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General Laboratory Medicine

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Acronyms and abbreviations that appear in this chapter include: Hb, hemoglobin; MCH, mean cell Hb; MCHC, mean cell Hb concentration; MCV, mean cell volume; PCV, packed cell volume; POC, point of care; POCT, point-of-care testing; RBC, red blood cells; TP, total protein; TP_{Ref}, refractometer total protein; TS, total solids.

1.1 Introduction to Laboratory Medicine

Laboratory medicine, more commonly referred to as clinical pathology (or bioanalytical pathology), is a distinct specialty that overlaps other medicine specialties such as internal medicine and oncology in the area of diagnostics. In contrast to internists, clinical pathologists practice a systems-based rather than problem-based approach when interpreting hematological and biochemical results. However, in addition to recognizing disease-associated changes, two other phenomena contribute to test interpretation: how test results are generated and how “normal” is defined. Artifacts due to sample preparation, sample condition or disease processes need to be identified and distinguished from true disease-associated changes. Similarly, test interpretation is always performed in context – the context of health. The accuracy and sensitivity of tests and the use of appropriately established reference intervals are essential to the ability to diagnose disease.

This chapter will provide selected information on hematological and biochemical test methodologies and validation, and will discuss the basic knowledge needed for generating and/or using reference intervals. The remainder of the book will address test interpretation using a systems-based approach.

1.2 Preanalytical Factors

Preanalytical factors that may affect test results should be minimized in order to ensure result accuracy [1]. Specimens should be collected according to standard practices and transported to the laboratory in a timely manner under conditions appropriate for the type of specimen and its stability. The minimum information on a specimen label for laboratory evaluation should include the full name of the patient (animal and owner), the patient signalment, and the specimen type (e.g., whole blood, serum or plasma). Especially for hematological evaluation, it is important that the patient’s signalment be correct as analyzer settings vary with respect to species.

Anticoagulated specimens for hematology that have visible macroclots in the tube will produce variably erroneous results. Because the degree of inaccuracy cannot be predicted, clotted specimens are unsuitable for analyzis and these specimens should not be analyzed or submitted for analysis.

Blood films and cytology smears should not be refrigerated and should be protected from condensation and freezing during transport to the laboratory to avoid condensation artifact (Figure 1.1). Failure to fully dry blood films or cytology preparations before placing them into slide holders can also result in moisture artefact.

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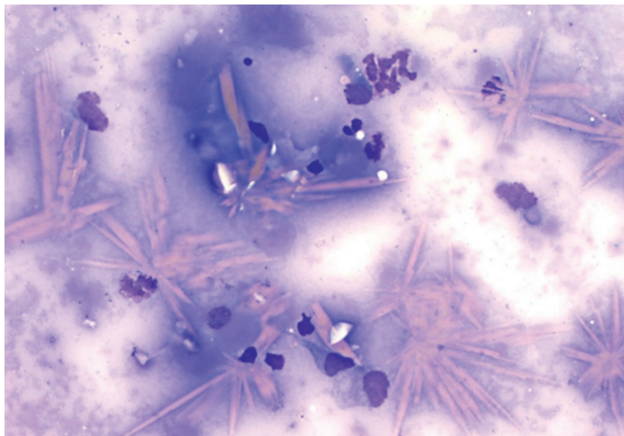

Figure 1.1 Condensation artifact caused by exposure of unfixed slides to moisture. The nucleated cells are lyzed and many hemoglobin crystals are present, formed from erythrocytes.

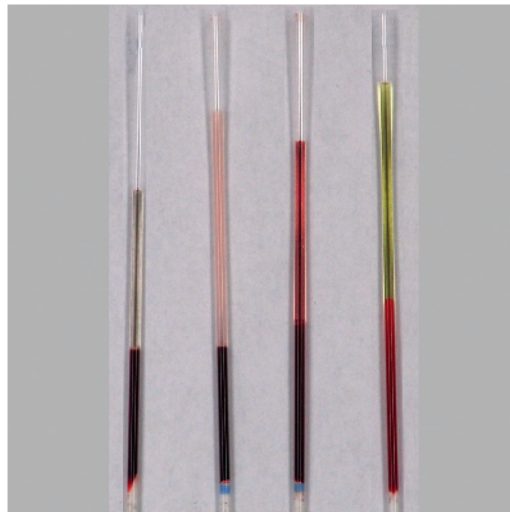

Figure 1.2 Evaluation of plasma. From left to right: normal plasma color and consistency; lipemic and slightly hemolyzed plasma; hemolyzed plasma; icteric plasma.

