GUIDELINES FOR THE ESTABLISHMENT OF REFERENCE RANGES IN VETERINARY MEDICINE

HUTCHISON[1], J.M., SALMAN[1], M.D., FOWLER[2a], M.E., ZINKL[2b], J.G., GARRY[1], F.B.

The establishment of reliable reference ranges for hematological/biochemical parameters in veterinary medicine is of particular importance when dealing with little-known species such as the llama. This paper will review the principles and procedures of data collection and analysis involved in establishing reference ranges, using data recorded from 174 llamas (Fowler et al, 1989) as an illustration.

Reference ranges include the middle 95% of values from a normal (reference) population. The lower and upper limits are estimates of the 2.5th and 97.5th percentile, respectively. Reference ranges should be critically evaluated before use.

THE ESTABLISHMENT OF REFERENCE RANGES

The reference population and selection of individuals

Age, breed, sex, reproductive status, nutritional status, management practices, geographical location (altitude) and activity can alter biological parameters in the reference population. Ideally, separate reference ranges would be established for various categories of animals. If this is not possible, the reference population should be stratified so that the distribution amongst the animals of these factors is representative of the general population.

The selection of individuals for inclusion in the reference range can be performed in two ways. (PetitClerc et al, 1984) In both cases, the inclusion/exclusion criteria should be stated. The first method is retrospective and involves sampling large numbers of individuals either randomly or nonrandomly (recording all pertinent information) then applying inclusion/exclusion criteria to the sample in order to obtain the representative reference population. This *'a posteriori'* method is not commonly utilized in veterinary medicine due to the large numbers of samples (> 2000) recommended. The second *'a priori'* method requires application of

[1]Department of Clinical Sciences, Colorado State University, Fort Collins, CO 80523

[2]Departments of Medicine[a] and Clinical Pathology[b], School of Veterinary Medicine, University of California, Davis, CA 95616

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inclusion/exclusion criteria to the general population, then sampling a selection of individuals which meet the requirements.

Number of samples required

Small sample sizes from a carefully defined reference population may be adequate where much information is available from other sources regarding the parameters concerned. In little-known species such as the llama and for parameters in any species which have not been studied, far greater numbers are required in order to define the shape of the underlying distribution and to give reasonable estimates of the 2.5th and 97.5th percentiles.

It has been suggested that a minimum of 120 animals be sampled. (Reed et al, 1971) This is the smallest number which allows 90% confidence intervals for the 2.5th and 97.5th percentiles to be established. If a reference population is to be subdivided (for example, to give ranges for various ages or sexes of animals) then a minimum of 120 animals in each subdivision should be sampled.

Data collection

Strict protocols have been established in human medicine for the preparation of subjects and the collection of blood samples. (Alström et al, 1975) Uniform management of animals prior to sample collection is unlikely to occur, however, consistency in restraint techniques, collection procedures and sample handling should be achievable. Protocols should be recorded.

Assessment of data

Many biological parameters do not follow the normal (Gaussian) distribution. (Elveback et al, 1970) For example, data collected from 174 llamas showed that 16 of 26 biochemical and 10 of 18 hematological parameters were not normally distributed. In this case, the computer statistical package *SAS*[3] was selected to test the null hypothesis that sample values were a random sample from the normal distribution. The test statistic, W ($0<W\leq1$), was computed. Small values of W lead to rejection of the null hypothesis. (Shapiro et al, 1965) The null hypothesis was rejected at $P<W \leq 0.01$.

Description of data

The mean ± 2SD may used to estimate the limits of the reference range for Gaussian data. Non-Gaussian data are described using non-parametric statistical techniques, which do not require knowledge of the underlying distribution. The data are ranked, and the sample 2.5th and 97.5th percentiles are used as estimates of the population values. More refined non-parametric statistical

[3]SAS Institute Inc., Cary, NC

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techniques are available, but add little to the precision of the estimates. (Shultz et al, 1985)

The problem of "outliers" (very extreme values) complicates both parametric and non-parametric statistical approaches. If parametric approaches are used, outliers greatly distort the mean and the standard deviation. Omission of these values makes the data appear "more normal", but this approach assumes that the values are truly aberrant and the distribution is Gaussian. Outliers result in very wide reference intervals when the non-parametric percentile method is used, particularly when sample sizes are small. In both cases the solution involves refinement of the inclusion/exclusion criteria (to ensure that no "suspicious" individuals are sampled), and increasing the sample size.

Summary

The following steps should be followed when a reference range is prepared:

1. Describe the reference population
2. Define the inclusion/exclusion criteria
3. Decide how many animals will be sampled
3. Collect the samples, using consistent techniques
4. Assess the data
5. Use an appropriate statistical technique to describe the data

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