Reference intervals for serum haptoglobin, cortisol and lysozyme in immediate post-partum and lactating dairy goats

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INTRODUCTION

The application of specific Reference Intervals (RIs) for each species and sometimes for each productive category is mandatory in veterinary clinic and welfare assessment, in order to avoid misdiagnosis. Unfortunately, accurate and up-to-date RIs are not available for all laboratory tests performed on animal population and in particular for immunological and stress parameters. Calculation of RIs requires a specific selection of animals and, where needed, a differentiation in production categories.

In recent years intensive production systems for small ruminants have spread through the Northern countries of the Mediterranean basin and specialized dairy herds have increased in size. This, in addition to the increasingly importance of animal welfare evaluation, has also lead to an increase in the use of specific laboratory parameters linked to the immune or stress response of animals. Among different immunological and stress parameters, lysozyme, haptoglobin and cortisol are the most commonly used in our laboratory to evaluate the general level of stress and immunity in ruminants.

Lysozymes are defined as 1,4-fl-N-acetylmuramidases cleaving the glycosidic bond between the C-1 of N-acetylmuramic acid (Mur NAc) and the C-4 of N-acetylglucosamine (GlcNAc) in the bacterial peptidoglycan. Some lysozymes also display a more or less pronounced chitinase activity (EC 3.2.1.14) corresponding to a random hydrolysis of 1,4-fl-N-acetylglucosamine linkages in chitin. Cleavage of the protective peptidoglycan layer by lysozyme causes leakage of the cell’s interior components and results in cell lysis. In ruminants lysozyme acts as an antibacterial by hydrolysing bacterial cell wall mucopeptides within neutrophil and macrophage granules, resulting in lysis of some Gram-positive bacteria, but also has been demonstrated to kill gram negative bacteria. It has been used to measure non-specific immunity in different ruminants such as dairy cattle1, calves3, water buffalo, lambs7 and goats1.

Haptoglobin (Hp) is a plasma α2-glycoprotein, produced in the liver. Together with many other proteins it forms a group of positive acute phase proteins (APP) whose concentration changes in response to damaging factors, being part of the inflammatory reaction or as a consequence of surgical trau-
ma or stress. Their concentration rises in many infectious diseases in humans and animals, including goats. In sheep and goats Hp is considered a major APP with increases that can reach 80 fold in inflammatory conditions, with its value almost negligible in healthy animals.

The concentration of cortisol in blood is widely used as an indicator of stress, such as isolation in lambs although an increase does not occur with every type of stressors. Serum cortisol concentrations have been used as a reliable indicator of short-term physical stress and of transport stress in goats.

To adequately use these parameters both on routine veterinary checks and in research there is a need to define proper Reference Intervals (RIs) to avoid misinterpretation and misdiagnosis. In order to provide maximal utility, a reliable and accurate RI should incorporate parameters such as species, gender, production category and breed, where appropriate. While the concept of RIs and their utility appears straightforward, the process of establishing them is quite complex particularly in veterinary medicine.

RIs, first introduced as a philosophy, have gained universal acceptance as one of the most powerful tools in laboratory medicine to aid in the clinical decision-making process. Current guidelines define RI as “limiting values within which a specified percentage (usually 95%) of apparently healthy individuals’ results would fall”. Essentially, these are values within the 2.5th and 97.5th centiles of the test results distribution for a reference (healthy) population. RIs provide valuable information to medical practitioners in the interpretation of quantitative laboratory test results and are critical in the assessment of patients’ health and in clinical decision making.

The aim of this study was to establish RIs for serum lysozyme activity, haptoglobin and cortisol in goats reared for milk production in two critical points: immediate post-partum and peak of lactation.

**MATERIALS AND METHODS**

**Animals**

The study was carried out in spring in fifteen commercial intensive farms of Northern Italy raising dairy goats. The dimension of the farms varied from small (16 animals) to big (835 animals) and raised Saanen, Chamois Colored, Anglonubian or Crossbreeds goats. During veterinary regular blood samplings to check herd health, 8 to 10 healthy animals/farm from one to six years of age with a normal body condition score and a clinical inspection were selected in each farm.

Blood was sampled in vacuum tubes without anticoagulant (Vacutainer®) from the jugular vein in 132 animals (Saanen and Chamois Colored). Animals were bleded in the morning between 9 and 11 am. Each animal was sampled immediately postpartum (2-4 days after delivery) and 45 days later, at peak of lactation, during routine veterinary monitoring.