1.3 Basic Hematological Techniques

1.3.1 Packed Cell Volume and Plasma Evaluation: Disease and Artifacts

Measurement of the percentage of red blood cells in whole blood can provide more information than simply the packed cell volume (PCV). In addition to the packed erythrocytes at the bottom of a microhematocrit tube, there is the white buffy coat layer and a plasma layer. The size of the buffy coat is related to the white blood cell (WBC) (and platelet) count; a thick buffy coat would indicate a high leukocyte (and/or platelet) count, whereas a scant buffy coat suggests leukopenia. The character of the plasma can also yield valuable information pertaining to a disease process, as well as contributing to spurious results. The plasma can appear hemolyzed, icteric or lipemic (Figure 1.2).

Hemolysis in samples from horses usually indicates an *in vivo* phenomenon due to toxins or immune-mediated disease (see Chapter 4). However, hemolysis can also occur during blood collection if excessive force or too small needle gauge is used in phlebotomy. Whether *in vivo* or *in vitro*, hemolysis produces a color change that can make refractometer readings difficult or interfere with spectrophotometric tests.

Icterus indicates hyperbilirubinemia that usually exceeds 1.5 mg/dL (see Chapter 5). However, in herbivorous animals yellow-colored plasma is not a reliable indicator of hyperbilirubinemia due to the presence of diet-associated carotene pigments, which impart a yellow color to plasma. Icterus has not been demonstrated to interfere with refractometer readings [2]. Depending upon the chemistry analyzer, icterus can cause interference with some serum chemistry tests.

Lipemia is visible to the eye as increased turbidity in plasma or serum at triglyceride concentrations >300 mg/dL. Whether physiological (postprandial) or pathological (see Chapter 9), lipemia can cause spuriously high refractometer readings and will interfere with many chemistry tests.

1.3.2 Protein Measurement by Refractometer

Protein can be rapidly and accurately measured by handheld refractometers. Because refractometers measure protein via a total solids-based technique, the total dissolved solids in the sample affect light refraction. In addition to protein, total solids include electrolytes, glucose, urea, and lipids. The term “total solids” has caused much confusion in the reporting of refractometric protein results. Total protein (TP) and total solids (TS) are not synonymous. Currently, the vast majority of refractometers incorporate a conversion factor in their design so that the scales report TP and not TS. Contributing to the confusion is the fact that at least one refractometer is named the “TS meter” (AO Corporation) when it is in fact calibrated to report TP. While the altered refraction of plasma is mostly due to protein content, increases in lipid, glucose or urea content interfere with refractometric protein measurements. However, marked increases in urea or glucose (273 and 649 mg/dL, respectively) are needed to increase protein measurement by 0.4–0.5 g/dL. Increases in plasma cholesterol of 39 mg/dL are shown to increase the refractometer TP (TP_{Ref}) by 0.14 g/dL [2].

Another potential cause of erroneous refractometer readings is the addition of EDTA from K_3EDTA anticoagulant tubes. At the standard concentration of EDTA (5 μmol/mL), K_3EDTA by itself has minimal effect on the plasma's refraction (≤0.1 g/dL increase). This is not true for peritoneal fluid, however, where overestimation of TP_{Ref} by 0.7 +/− 0.1 g/dL was reported in one study (see Chapter 18). At higher concentrations of EDTA (10 and 20 μmol/mL), EDTA can increase TP_{Ref} by 0.9–1.0 g/dL. Underfilling of EDTA tubes has the effect of increasing the EDTA concentration and will cause spurious increases in the TP_{Ref} [3]. Some commercial tubes with K_3EDTA anticoagulant may also contain additives to prevent crystallization of the EDTA. Tubes that contain the additive may increase TP_{Ref} readings by up to 0.9 g/dL, even when properly filled. In general, polypropylene (plastic) tubes are more likely to include additives to prevent evaporation than glass tubes [4]. While sodium heparin anticoagulant has no effect on TP_{Ref}, heparin has deleterious effects on cellular morphology and is not recommended for samples that will be evaluated cytologically.

