Određivanje zasebnih intervala za muške i ženske životinje se pokazalo nepotrebnim kod većine istraživanih parametara.

Ključne riječi: referentni raspon, bootstrap, biokemija, hematologija, ovca, pramenka