Interpretation of Equine Laboratory Diagnostics

Edited by **Nicola Pusterla and Jill Higgins**

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Preface

As equine practitioners, we are blessed to be in a field that is constantly and rapidly advancing. With the discovery of emerging or re-emerging diseases, equine practitioners, like ourselves, are challenged to not only understand these new entities, but also be able to use appropriate diagnostic tests and adequately interpret their results. The objectives of this book are to help equine veterinarians with the interpretation of equine laboratory diagnostics as they apply to hematology, clinical chemistry, serology and molecular diagnostics. There are currently incredible equine medical texts that address the laboratory diagnostic approach of certain diseases. However, much of their focus is devoted to the clinical presentation, pathophysiology of the disease, and treatment options. This book represents a unique compilation of both established and new diagnostics that are routinely offered by diagnostic laboratories across North America in an easy-to-use manual written by leading experts in the various fields. Our goal was to link the principles of clinical pathology in hematology, clinical chemistry, serology, molecular diagnostics, and genetics with clinical understanding and then translate the results of such investigation into a format that is readily usable by the busy equine veterinarian. This book is a quick reference guide for veterinary students, veterinary technicians and equine veterinarians, who have a need for laboratory diagnostics in their daily equine practice and studies. One of the main challenges in the veterinary profession we've experienced is locating accurate information on the meaning of certain diagnostic laboratory results taking into account their potential advantages and pitfalls. Who has the time to stay up-to-the-minute current with the newly-developed assays and to develop a level of comfort with their use without having any specific experience in interpretation of their results? This book offers an easy and practical introduction to currently available tests, helps with understanding of the results by providing examples, presents comparative data on test performance, and makes reference to the laboratories that offer such testing.

It is our personal goal to make professional life a little bit easier for our fellow hardworking equine veterinarians who are also doing double time being great moms and dads, husbands and wives, and all of the other roles that we play. Our hope is that this manual will become your go-to, quick reference guide that frees up your time to do the things that really matter… caring for horses, training the next generation of equine veterinarians, and making a difference in this world. Thank you to everyone who believed in the idea behind this book and to our families for allowing us the time and opportunity to work on it together. It has been a fun adventure!

Nic and Jill

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Veterinary Diagnostic Testing

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1.1 Introduction

Most veterinary diagnostic laboratories have websites or booklets describing requirements for diagnostic sampling. These resources have descriptions of the sample needed, volume, temperature requirements for shipping, and other valuable information to assist the referring veterinarian.

Obtaining diagnostic samples from animals may present zoonotic disease exposure to the veterinarian. The veterinarian should always be aware of zoonotic diseases, transboundary diseases and even potential bioterrorism acts when collecting diagnostic samples. One of the most recognized potential zoonotic exposures for veterinarians is rabies and this should be on the differential in any neurological case. Any neurological case should be carefully handled when obtaining brain or any samples from the horses.

Additionally, foreign animal diseases (FAD)/transboundary diseases should be on the differential when clinical signs suggest such. International movement of horses legally and illegally may introduce FADs into the United States and consultation with the USDA and state veterinarians should be done prior to any sampling should veterinarians have any concerns about these possibilities.

Veterinary diagnostic testing utilizes many of the rapidly developing testing platforms including PCR, sequencing, multi-array, and MALDI-TOF to assist in diagnosis. Testing procedures are changing frequently and veterinarians must familiarize themselves with their referral laboratories' website or contact the lab to stay abreast of new sampling requirements, and tests.

Many large state veterinary diagnostic laboratories are full-service laboratories and provide assistance to veterinarians in diagnostic plans, choosing tests and samples for suspected illnesses. State veterinary laboratories may be accredited by the American Association of Veterinary Laboratory Diagnosticians (AAVLD), which is an organization that promotes the improvement of veterinary diagnostics and standards for testing (see www.aavld.org/mission-vision-core-values). Veterinarians should work closely with their laboratory to be assured that they are familiar with the most current and correct sample collection and handling required by the laboratory.

Most laboratories have specialized sections for testing which include: clinical pathology, anatomical pathology, endocrinology, coagulation, bacteriology, virology, molecular diagnostics, and toxicology. Referral to other laboratories is routinely done by large laboratories due to the extensive testing requirements and recognized expertise of other laboratories.

1.2 Diagnostic Sampling

1.2.1 Whole Blood

One of the most frequently tested body fluids in the equine is blood.

- Most veterinary blood tests are done on whole blood, plasma or serum.
- A number of different blood tubes, transport vials, and so on, should be available to veterinarians at all times to obtain diagnostic samples such as CBCs and blood chemistries.
- Some blood tests require specialized collection tubes or containers that are not routinely stocked at veterinary practices and may be purchased from the laboratory.
- Consultation with your laboratory or review of their website should be done prior to blood sample collections to ensure quality and diagnostic samples.
- Special attention should be made to the specimen, the manner of collection, appropriate transport container, temperature requirements, correct test requests, and

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complete paperwork. Most laboratories welcome assisting veterinarians to help ensure the correct samples are collected.

1.2.2 Order of Draw

The order in which blood samples are drawn when multiple blood collection tubes are being collected from the animal is called "order of draw." Although this is not routinely practiced in veterinary medicine, it is suggested to follow the order of draw. Advanced techniques and the improved detection levels in diagnostic tests may cause inaccurate results from carry over between tubes with additives. It has been determined which additives affects test results and drawing the blood in the correct order is necessary, but some researchers feel the difference is minimum. The order of draw for most veterinary applications is: sterile tubes (blood cultures), light blue, red top, or SST, dark green, and purple (Box 1.1). If additional tubes are going to be drawn consultation with the lab should be done.

1.3 Collection, Preparation, and Handling

1.3.1 Blood Collection Tubes

Various types of evacuated blood-drawing supplies should be kept on hand in a clinic or in an ambulatory vehicle for equine diagnostic testing. Additional blood collecting supplies may include specialized blood-drawing needles, needle holders, and butterfly collection device needles.

There are numerous specialized blood collection tubes that are used in human medicine that can be used in veterinary diagnostic testing for special and routine tests (Figure 1.1). These tubes include: (1) trace element tube (royal blue cap), (2) thrombin based clot tube with activator gel for serum separation (orange cap), (3) glucose determinations (gray cap), (4) lead determination (tan caps), purple/lavender caps, and (5) blood culture collection tubes and DNA testing tubes (yellow capped with sodium polyanethol sulfonate (SPS) and others for specified tests.

Important facts about evacuated blood collection tubes:

- Expiration date
  - Blood collection tubes expiration dates are stamped on the tubes.
  - Out of date tubes may lose vacuum because of dried out stoppers and cause incomplete seals, incomplete filling of tube, and additives may become inactive over time.
  - Plastic collection tubes may not maintain the same shelf life as glass.
- Tube size and complete fill
  - Evacuated tubes are designed to auto-fill to a designated amount and should be allowed to fill until blood stops flowing automatically.
  - Under-filling tubes with additives will adversely affect results.
  - If there is a likelihood that a tube will not be filled to the correct volume, smaller tube sizes should be used to ensure the correct dilution of blood to the additive. Blood collection tubes/containers come in various sizes.

Box 1.1 Key points of blood sampling.