1.4 Point-of-Care Testing

Point-of-care testing (POCT) is defined as testing done at or near the patient with the expectation that results will be available quickly to facilitate immediate diagnosis and/or clinical intervention [5]. Whilst POCT provides quick, relatively inexpensive results with small volumes of blood, it also comes with its own set of risks. The major sources of error associated with POCT were categorized in one study as most often due to operator incompetence, nonadherence to test procedures, and the use of uncontrolled reagents and testing equipment [6]. Instrument calibrations and quality control measures may be omitted due to ignorance or the need for fast results. And, in veterinary medicine, analyzers may be used with species for which the instrument has not been validated. It should also be noted that diagnostic instruments for veterinary use are not subject to government regulations as they are for human use, which means that devices may not have been independently evaluated or tested [7]. Finally, poorly maintained instruments that are carried from one area to another may be a source of nosocomial infection or may transmit antibiotic-resistant bacterial strains [5].

As part of the process of ensuring accuracy in an analytical method, calibrators and controls are used. A *calibrator* is a material of known or assigned characteristics that is used to correlate instrument readings with the expected results from the calibrator (or standard). A *control* is a preparation of human or animal origin intended for use in assuring the quality control of the measurement procedure, not for calibration. Controls usually represent abnormal and normal concentrations of the measured analyte. Currently, there are some POC analyzers marketed as "maintenance free" that do not come with controls and some that do not have calibrators. These instruments should be used with caution as there is no way to verify assay accuracy.

1.4.1 Hematology Analyzers

1.4.1.1 Impedance Technology

Many POC hematology analyzers are based upon impedance methodology. Examples include the HM series (Abaxis, Union City, CA), the HemaVet 950 (Drew Scientific, Oxford, CT), the HemaTrue® (Heska, Loveland, CO), and the scil Vet abc™ (Scil, Gurnee, IL). Impedance technology employs an electric current that flows through a conductive liquid. When cells, which are nonconductive, pass through an aperture containing this fluid, there is an electrical impedance created for each cell that is proportional to the size of the cell. The impedance method facilitates measurement of the mean RBC and platelet volumes, as well as enumeration of WBCs, RBCs, and platelets. The WBCs (and any nucleated red blood cells) are counted separately from RBCs and platelets after cell lysis. Hemoglobin (Hb) concentration is also measured after RBC lysis. In the isotonic solution, nucleated cells are prevented from being counted along with RBCs and platelets because they are too big to pass through the aperture (Figure 1.3).

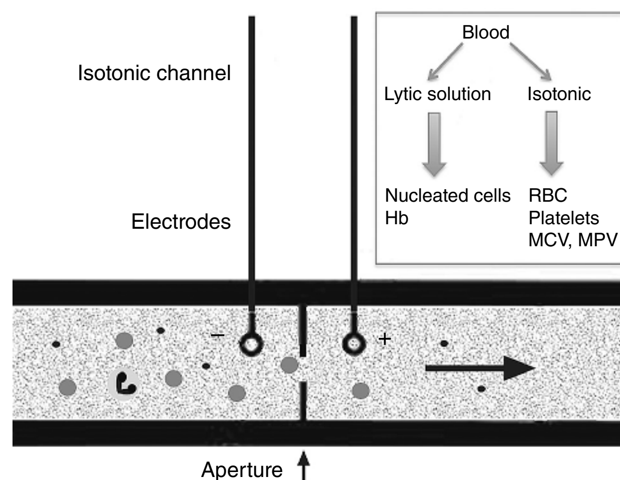

Figure 1.3 Schematic representing standard impedance methodology. Blood is directed into two chambers. In one chamber, a lytic solution is used to obtain the WBC count by evaluating bare nuclei and measuring the hemoglobin released from erythrocytes. The second chamber contains isotonic solution and an aperture of limited size through which erythrocytes and platelets are enumerated.

Failure of RBCs to lyze may result in their being counted as WBCs, thereby falsely increasing the WBC count. Similarly, large platelet aggregates may be erroneously counted as WBCs, resulting in spuriously low platelet and high WBC counts. Very large platelets may be miscounted as erythrocytes.

1.4.1.2 Centrifugal Hematology Analyzers

Centrifugal analyzers operate by taking quantitative measurements on the cell layers below and within the buffy coat. The quantitative buffy coat (QBC) VetAutoread™ (IDEXX Laboratories Inc., Westbrook, ME) is an example of a centrifugal hematology analyzer. Granulocytes, mononuclear cells (monocytes and lymphocytes), erythrocytes, and platelets are separated into layers in an enlarged microhematocrit-like tube using a cylindrical float to further expand the buffy coat layer. Cells separate into layers upon centrifugation according to relative density and fluorescent staining differentiates layers. Centrifugal analyzers can also provide fibrinogen concentrations by re-reading the sample after incubating in a precipitator.

