Chapter 1
General Laboratory Medicine

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Acronyms and abbreviations that appear in this chapter:

- 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

General 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 hematologic 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...
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performed in context—the context of “health.” The accuracy of the test methodology and the reference intervals generated from the methodology are essential to the ability to diagnose disease.

This chapter provides information on hematologic and biochemical test methodologies and validation, and discusses the basic knowledge needed for generating and/or using reference intervals. The remainder of the book addresses test interpretation using a systems-based approach.

Basic hematologic techniques

Packed cell volume and plasma evaluation: Disease and artifacts

Measurement of the packed cell volume (PCV) can provide more information than simply the percentage of red blood cells in whole blood. 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 (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 contribute to spurious results. The plasma can appear hemolyzed, icteric, or lipemic (Figure 1.1).

Hemolysis in samples from horses usually indicates an in vivo phenomenon due to toxins or immune-mediated disease (see Chapter 3). However, hemolysis can also occur during blood collection if excessive force or a needle gauge that is too small 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 4). 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. Depending on 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 physiologic (post-prandial) or pathologic (see Chapter 8), lipemia can cause spuriously high refractometer readings and will interfere with many chemistry tests.

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 all 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 mg/dL 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<sub>Ref</sub>) by 0.14 g/dL.²

Another potential cause of erroneous refractometer readings is the addition of EDTA from K₃EDTA anticoagulant tubes.¹ At the standard concentration of
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EDTA (5 μmol/ml), K$_3$EDTA by itself has minimal effect on the fluid’s refraction (≤0.1 g/dL increase). 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$_{ref}$. Some commercial tubes with K$_3$EDTA 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. 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.

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. While POCT provides quick, relatively inexpensive results with small volumes of blood, it also comes with its own set of risks. Instrument calibrations and quality control measures may be omitted out of ignorance or the need for fast results. Furthermore, 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 governmental regulations as they are for human use, which means that devices may not have been independently evaluated or tested. 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.

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.

Hematology analyzers

Impedance technology

Many point-of-care (POC) hematology analyzers are based on 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
Figure 1.2  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 to measure the hemoglobin released from erythrocytes. The second chamber contains isotonic solution and an aperture of limited size through which erythrocytes and platelets are enumerated.

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 white blood cells (WBCs), RBCs, and platelets. The WBCs (and any nucleated RBCs) 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 (see Figure 1.2).

Failure of RBCs to lyse 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.

**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, 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 rereading 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
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Figure 1.3  Schematic representing the principle of hematologic 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.

...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.

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 1.3). 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. The presence of band neutrophils, toxic change, or reactive lymphocytes can result in poor separation between leukocyte groups, adversely affecting the instrument differential (Figure 1.4). A manual differential from a blood film is still recommended to verify instrument differentials. The most common examples of POC hematology analyzers using light scatter are the ProCyte® and LaserCyte® analyzers (Idexx Laboratories).

Clinical chemistry analyzers
Dry reagent analyzers
The majority of in-clinic chemistry analyzers are based on dry reagent technology, which uses reflectance photometry. Similar to absorbance photometry, a
Figure 1.4 Laser-generated leukocyte differentials from the ProCyte Dx point of care hematology analyzer (Idexx Laboratories, Westbrook, ME). The scatterplot is based on 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 and are thus 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 neutrophil bands both have increased RNA content relative to normal mature neutrophils. Note how the increased RNA staining causes the neutrophil plot area to move up on the y-axis, blending into the lymphocyte region.

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. Analyzers will convert transmittance into absorbance because of the linear relationship between concentration and absorbance. Thus, concentration can be directly calculated from the absorbance.

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Absorbance = 2 - \log \%T \quad (1.1)
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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 Corporation), VetTest (Idexx Laboratories), and RefloVet Plus (Sci! Animal Care Company, Grayslake, IL).

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 manifesting as a color change in the liquid occurs. Light of a specific wavelength is then passed through the liquid; the wavelength used is usually the wavelength 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 Analyst (Hemagen Diagnostics, Columbia, MD). Just as with dry reagent systems, most common chemistry analytes, with the exception of electrolytes, can be measured.

Electrochemistry
In order to measure ion concentration, electrochemistry (also known as ion selective electrode methodology) is employed in POC analyzers. Examples include the VitalPath (Heska Corporation), VetLyte and VetStat (Idexx Laboratories), and EasyLyte Plus (Hemagen Diagnostics). Ion selective electrode (ISE) technology relies on 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 1.5). 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.

Test validation and reference values
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
Figure 1.5  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.

In the 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. 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 new 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. 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
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 (intra-assay precision). Estimating day-to-day precision (inter-assay precision) requires running aliquots of the same sample over 20 days.

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.

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 1.6).

Analytic 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.

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”
Figure 1.6 Serial dilutions of high and low concentrations of sorbitol dehydrogenase (SDH) to determine assay sensitivity. (A) There is a 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.

standard. “Normality” itself is also relative. What would be considered usual values for a racehorse may vary significantly from values for 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 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 instrument should not be used interchangeably with those for another instrument, especially when different methodologies are involved. Reference values must first be validated before being applied to a second instrument, especially with POCT.
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 another instrument; if two or fewer subjects are outside of the candidate RI, it is considered transferable. If 3 or 4 values fall outside the RI, another 20 patients can be tested and interpreted in the same manner as the original 20 samples. If more than 4 of the original 20 values fall outside the candidate RI, transference is rejected for that analyte and an alternate RI must be used.

References