- Review the referral laboratory website or contact the lab to obtain information.
- Required sample type: plasma, serum, whole blood, etc.
- Animal preparation: fasting, at rest, after exercise, after medications, etc.
- Volume of required sample. The minimum volume allows one single analysis including instrument dead volume.
- Collection tube type and size: EDTA, heparin, citrate, glass, plastic tube, microtube, etc.
- Sample handling after collection: clotting time, centrifugation, temperature requirements.
- Shipping and handling requirements: receipt at the laboratory within stated time, chilled, frozen, room temperature, and so on.
- Do not freeze sera in glass tubes.
- Storage temperature is specified as room temperature (15–30 °C), refrigerated (2–10 °C), or frozen (−20 °C or colder).
- Samples after collection should immediately be placed in appropriate temperature holding areas until testing is begun or until prepared for shipping to referral lab.
- An air-dried blood smear should accompany EDTA samples for hemogram if testing not performed within 3–5 h post collection.
- Slides should be labeled with a pencil or diamond point pen.
- Cells in collection tubes with anticoagulants/additives may develop artifactual changes; therefore, air-dried slides should be made to prevent these changes.
- Slides should be placed in slide mailers away from moisture and formalized tissues/samples. Formalin fumes affect air dried slides and may render cytology smear nondiagnostic.

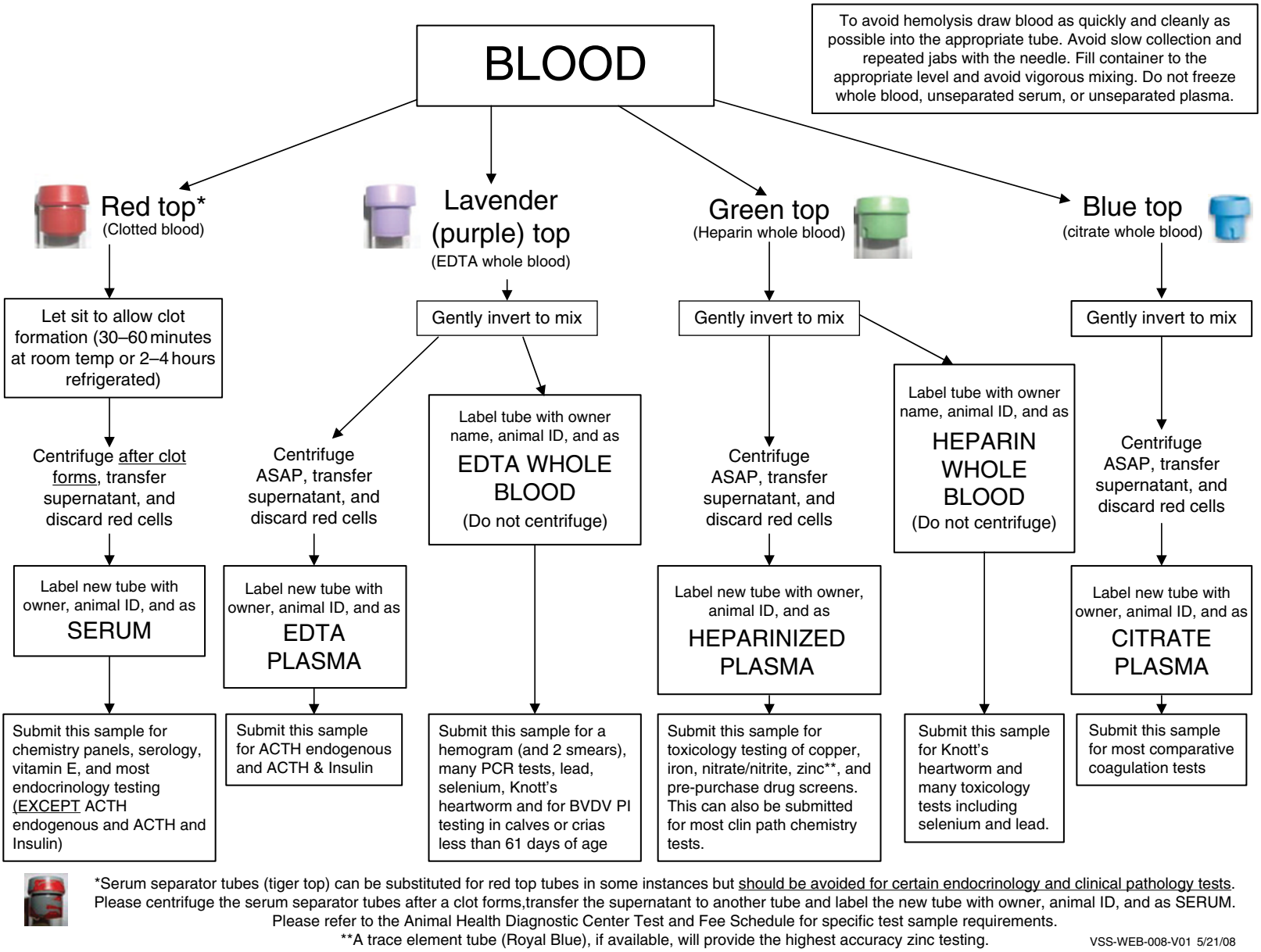

Figure 1.1 Blood flow chart. *Source:* Courtesy of Linda Mittel.

- Adhere to volume requested by laboratory because requested volume is used for verification of results, add-on tests, and parallel (acute and convalescent serology) testing.
- Necessary volume should be calculated prior to collecting samples.
- Mature normal sized horses should yield 4 ml of serum from each 10 cc blood drawn: 5 ml of plasma should be obtained from 10 ml of whole blood.
- These volumes may vary with hydration, health status (anemia) and other conditions.
- Foals or seriously anemic animals may require that smaller volumes of blood be taken.
- Microtubes ranging from 200 to 600 microliters and other blood collection tubes are readily available ranging in sizes from 2 to 10 ml.
- Butterfly collection lines/winged infusion sets may be used to obtain blood samples in the case of inaccessibility to the jugular veins, small vessel size, fractious animals, or difficult approaches.
- Butterfly collection lines/winged infusion sets can be placed directly into the blood collection tube, but a syringe should be attached to butterfly lines to obtain the blood to prevent vessels collapse from undue pressures. Note that butterfly collection needles/winged infusion sets have been recognized to be one cause of a large number of needle sticks to technicians and staff. Appropriate care should be done to prevent this.
- Blood may be drawn directly into syringes and transferred to appropriate tubes. Special transfer devices are available to transfer blood from syringe to collection device/tube.
- Special handling of these samples drawn by syringes must be done to prevent hemolysis, damage to the cells, and under/over filling of the tubes. The needle should be removed from syringe carefully (do not recap) and push the plunger steadily, but gently to prevent hemolysis and run the blood down the side of the opened tube. The correct volume should be placed in the tube and immediately stoppered and inverted as required.

1.4 Blood Sample Handling after Collection

All blood samples should be collected and gently mixed by inverting the tubes immediately after collection. The inside of certain tubes is sprayed with additives and sample must be inverted multiple times to allow contact with the additive and mixing of the blood.

- EDTA, heparin and other additive invert 8–10 ×
- SST, red top and plastic serum tubes invert 5 ×
- Sodium citrate tubes invert 3–4 ×
- Blood culture vials invert 8–10 ×

Plasma and serum are obtained from different types of tubes. Plasma is obtained from whole blood with an additive/anticoagulant. Serum is obtained from clotted blood.

1.5 Centrifugation of Blood Samples

Blood samples and other diagnostic samples may need to be centrifuged to separate components.