Only the spun hematocrit is measured with centrifugal analyzers. Since erythrocyte counts are not determined, the MCV cannot be calculated. The Hb can be estimated assuming a constant relationship between hematocrit and Hb. From Hb and hematocrit, MCHC can be calculated. Estimated WBC counts are obtained from the thickness of layers by assuming an average cell size.

1.4.1.3 Laser Technology

Laser hematology analyzers generate both cell counts and differentials using light scatter. Single cells pass through a laser beam and scatter light at forward and side angles from the cell, which is picked up by photoreceptors (Figure 1.4). Forward, right-angle, and side light scatter represent cell size and complexity.

While this technology affords the opportunity to generate leukocyte differentials, in general there is not good precision with differential leukocyte counts [7, 8]. The presence of band neutrophils, toxic change or reactive lymphocytes can result in poor separation between leukocyte groups, adversely affecting the instrument differential (Figure 1.5). A manual differential from a blood film is still recommended to verify instrument differentials. Examples of POC hematology analyzers using light scatter are the ProCyte® and LaserCyte® (IDEXX) and ElementHT5® (Heska).

1.4.2 Clinical Chemistry Analyzers

1.4.2.1 Dry Reagent Analyzers

The majority of in-clinic chemistry analyzers are based upon dry reagent technology, which uses reflectance

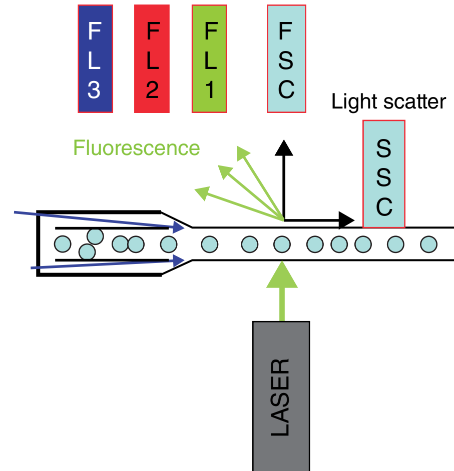

Figure 1.4 Schematic representing the principle of hematological analysis using laser methodology. Light passing directly through the cells (forward scatter; FSC) and light deflected 90° (side scatter; SSC) is captured by detectors. FSC and SSC correspond to cell size and complexity, respectively. Complexity refers to the character of the cytoplasm (e.g., presence or absence of granules). Fluorescence detectors capture fluorescence from dyes that stain RNA, myeloperoxidase or reticulum to differentiate leukocytes or to count reticulocytes.

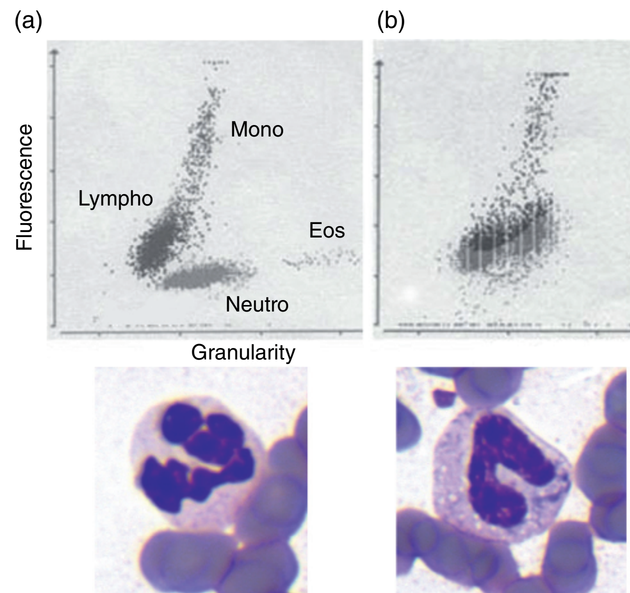

Figure 1.5 Laser-generated leukocyte differentials from the ProCyte Dx POC hematology analyzer (Idexx). The scatterplot is based upon side scatter (granularity) and fluorescence from a fluorescent polymethine dye that stains nucleic acids. (a) Scatterplot from a healthy horse. Neutrophils have the least amount of cytoplasmic RNA, thus are located at the base of the y-axis. (b) Scatterplot from a horse with toxic change in neutrophils and a left shift to band neutrophils. Neutrophils with toxic change and bands both have increased RNA content relative to normal mature neutrophils. Note how the increased RNA staining causes the neutrophil plot area to move upwards on the y-axis, blending into the lymphocyte region.

