Reference intervals for organically raised sheep: Effects of breed, location and season on hematological and biochemical parameters

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ABSTRACT

Hematological and biochemical tests are widely used for assessment of health status and diagnosis of disease in domestic animals. They are almost indispensible in health management of organic flocks, but environmental conditions and food supply can be highly variable in organic farming, which may cause levels of many analytes to lie outside the published reference intervals. We investigated key hematological and biochemical parameters in organically raised sheep in Croatia. Different (mostly indigenous) breeds raised in various geographic regions were evaluated throughout the year. We identified key parameters that varied under different conditions using classification based on machine learning. Our findings suggest that location (which determines climate and food availability) has a profound effect on hematological and biochemical values in organically raised sheep. The effect of location was more pronounced than that of breed and season. The results clearly show the merit of establishing specific reference intervals for each flock, especially in organic farming. We demonstrate feasibility of this approach by successful calculation of reference intervals from a small sample (30 individuals) using robust statistical methods.

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1. Introduction

Hematological and biochemical tests are an important tool for evaluation of nutritional and health status of farm animals and almost indispensable in organic farming, where allowed veterinary interventions are strictly regulated and limited in scope. Hematological (Polizopoulou, 2010) and biochemical (Braun et al., 2010) analyses have been extensively discussed as an essential part of a clinical examination, often pointing to a specific differential diagnosis or suggesting a prognosis (Braun et al., 2010). Influence of breed (Binev et al., 2007), age, lactation, pregnancy (Baumgartner and Pernthaner, 1994) and certain preventive procedures (Lepherd et al., 2009) on blood analytes has been described in healthy sheep.

Interpretation of hematological and biochemical test results is critically dependent on accurate reference intervals (RI), which represent the range of values considered normal in healthy animals. Formally, RI are defined as the central 95% of the healthy population, between quantiles 2.5 and 97.5 (Henny et al., 2000). The non-Gaussian nature of most parameters (Gräsbeck, 2004) makes estimation of RI nontrivial.

One of the key problems in determining reliable RI is the selection of the reference population – the set of

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apparently healthy individuals from which RI will be estimated. Another way to state this problem is to raise the question of partitioning – which parts of a general group of interest (such as sheep) need their own RI (e.g. different breeds, age groups etc.). While physiological reasoning represents a good starting point for selection of reference populations, an exact method or algorithm is required to confirm the need for separate RI of a subgroup. The oldest and simplest method (Harris and Boyd, 1995; Lahti et al., 2002a, 2002b) is based on the so-called z-statistic and excessively relies on the Gaussian distribution of the analyzed data. A relatively recent method (Lahti et al., 2004) represents a step forward in sophistication, but still does not guarantee a clear-cut answer for all data sets. A radically different approach based on machine learning, already applied to reference intervals by other researchers (Čož-Rakovac et al., 2009), was used in this study. Essentially, a machine-learning algorithm (Quinlan, 1992) is used to classify hematological and biochemical data according to various criteria and the level of success of such classification is used to identify the best criteria for partitioning, thereby defining suitable reference populations. The algorithm elegantly hides the underlying complexity and gives answers using an approach completely different from classical methods, providing another way to search for objective partitioning criteria.

When the reference population has been defined, actual RI need to be calculated. Classical parametric and non-parametric methods for determination of quantiles require at least 120–150 individuals in the reference population. Such a number is rarely available in typical organic farming settings, especially after partitioning of the reference population. A relatively straightforward method based on bootstrapping has been successfully used for RI calculation from smaller samples (Dimauro et al., 2009; Vojta et al., 2011). However, a method based on robust statistics (Horn et al., 1998), which reportedly excels with small samples, giving reasonable RI while maintaining accuracy, has been recommended by professional organizations (CLSI, 2008). This arguably superior method has yet to gain popularity and more widespread use, probably because it is relatively difficult to implement.

The objective of this study was to identify feasible criteria for selection of reference populations in organic farming using imported and indigenous sheep organically raised in different parts of Croatia. Further, a method for calculation of reliable RI from small samples, based on robust statistics,

was implemented and tested in order to assess its applicability and merits in a typical organic farming scenario.

2. Methods

2.1. Sheep

The research was carried out on 270 sheep divided into 9 flocks, with about 30 animals (26–35) sampled from each flock; see Table 1 for details. The flocks were from small organic production units (family farms) typical for their respective area. All studied animals were clinically healthy, which was confirmed by the attending veterinarian. Sheep were fed by free grazing with the addition of hay and grain when necessary (mainly between November and April). Climate for each location was determined according to Šegota and Filipčić (2003) and Peel et al. (2007).

2.2. Blood sampling and analysis

Blood was sampled four times from the same animals, in early October (fall), December (winter), March/April (spring) and late June (summer). From each animal, two 5 ml blood samples were taken from the jugular vein using the Vacutainer system with EDTA-containing (purple-stoppered) tubes for hematological and SST II gel (yellow-stoppered) tubes for blood biochemical analyses. Samples for biochemical analyses were centrifuged immediately and frozen at $-20\,^{\circ}C$ until processing. Hematological samples were kept at $4\,^{\circ}C$ and analyzed within 24 h from collection.

Hematological parameters were determined on a Beckman Coulter ACT diff Hematology Analyzer (Beckman-Coulter, USA) running veterinary software. Analyzed parameters included total white cell blood count (WBC), total red cell blood count (RBC), hemoglobin (HGB), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and platelet count (PLT).

Blood biochemical parameters were determined using 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 the biochemistry panel were calcium, phosphate, magnesium, glucose (GLU), urea, aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), total protein (TP), creatinine (CRE) and albumin (ALB).

2.3. Classification based on machine learning

Classification models were constructed using the implementation of C4.5 decision trees algorithm in the WEKA package (Witten and Frank, 1999). Accuracy of the prediction by the constructed models was assessed by tenfold cross-validation. Decision trees were visualized with the Graphviz program (Ellson et al., 2003). Relative merit and ranking of parameters considering their importance for the tree model was estimated using the RELIEF algorithm (Kononenko et al., 1997).

2.4. Reference interval estimation

The robust method for reference interval estimation was implemented with the R language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria; R Development Core Team, 2011) according to the CLSI guidelines (CLSI, 2008). The R package "boot" (version 1.2-43, by Angelo Canty and Brian Ripley; Davison

Table 1
Essential data describing the nine flocks on which the study was conducted. Each flock was of the size indicated, but only about 30 animals were investigated from every flock. Climate type is according to Köppen's classification. Elevation is given in meters. Types are Merino (M), Pramenka (various types of the Pramenka breed) and indigenous island sheep (I).

Location	Elevation	Climate	Region	Breed	Type	Flock size
Čakovci	112	Cfb	lowland	Merinolandschaf (purebred)	M	150
Ponor	310	Cfb	continental	Pramenka (crossbreed)	P	150
Radučić	270	Cfa	hinterland	Dalmatian pramenka	P	70
Jošani	700	Cfb	continental	Pramenka (Travnik, Kupres)	P	550
Cres (Verin)	90	Cfa	island	Cres sheep	I	40
Sjeverovac	140	Cfb	lowland	Merinolandschaf (purebred)	M	200
Goljak	450	Cfb	continental	Merinolandschaf (purebred)	M	200
Oklaj	250	Cfa	hinterland	Dalmatian pramenka	P	500
Brač (Postira)	140	Csa	island	Brač sheep (pramenka)	I	190