- The normal waiting time for blood to clot is ~30 min.
- Centrifugation of clotted blood to obtain serum or anticoagulated blood to obtain plasma is typically done at 1000–1200 g for 10–15 min. Some blood collection tube manufacturers have specific centrifuge speed requirements and review of these requirements may be necessary prior to sample handling.
- Temperatures during centrifugation should be between 20–22 °C. If analytes are temperature-labile, centrifugation should be done at 4 °C or refrigerated.
- Serum collection
  - Serum collection tubes should be handled as suggested by tube manufacturer and as required by the laboratory.
  - Blood collected in a plain red top tube, serum separator tube (SST), or a tube to obtain serum should be allowed to clot at room temperature for a minimum of 30 min and no longer than 2 h before centrifugation and removal of the clot.
  - Special serum collection tubes are available to expedite clotting within 30 min. Orange capped SST tube with thrombin manufactured by Becton Dickson allows for clotting in 5 min and is commonly used in emergency situations.
  - The premature spinning of samples prior to full clotting will cause difficulty in separating the clot from the sera and may cause hemolysis, change in electrolytes, and analytes that may adversely affect results.
  - Refrigeration prior to the clot formation may affect results and cause spurious values particularly potassium levels.
  - Hemolyzed blood can adversely affect blood chemistry analytes.
  - Blood potassium, and total bilirubin can be affected by hemolysis.
  - Tubes should be spun in a centrifuge after clotting and serum should be promptly removed with a disposable pipette and placed into another plain red top tube or transport vial and stored at designated temperature.

  - Vitamin E and bilirubin are light sensitive and should be wrapped in aluminum foil or stored in dark container as should all light sensitive samples.
  - Ammonia, certain blood coagulation testing, and ACTH are temperature sensitive. These analytes must be collected and chilled/frozen immediately.
  - Some serum samples for serological testing may be kept at room temperature, but it is best to refrigerate or freeze sera after collection and during shipment.
  - Sera should be frozen to prevent protein breakdown and bacterial contamination,
  - particularly when samples will be held longer periods of time (2–3 weeks) for parallel testing of acute and convalescent serum samples.
  - Serum separator tubes (SST) or "tiger tops" (ref) have a special gel that allows for easier separation of the sera from the clot after centrifugation. This gel does not make a complete seal between the cells and serum (or plasma) and the tube should be centrifuged and serum removed from the cells to prevent changes in analyte values.
  - SST tubes should not be re-centrifuged because potassium values will be spuriously elevated.
- Plasma collection
  - Plasma is obtained from whole blood tubes with additives or anticoagulants such as EDTA, heparin, and citrate.
  - Whole blood is spun in a centrifuge and the supernatant, plasma, is removed with a disposable pipette. There are plasma collection tubes with gel to aid in the separation of the plasma from the cells. Plasma is collected and placed in a transport vial or plain red top tube. It is imperative to identify the plasma source (EDTA plasma, citrated plasma, etc.) because the additives/anticoagulant may affect the test and some tests are validated with a specific plasma type and it required for testing.

1.6 Blood Culture Sampling

- Blood cultures are used in veterinary medicine in cases of sepsis, fevers of unknown origins, and other potential bacteremia/fungemic conditions. Use of blood cultures will assist in identification of the infectious agent associated with the illness and decrease the overuse of antibiotics.
- Specialized blood culture vials/tubes are required.
- A set of both anaerobic and aerobic blood culture samples should be drawn at the same time.
- Three sets (aerobic and anaerobic) should be drawn over a 24-h period. Sampling should occur prior to initiating therapy. In critically ill animals in need of antimicrobial therapy, two sets of blood cultures can be drawn within 15 min of each other and antimicrobials administered afterward.
- Samples should be drawn as a fever is rising to optimize isolating of bacterial organisms.
- Aseptic collection techniques are critical to prevent sample contamination and subsequent confusion on the interpretation and validity of results.
- Blood culture vials are available with resins to remove antimicrobials from blood for culture.
- Inoculated blood culture vials should be protected from temperature extremes, bright light and never be chilled.
- Blood culture vials should be taken to the laboratory as soon as possible (within 3 h) after collection, but if shipped to a referral lab, vials should be maintained at room temperature prior to and during shipping. Samples must be shipped overnight.
- Ship the blood culture vials in an insulated container to prevent temperature extremes.
- Patient identification should be noted on vial, time, and location of draw (which vein used, etc.) to prevent future resampling blood cultures in the same area.

1.7 Laboratory Validation of Blood Samples

Blood tests are validated on specific types of blood samples or products (plasma or serum) and reference values are established using these validated samples. Certain tests are required to be done on specific specimens; that is, CBCs must be done on whole blood from EDTA tubes or capillary tubes with EDTA anticoagulant in the tube.

- Heparinized whole blood causes distortion of the RBCs and is not acceptable for a hemogram.
- The specific type of plasma should be identified, that is, heparinized plasma, citrated plasma or EDTA plasma and noted on transport tube along with other animal identification, date, and initials of the person who drew the sample.
- Heparinized plasma is used for some toxicology testing.
- EDTA plasma is used for testing ACTH.
- Citrated plasma is used in coagulation studies.

Each laboratory may have their own specific requirements and this should be reviewed prior to sampling. Every veterinary laboratory does not have the same requirements.

1.8 Specimens, Transport Containers, and Media for Various Disciplines

1.8.1 Clinical Pathology

Emergency testing and routine tests are the norm for this section and turnaround times are usually quick. There are many routine tests that are performed in this section, but sophisticated testing is also done such as flow cytometry, immunophenotyping, and body fluid analysis (synovial fluid, pulmonary fluid, abdominal fluid, cerebrospinal fluid). Clinical pathology testing compliments most other laboratory sections and is often one of the first tests requested in diagnostic workups.

The section on blood collection and testing in this manual applies to most of the blood sample submissions to clinical pathology. Other equine samples that are processed in clinical pathology require special handling/collection include:

- Tracheal wash fluid, joint/synovial fluid, cerebrospinal fluid, bronchoalveolar lavage fluid, bone marrow, urine, and various needle aspirates submitted for cytological evaluation and analysis require submission in a sterile red top/EDTA tube.
- If cytological evaluation is requested, air dried smears of the fluid from either the red top tube or the EDTA tube should be sent with the sample in order to preserve the cellular components from breakdown and facilitate interpretation. These slides should be sent in slide mailers and kept dry.
- If culture will be requested on body fluid samples, a sterile red top tube or vial should be sent in addition to the EDTA sample. EDTA is bactericidal and not acceptable for aerobic or anaerobic bacterial culture or fungal culture. A sterile red top tube or sterile transport vial is required for aerobic fluid culture.
- Anaerobic culture on fluid samples requires the use of an anaerobic transport media. Anaerobic vials, large mouth screw top lids, vials with septum for needle injections or bottles are available for fluids, and tissues. Saturated swabs are not the preferred sample.
- Synovial fluid should be placed in a sterile red top and EDTA tube. Air dried slides should be made and submitted. Samples with small number of cells may require that the sample be cytospinned and slides made from the pellet on arrival at the lab to obtain a good representation of the cellular components.
- Bronchoalveolar lavage (BAL) samples should be sent in an EDTA blood collection tube and a plain red top tube chilled for overnight delivery. Air dried smears made direct from the EDTA tube should be submitted with the fluids (cells in a low protein fluid such as the saline lavage fluid may breakdown and become difficult to identify).
- Tracheal wash samples should be placed in both, a sterile red top tube for culture and an EDTA tube for cytological evaluation. Air dried smears should be made from the EDTA sample tube.

1.8.2 Microbiology

This lab section is responsible for the growth, identification, and antibiogram of bacteria, yeasts, and fungal agents. The advent of new technology has allowed for quick and novel bacterial identification. The MALDI-TOF™ machine has revolutionized the identification time of bacteria to minutes versus days. PCR and sequencing are other testing platforms that are used for bacterial and fungal identification. Collaboration with the molecular section of the laboratory is done many times to assist in identifications.