**Laboratory analysis**

Samples were submitted refrigerated to the laboratory where tubes were kept at room temperature for 2 h and then centrifuged at 4°C for 10 min at 500x g; serum was separated and stored in aliquots at −80°C until analysis.

Serum lysozyme was assessed by the lyso-plate assay as previously described and modified as follows. Briefly, serum samples were reacted with a suspension of Micrococcus lyso-idekticus inside an agar gel in 10 cm Petri dishes. Serum samples were distributed in duplicate in 3 mm holes, 2 cm apart, at a regular distance of 1.5 cm from the dish edge. Contrary to the original protocol, the reaction was carried out at 37°C for 18 h, in a humidified incubator. The diameter of the lysis areas around serum samples and lysozyme standards of known concentration in phosphate buffer 0.066 M pH 6.3 was assessed by calipers or rules. Under these conditions, lysozyme concentration (µg/ml) is proportional to the diameter of lysis areas and is determined from a standard curve created with reference preparations of egg white lysozyme (Sigma-Aldrich, St. Louis, MO, USA). Serum haptoglobin was measured by a colorimetric kit (Phase Haptoglobin Colorimetric Assay, Tridelta Development Ltd, Kildare, Ireland).

Serum cortisol was measured by a solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite® 1000 Cortisol) with the DPC Immulite 1000 Analyzer.

**Statistical analysis**

Data were analyzed using Reference Value Advisor, a software for Microsoft Excel for Windows v.2003, to obtain RIs for each of the three analytes (lysozyme, haptoglobin, cortisol) in the two production categories considered (immediately postpartum and peak of lactation). Data were processed separately for two different breeds. For each series of data outliers detected according to Tukey’s or Dixon’s tests and considered to be aberrant observations were deleted. As in all the cases analyzed our samples sizes were between 40 and 120, a parametric methods was utilized. Box-Cox transformed data were used, in particular standard transformed data for Gaussian distribution and robust transformed data in absence of Gaussianity, but only after checking the symmetry of distribution. In alternative, if data were non Gaussian and not symmetrically distributed we used nonparametric method with CI (Confidence Interval) determined using bootstrap method.

**RESULTS**

The results of the analysis (RIs and CIs of the limits) are reported in Table 1 for Chamois Colored and in Table 2 for Saanen goats.

**DISCUSSION**

RIs determination is difficult, time consuming and expensive and it is unrealistic to expect that each laboratory to develop its own for all animal species and parameters analyzed. Options for their determination have been already used also in pediatric and veterinary medicine and could be applied as an alternative approach in specific cases to reduce the number of animals required.

However the International Recommendations, recently updated by IFCC and the CLSI state that a nonparametric method is preferred when the number of reference individuals within one group is at least equal to 120, even if for...
smaller reference samples an alternative robust method could be used, preferably after transformation of the data to a distribution to Gaussian or normal. This could explain why, despite their common use in veterinary medicine and research, few RIs have been published up to now for these parameters, i.e range of reference for lysozyme but not RIs have been proposed only for Holstein Fresian Cattle by Amadori et al.2. It is interesting to note that Saanen goats (0.6-4.7 µg/mL) had lower RIs for lysozyme compared with those of Chamois Colored goats (0.7-6.4 µg/mL) in the immediate postpartum. To the author’s knowledge studies on different cattle breeds are available17 about serum lysozyme levels at the delivery and at the peak of lactation but not on different goats breeds. According to Trevisi et al.17 high-yelding Fri-sian dairy cows show lower serum lysozyme levels compared with other less productive dairy breeds or beef breeds. It is interesting to note that also Saanen goats, with 601±240 L of milk/lactation, are more productive than Chamois Colored goats 566±226 L of milk/lactation. Serum cortisol increased around parturition in both the breeds because delivery is considered as a physical stress that change the homeostasis of the goats. Saanen goats cortisol RIs were wider than those of Chamois Colored goats; this evidence is in disagreement with Kitts12 which found that maternal cortisol was higher at the term in all the pregnant ewes considered, but not different between the three breeds studied. Haptoglobin RIs have been established for Merino lambs and goats. Compared to published haptoglobin RIs in goats (0.399-1.242 mg/mL)10 our RIs have minor lower limits both at delivery and at peak of lactation, probably due to the higher number of animals included in our study and the applied stratification of samples in the two different categories of goats and in two different breeds. An increase of acute phase proteins (APP) at parturition is well documented in healthy cows, mares and dogs19,20,21. Saanen goatshad high levels of serum haptoglobin both immedia-tely postpartum and at peak of lactation compared to the Chamois Colored.