photometry. Similar to absorbance photometry, a chemical reaction (occurring within a dry fiber pad or multilayer film) results in a product that absorbs a portion of the light that illuminates it. The remaining reflected light reaches a photodetector that measures its intensity relative to the original illuminating light or a reference surface. There is an inverse relationship between reflected light (transmittance) and absorbance, where T is the percent transmittance (Equation 1.1). Analyzers will convert transmittance into absorbance because of the linear relationship between concentration and absorbance. Thus, concentration can be directly calculated from the absorbance.

$$\text{Absorbance} = 2 \quad \log\%\text{T} \qquad (1.1)$$

Dry reagent technology has the advantage of minimal interference from hemolysis, lipemia, and icterus relative to wet chemistry analyzers. While most of the common chemistry analytes can be measured with dry chemistry systems, electrolytes cannot. Common in-clinic analyzers using this methodology include the Spotchem® (Heska), VetTest® (IDEXX), and RefloVet® Plus (Scil Animal Care Company, Grayslake, IL).

1.4.2.2 Reconstituted Liquid Chemistry Analyzers

Liquid chemistry analyzers operate via absorbance photometry. Reconstituted liquid systems use lyophilized rather than liquid reagents in cuvettes attached to rotors so that centrifugation mixes the sample with the reagent. Similar to reflectance photometry, when the sample is added to the reagents a chemical reaction occurs, manifesting as a color change in the liquid. Light of a specific wavelength is then passed through the liquid; the wavelength used is usually the one at which maximum absorbance for the substance being measured occurs. The light transmitted through the fluid post reaction is measured and converted into absorbance. Liquid chemistry systems are affected by hemolysis, lipemia, and bilirubinemia more than dry reagent systems. If not already known, determining the effect of substances such as these on the measurement of specific analytes should be part of the validation of a methodology.

Examples of this type of chemistry analyzer include VetScan® (Abaxis) and Hemagen Analyzt® (Hemagen Diagnostics, Columbia, MD). Just as with dry reagent systems, most common chemistry analytes, with the exception of electrolytes, can be measured.

1.4.2.3 Electrochemistry

In order to measure ion concentration, electrochemistry (also known as ion selective electrode [ISE] methodology) is employed in POC analyzers. Examples include the VitalPath™ (Heska), VetLyte® and VetStat® (IDEXX), and EasyLyte® Plus (Hemagen). ISE technology relies upon development of a membrane potential for the ion being measured. This is achieved by using an electrode with a membrane selective for the ion being measured. The membrane potential that develops when the membrane is in contact with the sample is then proportional to the activity of the ion of interest (Figure 1.6). This is compared to the reference electrode to calculate the ion concentration using the Nernst equation. Unlike flame photometry methods to measure electrolytes, ISE is not affected by lipemia or hyperproteinemia.

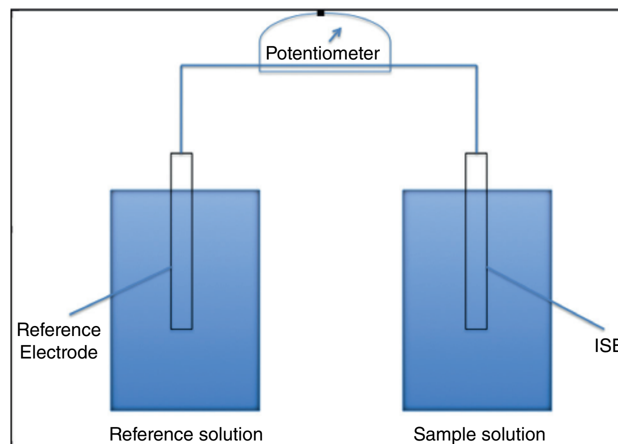

Figure 1.6 Ion selective electrode (ISE) methodology. When a sample is in contact with the membrane selective for the ion to be measured, a membrane potential proportional to the activity of the ion develops. The ion concentration is calculated using the Nernst equation by comparing the sample potential to the potential generated from a reference electrode in a reference solution.