- Bacterial sampling and transport media
  - Sampling for isolation of bacteria and fungi may require specialized transport media (TM) to allow shipping/transfer to a referral laboratory.
  - Anaerobic and aerobic blood culture has specialized collection media. Amies transport media with or without charcoal and modified Stuart's medium are three of the commonly used aerobic bacterial TM. Amies TM with charcoal is used in veterinary medicine for the isolation of fastidious organisms such as *Taylorella* sp. and is required in contagious equine metritis regulatory testing.
  - Specialized enteric TM are available for assisting in the recovery of enteric organisms such as Para Pak™ transport media. This TM does not need refrigeration after inoculation for shipment.
  - Anaerobic vials, jars with large mouth lids, and tubes are available for fluids, tissue samples, and swabs, respectively. Some manufacturers sell anaerobic culture tubes with screw top tubes with special injection septum for liquid sample introduction or for swab introduction. Anaerobic transport media is required for swabs, body fluids, small pieces of tissue for anaerobic bacterial isolation. Anaerobic culture can be performed on fresh tissue that is > 2–3 cm in diameter (where the center of the tissue has maintained anaerobic conditions). The samples should arrive to the laboratory within 24 h of collection.
  - Tied off loops of bowel can be submitted for anaerobic enteric culture where laboratory will culture contents/tissue for anaerobes.
  - Fresh tissues samples must arrive chilled or frozen within 24 h after animal's death whereas inoculated

anaerobic transport media must be kept at room temperature for shipping and handling and arrive within 24 h.
  - *Clostridium* toxin tests can be done on fresh feces, but toxin proteins are extremely heat-labile and samples should be frozen as soon as obtained and shipped frozen within 24 h of collection.
  - Proper inoculation and handling of the anaerobic TM before inoculation and during is required to maintain anaerobic conditions. Tubes should be stored upright and when inoculating so to prevent loss of gas cap (see https://ahdc.vet.cornell.edu/docs/Anaerobic_Culture-Inoculation_of_Anaerobic_Transport_Media.pdf).
  - Inoculated anaerobic transport media must be maintained at room temperature.
  - Botulism PCR testing is done for the presence of *Clostridium botulinum* genes in feed, intestinal tissue and feces. This testing is done at the National Botulism Reference laboratory at the University of Pennsylvania, School of Veterinary medicine (www.vet.upenn.edu/veterinary-hospitals/NBC-hospital/diagnostic-laboratories/national-botulism-reference-laboratory).
- Fungal sampling and transport media
  - Transport media used for suspect systemic fungal infections is the same as for bacterial cultures. Consultation with the lab prior to suspect fungal submission is suggested. The use of molecular testing (PCR) for fungal identification directly from the clinical sample requires special handling and bacterial transport media cannot be used.
  - Dermatophytes do not require specialized TM.
  - Skin scrapings, hair, and horn/hoof samples should be sent in dry containers/paper envelopes to prevent moisture condensation and overgrowth with contaminants.
  - Skin, corneal fluid, tissue samples/biopsies should be placed into sterile screw top transport vials with a drop of sterile saline, chilled, and shipped for arrival to lab within 24 h.
  - Systemic fungal infection swab samples can be transported in aerobic and anaerobic bacterial media (Port a cul™) or the previously discussed anaerobic containers.
  - Inoculated bacterial transport media with fungal samples should be shipped and handled as discussed in the bacterial section.
  - Swabs obtained from the cornea, uterus/endometrium, and other locations should be inoculated into aerobic or anaerobic transport media and shipped chilled or room temperature, respectively. Actual tissue sample is preferred for culture.
  - All samples must be shipped overnight and arrive chilled to the laboratory to prevent overgrowth by contaminants.
  - If both fungal and bacterial testing is to be done, two swabs should be obtained to assure adequate sample volume.

1.8.3 Molecular Testing

The development of molecular assays has increased the breadth of testing for infectious pathogens. Molecular diagnostic laboratories utilize various molecular diagnostic modalities, including nucleic acid amplification techniques, and sequencing technologies.

- Universal viral transport medium (liquid) is available from various manufacturers and is room temperature stable for viral transport, maintenance, and long-term freeze storage.
- All body fluids including whole blood, serum, CSF, respiratory fluid samples, urine, and feces are acceptable samples for viral testing.
- Viral isolation is still very important even with the advent of PCR. Isolation allows for vaccine development, anti-viral treatments, and identification of novel agents. However, viral isolation requires that the sample contains at least a moderate viral load in order to successfully grow virus.
- Bacterial transport media cannot be used for viral PCR testing.
- Dacron- or rayon-tipped swabs are preferred for PCR and viral testing.
- Freezing tissues and samples can preserve samples for later viral testing, however, repeated freeze thaw is not recommended.

1.8.4 Parasitology

The parasitology section provides identification of parasites by various methods. These include direct fecal smear examinations, fecal flotations, fresh and fixed tissue samples for parasite identification, whole parasite identification, serological, and molecular testing.

- Fecal floatation testing requires 1–2 normally formed fecal balls (approximately 10 g of feces) from an average horse for quantification. Samples should be sent in a clean anaerobic leak-proof containers/plastic bag. Samples should not be submitted in an exam glove or rectal sleeve.
- Fresh feces submitted for fecal floatation must not be exposed to temperature extremes. Eggs may rupture/hatch in sample and the sample may become nondiagnostic.

- The McMaster, Wisconsin, and other modified methods may be used to obtain approximate numbers of strongyle egg counts and are frequently performed in private veterinary clinics.
- Testing fecal samples for tapeworms, *Anaplocephala perfoliata*, by floatation is not a reliable test due to the intermittent shedding of eggs by the adult tapeworms. Serological testing has been developed but has not gained favor due to the inability to interpret positive results in horses that have been successfully treated for tapeworms, but still remain seropositive.
- Fecal sampling for floatation to assist in determining resistance patterns using fecal egg count reduction test (FECRT) should be obtained 10–14 days post administration of an anthelmintic.
- Fecal samples that cannot be tested soon after collection (within 7 days or less) may be placed into TM such as 10% formalin or polyvinyl alcohol to assist in preserving the ova and the delicate trophozoites forms seen with enteric protozoal infections.
- Fecal samples for larval parasite and identification (strongyle family) should be fresh, kept at room temperature, and contain large numbers of ova on fecal floatation (>100 epg) to insure adequate numbers of larval hatching for identification.
- Lungworms, *Dictyocaulus arnfieldi* can be diagnosed in fresh fecal samples, but requires active floatation techniques and special sugar solutions. Clinical signs or suspect disease should be provided to parasitology lab to allow proper techniques to be performed. Baermann testing is used for diagnosis of lungworms if eggs are not found in fecal samples that have been tested by active floatation methods.
- Pinworms are not routinely found in fecal floatations and the "cellophane tape test" can be used to assist with diagnosis of pinworms ova (cellophane tape is stuck to a clear glass side and examined microscopically).
- Enteric protozoal infections are not thought to be pathological in apparently normal equine adults and foals, but antigen (fecal) ELISA detection tests are readily available for *Giardia*, and cryptosporidium.
- EPM causative agents, *Neospora hughesi* and *Sarcocystis neurona* antibody levels can be detected in serum and CSF by IFAT and ELISA. IHC and PCR are available for detection of the organisms in neurological tissue, but may not be rewarding due to the focal localized areas of infection.
- Skin scrapings and entire/partial parasites submitted for identification must be submitted in a clean escape-proof container such as plain red top blood collection tube or transport vial with screw top lid. Isopropyl alcohol in a red top tube/leak-proof vial can be used to transport and preserve ticks, mites, and other parasites.
- Skin scrapings should be obtained after lightly scraping the affected area until small drops of fresh blood are seen.
- Tissue samples or fresh tissue biopsies for parasite evaluations such as *Oncochera* sp. can be submitted in clean leak-proof containers with a few drops of sterile saline to keep samples moist and prevent dessication of parasite.
- Skin parasites maybe "washed out" during histological sample processing; therefore, a fresh biopsy in addition to the fixed sample should be submitted in a transport vial that prevents desiccation for parasite evaluation.