**CONCLUSION**

The present study complied with the requirements described in previous studies for the determination of a RIs de novo in reference individuals selected according to predefined criteria. Despite the limitations of the study, these results provide a well-established, statistically defined RIs for haptoglobin, ly-sozyme and cortisol for the two the most typical dairy breeds

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**Table 1** - Reference intervals for the different parameters in Chamois Colored goats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Number of goats sampled</th>
<th>Production category</th>
<th>Method</th>
<th>Lower and upper limit of reference interval</th>
<th>90% CI for lower limit</th>
<th>90% CI for upper limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme (µg/mL)</td>
<td>52</td>
<td>Immediately postpartum</td>
<td>Box-Cox std</td>
<td>0.7-6.4</td>
<td>0.6-0.9</td>
<td>5-8.3</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>Peak of lactation</td>
<td>Box-Cox std</td>
<td>1.3-4.5</td>
<td>1.2-1.4</td>
<td>3.8-5.4</td>
</tr>
<tr>
<td>Haptoglobin (mg/mL)</td>
<td>43</td>
<td>Immediately postpartum</td>
<td>Box-Cox std</td>
<td>0.0-0.2</td>
<td>0-0</td>
<td>0.1-0.2</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>Peak of lactation</td>
<td>Box-Cox std</td>
<td>0.0-0.3</td>
<td>0-0</td>
<td>0.2-0.4</td>
</tr>
<tr>
<td>Cortisol (µg/dL)</td>
<td>49</td>
<td>Immediately postpartum</td>
<td>Box-Cox std</td>
<td>0.1-3.4</td>
<td>0.1-0.2</td>
<td>2.8-4.1</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>Peak of lactation</td>
<td>Box-Cox std</td>
<td>0.1-2.9</td>
<td>0 -0.1</td>
<td>2.2-3.9</td>
</tr>
</tbody>
</table>

**Table 2** - Reference intervals for the different parameters in Saanen goats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Number of goats sampled</th>
<th>Production category</th>
<th>Method</th>
<th>Lower and upper limit of reference interval</th>
<th>90% CI for lower limit</th>
<th>90% CI for upper limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme (µg/mL)</td>
<td>66</td>
<td>Immediately postpartum</td>
<td>Box-Cox std</td>
<td>0.6-4.7</td>
<td>0.4-0.8</td>
<td>4.2-5.2</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>Peak of lactation</td>
<td>Non parametric</td>
<td>1.4-4.5</td>
<td>1.4-1.5</td>
<td>4.2-4.5</td>
</tr>
<tr>
<td>Haptoglobin (mg/mL)</td>
<td>53</td>
<td>Immediately postpartum</td>
<td>Non parametric</td>
<td>0-0.8</td>
<td>0-0</td>
<td>0.5-0.8</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>Peak of lactation</td>
<td>Non parametric</td>
<td>0-0.5</td>
<td>0-0</td>
<td>0.4-0.5</td>
</tr>
<tr>
<td>Cortisol (µg/dL)</td>
<td>68</td>
<td>Immediately postpartum</td>
<td>Box-Cox std</td>
<td>0.1-3.5</td>
<td>0.1-0.2</td>
<td>2.9-4.2</td>
</tr>
<tr>
<td></td>
<td>79</td>
<td>Peak of lactation</td>
<td>Box-Cox std</td>
<td>0-3.3</td>
<td>0-0.1</td>
<td>2.6-4</td>
</tr>
</tbody>
</table>
goats raised in Northern Italy in two critical stages of goat breeding. These RIs could be of value in evaluation health and welfare of goats submitted to our laboratory and laboratories using similar instrumentation and/or methods of analysis. We are therefore confident that they could be a reliable tool to be used in veterinary practice and animal welfare evaluation.

ACKNOWLEDGMENT

This study was financed by the Italian Ministry of Health PRC 2008014 “Influenza di alcuni parametri di tipo ambientale sul benessere di alcune specie (bovini, suini, tacchini, galline oviole, capre) nelle diverse realtà di allevamento italiano”.

Aliquots of blood to determine the analytes’ concentrations came from blood samples undertaken during routine veterinary checks.

References


Full bibliography available from the corresponding author.