1.5 Test Validation and Reference Values

1.5.1 Test Validation

Laboratory test method validation refers to the multitiered process of evaluating the performance of a new instrument or test methodology, often in relation to an instrument or methodology that is currently in use. In its broadest sense, method validation comprises the evaluation of test performance following a change in reagents, instruments, methodology, or – unique to veterinary clinical laboratories – introduction of a new species. The importance of test validation for different species cannot be overstated. As a result of the interspecies structural differences in any given analyte, a methodology that is adequate for one species may be inappropriate for another. Differences in expected reference values may affect whether a test has an appropriate

detection limit and analytical range. Species differences exist also in how lipid, hemoglobin or bilirubin interfere with analyte measurements [9]. Certainly, drug interferences could also be species specific. Thus, in the age of POC instrumentation, it is essential that the instrument be validated for the species in which it is used.

Before evaluating a test for a novel species, it is important to know whether the analyte to be measured is clinically relevant. For example, in equids there is little need to validate an alanine aminotransferase (ALT) assay for clinical purposes (see Chapters 5 and 10). The ultimate goal of method validation is to provide objective evidence that the evaluated method will show acceptable reproducibility and accuracy so as to be clinically applicable.

The major steps in test validation consist of estimating the following.

1) Precision
2) Accuracy
3) Sensitivity
4) Specificity
5) Reference intervals

Reproducibility of results is referred to as *precision*. Precision is measured as a coefficient of variation and reflects the amount of variation inherent in the method and is estimated by repeating measurements of the same sample at least 20 times (intraassay precision). Estimating day-to-day precision (interassay precision) requires running aliquots of the same sample over 20 days [10].

Accuracy or *bias* measures the amount of closeness in agreement between the measured value of an analyte and its "true" value. Accuracy is estimated by comparing the performance of the candidate method with that of a definitive or reference method (gold standard), by performing a recovery experiment, or by comparing the candidate method with the established method that is being replaced. Recovery experiments estimate the ability of an analytical method to correctly measure an analyte when a known amount of the analyte is added to authentic biological samples.

Sensitivity is related to precision and refers to a test's ability to detect both small quantities of the analyte and small differences between samples. A "sensitive" methodology has a high level of analytical sensitivity and a low detection limit. The detection limit and analytical sensitivity are related but not synonymous. The detection limit is defined by the International Union of Pure and Applied Chemistry (IUPAC) as the smallest quantity or concentration that can be detected with reasonable certainty. The detection limit depends on the magnitude of the blank measurements and is related to their imprecision [11].

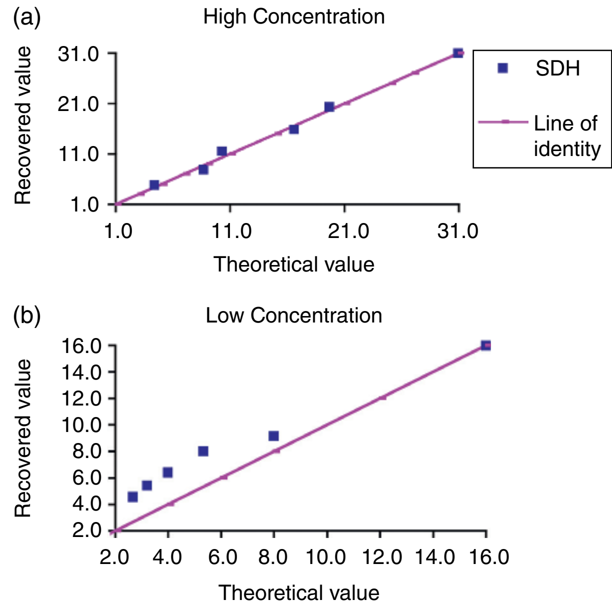

Figure 1.7 Serial dilutions of high and low concentrations of sorbitol dehydrogenase (SDH) to determine assay sensitivity. (a) There is very good correlation between the expected and recovered values in dilutions made from high SDH concentrations. (b) In contrast, at low concentrations of SDH the assay is less sensitive.

Sensitivity measures the change in signal relative to a defined change in the quantity or concentration of an analyte. This is usually accomplished by measuring a series of dilutions of a known amount of analyte (Figure 1.7).

Analytical specificity refers to the ability of a method to detect only the analyte of interest and is related to accuracy. Specificity may be affected by factors such as hemolysis, icterus or lipemia of serum or plasma, or by drugs and other substances that compete for reagents or affect the physical properties of the sample. Interference studies are performed by adding the interfering material directly and measuring its effects or by comparing measurements from hemolyzed, icteric or lipemic samples using the candidate method and one that is not affected by these factors.