1.8.5 Toxicology

Toxicology laboratories utilize various types of analytical equipment and instruments, techniques for the detection, identification, and quantification of organic, inorganic, and toxic compounds. Vitamins and mineral testing are often performed in these laboratories. The accurate diagnosis of a toxicosis like many other diseases is made by utilizing information made from criteria. Forensic and legal cases tested in toxicology have stringent requirements for sampling, identification, shipping, and handling. These should be reviewed prior to obtaining samples to prevent serious errors in sampling. Chain of custody may be necessary particularly in forensic cases and possible legal cases. This should be discussed with the laboratory and client that is requesting testing so that the samples are not compromised for use in legal cases.

Drug screens for regulatory, and pre-purchase drug screens have specific requirements such as (1) sample type (i.e., whole blood, urine), (2) blood tube collection types, including EDTA, heparin or serum, and (3) testing volumes. It is critical to follow the laboratory guidelines for testing and sampling since many of these drug screens are associated with legal repercussions and cannot be redrawn.

Ante-mortem samples may include whole blood (blood tube additives may vary on testing and should be discussed with toxicologist), serum, urine, hair, body fluid, reflux, and feces. If unable to contact toxicologist prior to testing whole blood, tubes with EDTA or heparin are generally acceptable. Certain drugs are protein-bound and necessary sampling tubes may vary with each compound; therefore, using both tubes would prevent errors on the part of the submitter.

Samples should be placed in individually identified containers such as plastic sealable bags, sterile urine sample cups or wrapped in aluminum foil for testing lipophilic toxins. Excess air should be removed from plastic bags. Samples should be frozen as soon as possible and kept frozen in a deep freezer (not frost free) until analyzed. Serum should be removed from the clot and frozen.

SST tubes are not appropriate for drug monitoring or toxicological analysis. The gel in SST extracts lipophilic substances which is most drugs; therefore, causing falsely low drug concentrations.

Testing plant materials and forage for possible toxicities should include part of the leaves, stems, flowers and roots. Forage samples should be kept cool and dry or even frozen. Photographs of suspect plants showing stems, roots, flowers, seeds, should be submitted along with plants if available.

Post mortem/necropsy cases should always include a complete "tox set" and be held frozen until needed for testing. This link describes the information and suggested samples for toxicological workups and drug screens (https://ahdc.vet.cornell.edu/docs/Toxicology_Submissions_and_Analytica_Screens.pdf). The "tox set" can be used if necessary after histopathology results are obtained or for use in ancillary testing. Tissue material from a necropsy should include brain, liver, kidney, fat, urine, aqueous humor or intact eyeball, skin (site of exposure), heart blood collected in lithium heparinized blood collection tubes, stomach, reflux, intestinal contents, and feces. Collect stomach, intestine, and feces last to prevent contamination of entire carcass. Each sample or tissue should be placed in individually identified container similar to the ante-mortem testing. Most toxicological samples should be frozen and stored in a non-frost proof freezer. Other samples to collect may include paint chips, soil, supplements, and feed, forages, water, and cohort blood and urine samples.

1.8.6 Virology

The virology diagnostic section provides testing for viral agent detection and monitoring in multiple species using viral isolation and serology as the mainstay of testing. The development of PCR and molecular testing has increased the breadth of testing, and this section now utilizes various diagnostic modalities including, nucleic acid amplification techniques, and sequencing technologies.

Fresh tissue samples, and body fluids and products in viral transport media are acceptable samples. Some viruses are unable to be cultured easily or even at all and PCR techniques are being used successfully with these viruses. Viral transport media may optimize viral isolation and can be used in PCR techniques.

- Viral isolation requires that sample has a high viral load in to grow virus. Low numbers of viruses in sample may cause false negatives.
- Multiple species tissue cell lines may be necessary to isolate viruses from various animal species.
- Viral isolation is still very important even with the advent of PCR. Isolation allows for vaccine development, anti-viral treatments, and identification of novel agents.
- Turnaround time with viral isolation may range from 3–30 days.
- Bacterial transport media cannot be used for PCR testing.
- Dacron or rayon flocked swabs are preferred for PCR and viral isolation.
- Acceptable samples for viral isolation or PCR includes nasal swabs, body fluids/discharges, and target tissue samples.
- Universal Transport Medium, is a room temperature stable viral transport media for collection, transport, maintenance, and freezer storage.

1.8.7 Immunology/Serology

This laboratory section is responsible for testing areas that include allergies, autoimmune diseases and presence of antibodies in serum or other body fluids such as CSF, peritoneal fluid, and aqueous humor. Testing includes various platforms such as serum neutralization (SN), hemagglutination inhibition (HI), complement fixation (CF), Western Blot, ELISA, flow cytometry, multiplex, indirect fluorescent antibody (IFA), agar gel immunodiffusion (AGID), microscopic agglutination, serum hemagglutination inhibition (SHI), and cytokines. Most serology tests use an antigen as a reagent to capture antibodies.

- Serum is the most common sample tested, but other body fluids that are validated can also be used such as peritoneal fluid, CSF, and so on.
- Serum should be obtained in a clot tube (SST or red top) and allowed to clot at room temperature and centrifuged. Serum should be removed and placed in a transport tube.
- Acute samples and convalescent sera should be submitted together for parallel testing. Convalescent sera should be drawn 10–21 days after illness depending on agent to be tested for.
- Leptospirosis MAT serum samples should be drawn approximately 10 days post beginning of suspected illness. Further, antimicrobial treatment may blunt leptospirosis antibody response.
- *Anaplasma phagocytophilum* IFA titers develop 5–7 days after infection with agent.
- Titers associated with *Borrelia burgdorferi*, EHV-1/-4, *S. neurona*, *N. hughesi* may produce lifelong antibodies and positive titers are not always associated with active illness.
- Vaccine titers do not correspond to disease-protective levels in animals.
- IgM is the first isotype to elevate after infection followed by IgG.

1.8.8 Anatomical Pathology

Surgical biopsies, post mortem gross examinations, and histology are the most frequently submitted cases. Histology is the most frequently requested test and supporting stains and tests assist in diagnosis.

- Formalin preservation of tissues or biopsy samples should be done as soon as possible to prevent autolysis. The minimum dilution of formalin to tissue should be 10:1.
- Small pieces of tissue are required to allow for fixation. Large pieces of solid tissue should be cut into pieces that are 0.5 cm thick to allow fixation of tissues.
- Formalin preserved tissue should not be allowed to freeze.
- Bouin's solution may be used for fixation of delicate tissues such as with ophthalmic, intestinal tissues and reproductive histological evaluation.

1.8.9 Endocrinology

This laboratory section tests reproductive and metabolic hormones, and vitamins in the horse. This includes progesterone, PMSG, testosterone, granulosa cell tumor testing, metabolic testing including ACTH, leptin, and thyroid tests.

- Serum is the preferred sample for the majority of equine tests except for ACTH testing.
- Serum should be removed after centrifugation from the blood collection tube after clot formation and placed into a plain red top tube or transport vial.
- Hemolyzed samples may adversely affect results.
- Blood collection tubes with activators, SST tubes and activators, or any additives are not acceptable for serum collection.
- Sera should be chilled/frozen after removal from clot and shipped to laboratory to arrive chilled.
- EDTA whole blood testing for Cushing's disease should be chilled immediately after collection and prior to centrifugation. Equine ACTH testing requires EDTA plasma that has been collected after gravitational separation should not be frozen, but chilled. Proteolytic enzymes that may be still in plasma may affect results and cause ACTH values to be inaccurate. Do not place EDTA whole blood too close to ice packs prior to plasma separation for the same reason. EDTA plasma must be frozen as soon as possible after removal from cells and placed in a plain plastic red top tube or plastic transport vial. EDTA plasma should not be placed back into EDTA tubes for transport to laboratory. If liquid additives have been used as the tube additive sample dilution may occur.

1.8.10 Coagulation

Vascular injury is the most common cause of hemorrhage in the horse, but there are various conditions in the horse that may cause hemostatic failure. Diagnostic testing can aide in this determination, but careful sampling techniques, proper collection and handling are necessary to obtain accurate meaningful results. If the animal is excited splenic contraction may occur and cause elevated blood cell counts and increased platelet counts, alcohol from the skin preparation, sedatives, and analgesics may also affect the results.

There are primary hemostatic (platelet plug tests) and secondary hemostatic and fibrinolysis assays (fibrin clot formation/coagulation) available to assist in the diagnosis.

- Primary hemostatic tests include platelet counts that can be obtained from a stained blood smear by examination of the feathered edge of a smear to detect platelet clumping. This can be done in a clinical pathology laboratory when a CBC is done.
- Routine EDTA tubes used for hemograms are acceptable for making blood smear for platelet evaluation. This requires a careful venipuncture (atraumatic and away from recent venipuncture sites) directly into an evacuated EDTA (purple cap tube), heparin (light green cap) or citrate (light blue cap) collection tube. Complete fill of the tube for the proper ratio for testing is required. After collection mix by inverting 8–10 times. The sample should remain at room temperature, and the smear prepared as soon as possible after collection.
- Secondary hemostatic and fibrinolytic assays are often done with POC units. Automated POC units are available in clinical settings for stall-side testing and require the same correct careful sampling handling as in primary testing. Sample must be collected into a citrate blood collection tube (light blue tube), allow complete autofill, and mix by inversion 8–10 times. Sample may be drawn through intravenous catheter, but sample must be obtained after the catheter has been flushed with 20 cc of sterile calcium free saline. Maintain citrate blood collection tube at room temperature until it is centrifuged. Following centrifugation, place the plasma into a plastic tube. Ship sample chilled or frozen to the laboratory. Hemolyzed samples are not acceptable.

Further Reading

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2

Basic Techniques and Procedures

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2.1 Introduction

A small fraction of microorganisms can be categorized as pathogens, or having the ability to incite infectious disease processes in a host. It has been shown that the term "pathogen" can be applied to very few microorganisms, even fewer if pathogenicity is defined as causing infectious disease at all times (Goldmann and Pier, 1993). Traditionally, accurate and prompt identification of pathogenic microorganisms in clinical samples has been a responsibility of microbiologists in a laboratory setting. The effectiveness of the microbiology laboratory depends on appropriate sample selection, collection, and transportation. So, the significance of microorganism detection in clinical samples needs to be discussed between microbiologists and clinicians with regard to maintaining sample quality as well as taking into consideration the roles of the host and environment.

Rapid, sensitive, and specific detection of pathogenic microorganisms is essential for the effective treatment of an infected host. Diagnostic methods in microbiology have a task to make microorganisms "visible" and "measurable," so the methods are either qualitative (presence/absence) or quantitative (absolute number of pathogens, colonies, plaques, or genes).

Until very recently, efforts to detect and identify microorganisms have depended on *in vitro* analysis, where bacteria are grown in culture. Based on Koch's postulates, a bacterium must be shown to grow outside the body in culture in order to prove that it causes disease. In the past, this criterion has posed a problem, as many bacteria are particular in their growth requirements. However, since at least a fraction of microorganisms is not so particular, these efforts have yielded an array of diverse microbial cultivation techniques. Microbial cultivation methods opened up an unsuspected world of microscopic life and presumed causative agents of infectious diseases (Relman, 1998). Over the years, researchers have pointed out two reasons why the majority of bacteria do not culture: (1) some bacteria only grow in specific conditions offered by the host including a very narrow pH, the right nutrient availability, and so on; and (2) certain bacteria only grow in the presence of certain other species of bacteria (Lamoth and Greub, 2010).

Current testing methods of detecting pathogenic microorganisms have to answer these three basic questions: (1) Is something there (qualitative testing)? (2) If there, how much is present (quantitative testing)? (3) If there, what is it (identification testing) (Moldenhauer, 2008)? Issues in detecting pathogenic microorganisms are as follow: *sensitivity* – detection volume and presence of interfering substances that reduce assay sensitivity; *specificity* – detection of the right organism, or group of organisms; *quantification* – precision and accuracy.

Commonly used methods of pathogen detection include: microscopy, cultivation, biochemical methods, bio-testing, immunological methods, and molecular methods (Murray et al., 1995a, Gracias and McKillip, 2004, Petti, 2007, Lazcka et al., 2007, Nayak et al., 2009). None of these methods are 100% efficient, so it is highly recommended to utilize more than one method, which depends on sample type, clinical symptoms, and suspected pathogen (Pickup, 1991). For bacterial detection, traditional microbiology has proved a time-consuming procedure. Organisms have to be isolated and grown, and usually a series of biochemical tests must be completed for identification (Kaspar and Tartera, 1990). Molecular methods are necessary if the traditional methods provide poor results. Techniques such as the polymerase chain reaction (PCR) used for the amplification of pathogen-specific DNA sequences have proved to be sensitive. However, when using environmental samples, a degree of sample preparation is required since impurities contained within the sample may inhibit the PCR. Furthermore, the

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use of small sample volumes means that the cells often has to be concentrated to obtain the desired sensitivity (Radstrom et al., 2004).

2.2 Essential Prerequisites for High-Quality Bacteria Detection

The effectiveness of the laboratory diagnostics depends on the appropriate sample selection, collection, and transportation. If sample collection and handling are not priorities for clinicians, the laboratory can do very little to advance patient care. It is the laboratory's responsibility to provide the necessary information, which should include specific criteria for safety, selection, collection, transportation, sample acceptability, and labeling.

During sample collection, the clinicians should abide by the *safety procedures* in order to protect themselves, the personnel handling the samples, and the sample integrity. All sample collection procedures must be performed while wearing gloves, laboratory coat, and, where appropriate, masks. All sample containers must be leak-proof within a sealable, leak-proof plastic bag. Leaking containers or syringes with needles must never be transported to the laboratory (OSHA, 2011, Gimenez-Marin et al., 2014, CLSI, 2011).

Sample(s) selection should be on the basis of signs and symptoms that represent the disease process, and should be collected before administration of antimicrobial agents. Some infectious diseases are distinctive enough to be identified clinically. Most pathogens, however, can cause a wide spectrum of overlapping clinical syndromes in animals. Conversely, a single clinical syndrome may result from infection with any one of many pathogens. The manifestations of an infection depend on many factors, including the site of acquisition or entry of the microorganism; organ or system tropisms of the microorganism; microbial virulence; the age, sex, and immunologic status of the patient; underlying diseases or conditions. The signs and symptoms of infection may be localized, or they may be systemic. Samples selected for microbiologic examination should reflect the disease process and be collected in sufficient quantity to allow complete microbiologic examination. For example, the number of microorganisms per milliliter of a body fluid or per gram of tissue is highly variable, ranging from less than 1 to 10^8 or 10^{10} colony-forming units (CFU). Swabs, although popular for sample collection, frequently yield too few cells for accurate microbiologic examination and should be used only to collect material from the skin and mucous membranes. Because skin and mucous membranes have a large and diverse indigenous flora, every effort must be made to minimize sample contamination during collection (Washington, 1996, Rabenau et al., 2010, Panel et al., 2010). If possible, samples should be collected before the administration of antibiotics. Above all, close communication between the clinician and the microbiologist is essential to ensure that appropriate samples are selected and collected and that they are appropriately examined.