Reference values are typically generated at the end of the method validation process and should be included with an instrument after the manufacturer has validated the methodology. When considering a POC instrument for purchase, if the manufacturer has truly validated the instrument for horses, species-specific reference values should be available.

1.5.2 Reference Values

The use of reference values to diagnose or screen for disease implies that health is a relative concept; clinical

examination, evaluation of laboratory data, and diagnostic imaging findings all require comparison to a "normal" standard. "Normality" itself is also relative. What would be considered usual values for a racehorse may vary significantly from values from a cold-blooded working horse. Because health and disease are defined against "normal" or reference standards, the importance of appropriate reference values cannot be overstated. A few general principles regarding the use of reference values should be common knowledge for all veterinary practitioners.

1) When laboratory-specific or instrument-specific reference values are not available, published reference intervals (RI) should be used with caution. Published reference values should provide basic information regarding how health was defined for the population, as well as the general characteristics of the population (including number of animals sampled) and the instrumentation from which the values were derived. The practitioner should attempt to match the population and instruments from which the values were generated as closely as possible to the patient to which they are being applied.
2) Reference values obtained from one type of POC instrument should not be used interchangeably with those for another instrument, especially when different methodologies are involved. Similarly, using RIs generated from diagnostic laboratories analyzers to interpret data from your POC analyzer can be like comparing apples and oranges. If your POC analyzer does not come with RIs provided by the company from which you bought it, look for published RIs which are for similar POC analyzers. If your POC analyzer *does* have RIs provided from the manufacturer, take the time to find information on where the RIs came from. Some POCs may be designed for humans and the RIs provided may not even be from a veterinary species or may pertain only to a given species.
3) If you plan to replace a POC analyzer with a similar but different instrument and want to use the old RIs from your original analyzer, the old RIs should be validated for the new instrument. Validation can be achieved using a small sample (n = 20) of "normal" individuals. The values obtained from these healthy individuals can be tested against the RI to be used with the new instrument; if two or fewer subjects are outside the candidate RI, it is considered transferable. If three or four values fall outside the RI, another 20 patients can be tested and interpreted in the same manner as the original 20 samples. If >4 of the original 20 values fall outside the candidate RI, transference is rejected for that analyte and an alternative RI must be used [10].

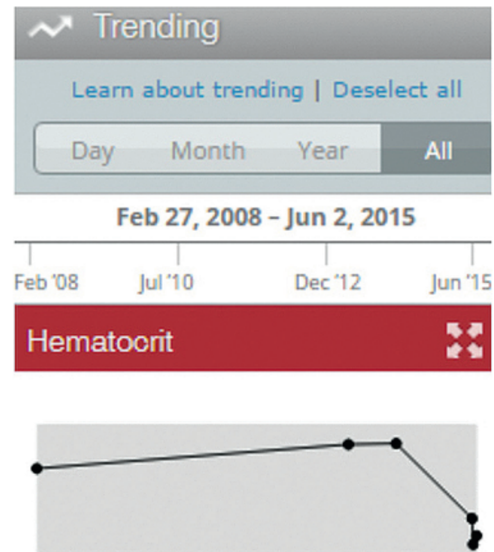

Figure 1.8 A graph from Idexx Laboratories' VetConnect® platform depicting a patient's hematocrits over the course of seven years. The gray zone represents the reference limits for hematocrit. Although the last three values on the graph are within the reference limits for "normal," these values are clearly abnormal for this individual.

Reference intervals used for interpretation of laboratory data are population based, using cross-sectional data typically representing 95% of the population chosen. Thus, by definition, any given RI implies that there will always be about 2.5% of the population whose values will normally fall above or below the RI. This fact should be considered when interpreting abnormal data that do not fit the clinical picture.

A population-based RI may not be sensitive enough to detect change in an individual if it is not marked. While this can be true for any analyte, some analytes are much more prone to this effect than others [12]. For these analytes, using the individual as its own normal can be much more effective in identifying abnormalities, especially with particular analytes (Figure 1.8). Patient-based RIs are generated from the individual patient's longitudinal data, if available, and can be assessed by looking at how the data trend from that patient in health. Some diagnostic laboratories and practice information systems provide graphing tools to follow patient data over time. In this manner, significant changes in an analyte can be detected before the values fall outside the RI.

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