Although sample processing is usually well standardized in the diagnostic laboratory, *preanalytic procedures* outside the laboratory usually follow with considerable variability. It has been shown that preanalytic errors make up to 85% of all laboratory errors, with 95% of them occurring outside the laboratory. For example, false negative results may occur due to sample degradation during inadequate transport. Additionally, contamination in the field/during sample collection may cause false-positive results, which could have severe consequences. The main issues of concern during sample transport and storage include sample integrity, contamination, sample identity, and the risk of environmental hazards due to infectious material (Endler and Slavka, 2010, Gimenez-Marin et al., 2014). Samples collected from potentially infected animals are considered hazardous, so the transport outside of the laboratory is subject to national and international regulations. All samples must be *promptly* transported to the diagnostic laboratory, preferably within 2 h. In general, samples for detection of bacterial agents should not be stored for more than 24 h, and no more than 2–3 days at 4 °C for viruses (Miller and Holmes, 1995).

Detection methods depend more and more on the quality of the *sample preparation*. Samples are very heterogeneous (stool, plasma, CSF), so preparation must be a factor to consider in order to achieve results within a reasonable amount of time. Thus, the increasing need for speed and precision in new detection methods illustrates the importance of sophisticated methods for sampling and sample preparation within the overall process. The proper development and adaptation of sample preparation toward the endpoint detection method applied is essential for exploiting the whole potential of the complete workflow of any diagnostic method. The overall goals of sample preparation are: (1) to concentrate the target bacteria, as bacteria may be at low concentrations and detection methods commonly use only small sample volumes; (2) to remove or reduce the effects of inhibitory substances, as sample matrices may be incompatible with the analytical methods; and (3) to reduce the heterogeneity of samples in order to ensure negligible variations between repeated sampling. If homogenized thoroughly, the pooling of samples will facilitate a high throughput analysis.

Quality assurance and *quality control* in the routine diagnostic laboratory must be taken into consideration. Unfortunately, quality assurance and quality control guidelines are not always properly developed or they are highly diverse between laboratories as only a limited number of standards and reference materials are available. Components that are required for validation of used microbiological diagnostic tests or test systems should include: (1) internal and external run control; (2) participation in inter-laboratory test results comparison; (3) validation of employee competence; (4) calibration and maintenance of instruments, and (5) correlation with clinical findings (Raggam et al., 2010, Madej et al., 2010).

2.3 Microbiological Methods for Pathogen Detection

When a new and promising microbiological diagnostic technique is developed, microbiologists quickly incorporate it into pathogen detection methods. For example, the advent of PCR as a DNA amplification technique led to the emergence of methods that rely on PCR for the detection of various pathogens. Although these methods are PCR-based, they normally incorporate other familiar techniques such as culturing and microscopic examination. Microscopy, culture, biochemical, biosensors, immunological, and molecular techniques are used in various pathogen detection methods. Most pathogen detection methods include enrichment (a culture technique) and enzyme-linked immunoassay (an immunological technique) or polymerase chain reaction (a genetic technique) (Yousef, 2008).

2.3.1 Microscopy

The first step in processing clinical material is microscopic examination of the specimen. Direct examination is a rapid, cost-effective aid to identify microorganisms and enumerate cells. Visible microorganisms may denote the presumptive etiological agent, guiding the laboratory in selecting appropriate isolation media and the clinician in selecting empirical antibiotic therapy.

Modern microscope instruments are designed to produce magnified visual or photographic images of objects too small to be seen with the naked eye. The microscope must accomplish three tasks: (1) produce a magnified image of the sample, (2) separate the details in the image, and (3) render the details visible to the human eye or camera. For microscopic examination, it is sufficient to have a compound binocular microscope equipped with achromatic objectives, wide-field oculars, a mechanical stage, a sub stage condenser, and a good light source. For examination of wet-mount preparations, a dark field condenser, or condenser and objectives for phase contrast, increases image contrast. An exciter barrier filter, dark field condenser, and ultraviolet light source are required for fluorescence microscopy. Microscope objectives are the most important components of an optical microscope because they determine the quality of the images that the microscope is capable of producing. There is a wide range of objective designs available that feature excellent optical performance and provide for the elimination of most optical aberrations. Standard bright-field objectives, corrected for varying degrees of optical aberration, are the most common and are useful for examining samples with traditional illumination techniques. Other, more complex, methods require specific objective configurations, which often include placement of a detector on or near the rear focal plane (Chapin, 1995, Drent, 2005, Abramowitz et al., 2002).

Microscopes can be separated into several different classes. One grouping is based on what interacts with the sample to generate the image, light or photons (optical microscopes), electrons (electron microscopes), or a probe (scanning probe microscopes). Alternatively, microscopes can be classed on whether they analyze the sample via a scanning point (confocal optical microscopes, scanning electron microscopes and scanning probe microscopes) or analyze the sample all at once (wide-field optical microscope and transmission electron microscopes). The most commonly used are *compound microscopes* that are light illuminated. The image seen with this type of microscope is two-dimensional (2D) enabling views of individual cells, even living ones. It has high magnification but low resolution (Conchello and Lichtman, 2005, Centonze Frohlich, 2008). *Fluorescence microscopy* is the most rapidly expanding microscopy technique employed today, both in the medical and biological sciences, a fact which has spurred the development of more sophisticated microscopes and numerous fluorescence accessories. Epifluorescence, or incident light fluorescence, has now become the method of choice in many applications (Michalet et al., 2003). A *dissection microscope* is light illuminated and the image appears in 3D. It is used for dissection utilizing a laser light. Laser light scans across the sample with the aid of scanning mirrors and then the image is placed on a computer screen (Foldes-Papp et al., 2003). The *digital microscope* uses the power of the computer to view objects not visible to the naked eye. The computer software allows the monitor to display the magnified sample. An advantage of digital microscopes is the ability to email images, as well as comfortably watch moving images for long periods. A *scanning electron microscope* uses electron illumination.

The image is seen in 3D and has high magnification and high resolution. The sample is coated in gold and the electrons bounce off to give the exterior view of the sample. The pictures are in black and white. A *transmission electron microscope* is electron illuminated and gives a 2D view. The electron beams pass through the thin slices of sample and have high magnification and high resolution (Grogger et al., 2000).

Microorganisms present in collected clinical or pathological samples are transparent and the best approach to distinguish them is to use dyes or biological stains. Differentially stained samples are the most helpful for presumptive identification of the majority of pathogens. The Gram stain and acid-fast stain are examples of differential stains. There are other miscellaneous stains such as periodic acid-Schiff, toluidine blue O, Giemsa, and Wright stains. In addition, the fluorescence stains aid in identification of microorganisms because of the specific attachment of the fluorochromes in the dyes to microorganism components.

2.3.2 Culture Techniques

Culturing in microbiology refers to the transfer of an organism from its ecological niche (e.g., organ, tissue, body fluid, exudate), transient vehicle (e.g., food), or storage medium (as in case of stock culture), into a growth-permitting laboratory medium. In many instances, isolating and culturing microorganisms either in artificial media or in a living host confirms the cause of an infection. Bacteria (including mycobacteria and mycoplasmas) and fungi are cultured in either liquid (broth) or on solid (agar) artificial media. The inoculated medium is then incubated at an optimum growth conditions and for a suitable length of time to allow cell multiplication, resulting in a culture of the organism.

Liquid media provide greater sensitivity for the isolation of small numbers of microorganisms; however, identification of mixed cultures growing in liquid media requires subculture onto solid media so that isolated colonies can be processed separately for identification. Growth in liquid media also cannot ordinarily be quantitated. Solid media, although somewhat less sensitive than liquid media, provide isolated colonies that can be quantified if necessary and identified. Some genera and species can be recognized on the basis of their colony morphologies. The laboratory medium could be non-selective, selective, or differential, depending on the goal of the culture technique.

Non-selective culturing relies on using growth-permitting non-selective media. "Enrichment," which is used extensively in pathogen detection methods, is a non-selective culture technique. Buffered peptone water, for example, is used to enrich environmental samples in *Salmonella* spp. (Thomason et al., 1977).

Selective media allow the growth of target bacteria, while inhibiting the growth of other microbial populations. For example, organisms that have the ability to utilize a given sugar are screened easily by making that particular sugar the only carbon source in the medium, allowing for the growth of the target microorganism only. Likewise, the selective inhibition of some types of microorganisms can be studied by adding certain dyes, antimicrobials, salts, or specific inhibitors that will affect the metabolism or enzymatic systems of the organisms. For example, media containing potassium tellurite, sodium azide or thallium acetate at different concentrations will inhibit the growth of all Gram-negative bacteria. Media supplemented with the antimicrobial penicillin or crystal violet inhibits the growth of Gram-positive bacteria. Tellurite agar is used to select for Gram-positive organisms, and nutrient agar supplemented with the antimicrobial penicillin can be used to select for the growth of Gram-negative organisms (Holt et al., 1994).

Screening is a culture technique used to distinguish target from non-target microorganisms. Laboratory media supplemented with differential agents are used in screening. These agents allow analysts to visually detect the target microorganisms in a microbial population. Acid producing bacteria, for example, are distinguished from non-acid producers when suitable pH indicators are included in carbohydrate-containing agar media (Holt et al., 1994, Zhou et al., 2011).

Selection and screening are executed simultaneously using selective-differential media. For example, xylose lysine desoxycholate agar is a selective-differential medium often used in culture-based methods for detection of *Salmonella* spp. This medium contains sodium desoxycholate that selects for *Enterobacteriaceae*, and phenol red, a pH indicator that reveals acid production by non-*Salmonella* isolates. When executed properly, selection and screening, done sequentially or simultaneously, enable analysts to isolate target microorganisms as pure cultures (Holt et al., 1994).

Cultures are generally incubated at 35–37 °C in an atmosphere consisting of air, air supplemented with carbon dioxide (3–10%), reduced oxygen (microaerophilic conditions), or no oxygen (anaerobic conditions), depending upon requirements of the microorganism. Since clinical samples from bacterial infections often contain aerobic, facultative anaerobic, and anaerobic bacteria, such samples are usually inoculated into a variety of general-purpose, differential, and selective media, which are then incubated under aerobic and anaerobic conditions. The duration of incubation of cultures also varies with the growth characteristics of the microorganism. Most aerobic and anaerobic bacteria will grow overnight, whereas some mycobacteria require as many as 6–8 weeks.

2.3.3 Biochemical Methods

Although classification based on genetic divergence highlights the evolutionary relationships of bacteria, classification based on the morphological and biochemical features of bacteria remains the most practical way to identify these organisms. A definitive identification scheme for bacteria was presented in *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1994). Bacteria are classified on the basis of many characteristics: cell shape, nature of multicellular aggregates, motility, formation of spores, and reaction to the Gram stain. Important in the identification of a genus and species of bacteria are biochemical tests, including the determination of the kinds of nutrients a cell can use, the products of its metabolism, the response to specific chemicals, and the presence of particular characteristic enzymes.

The methods available use a combination of tests to establish the enzymatic capabilities of a given bacterial isolate as well as the ability of the isolates to grow or survive the presence of certain inhibitors. Enzyme based test are designed to measure the presence of a single enzyme as well as a complete metabolic pathway. Examples of single enzyme tests are as follows: catalase test, coagulase test, pyrase test, hippurate hydrolysis test, oxidase test, indole test, Dnase test, *ortho*-Nitrophenyl-β-galactoside test, urease test, methylene red test, and Voges Proskauer test. In addition, establishing inhibitor profiles are useful in identification of isolated microorganisms (Holt et al., 1994, Murray et al., 1995b, Lennox and Ackerman, 1984).

The classification systems frequently used for alternative methods are based on how the technology works; for example, growth of microorganisms, viability of microorganisms, presence/absence of cellular components or artifacts, nucleic acid methods, traditional methods combined with computer-aided imaging (which might also be considered automation of an existing method), and combination methods. *Growth-based technologies* are based upon the measurement of biochemical or physiological parameters that reflect the growth of the microorganisms. Examples of these types of methods include: ATP bioluminescence, colorimetric detection of carbon dioxide production, and measurement of change in headspace pressure, impedance, and biochemical assays. *Viability-based technologies* do not require growth of microorganisms for detection. Differing methods are used to determine if the cell is viable, and if viable cells are detected, they can be enumerated. Examples of this type of technology include solid phase cytometry, flow fluorescence cytometry, and optical imaging with NADH detection (Moldenhauer, 2008).

2.3.4 Biosensors

Conventional methods are used despite their long turnover times because of their high selectivity and sensitivity. Biosensors are particularly attractive as a means to detect and identify pathogenic microorganisms due to their specificity and sensitivity, the potential to shorten the time span between sample uptake and results, and the competitive cost. Biosensors also allow the analysis of complex sample matrices (Lazcka et al., 2007, Love and Jones, 2008). To provide protection, that is, timely warning of the presence of a pathogen, environmental samples are often analyzed using biosensors. This presents an additional problem, in that other microorganisms will also be present within the sample. The detector needs to be able to discriminate the pathogen of interest from the background, and this can be achieved in a number of ways. These include: (1) detection of an increase in the number of particles, (2) detection of an increase in biological particles, (3) detection of pathogenic biological agents, or (4) the specific identification of a biological agent. The use of biosensors for sensitive specific detection of a pathogenic microorganism still remains a significant challenge, and success is often dictated by the nature of the detection element (the specific ligand) and the choice of target analyte (Ivnitski et al., 1999).

The basic biosensor framework includes a substrate such as silicon, glass or polymers such as polymethyl methacrylate, polydimethyl siloxane, and so on, coated with a conductive layer like polysilicon, silicon dioxide, silicon nitrite, metal like gold, and metal oxides. A suitable detection system includes specific capture molecules like antibodies, enzymes, DNA/RNA probes, and phage-derived biomolecular recognition probes. Highly sensitive sensors (e.g., thickness shear mode and immunosensor) can be fabricated using piezoelectric materials such as quartz crystal, potassium sodium tartrate, lithium niobate, and so on as a substrate, coupled with electromechanical detectors (Nayak et al., 2009). High sensitivity, ease of operation, high accuracy and wide detection capacity can be achieved with optical biosensors that utilize fiberoptics, optoelectronic components, complementary metal oxide semiconductors, and fluorescence/phosphorescence, reflectance, chemiluminescence, light scattering, or refractive index for the detection purpose (Lazcka et al., 2007, Velasco-Garcia, 2009).

The expeditious growth in the development of biosensors and the involvement of multidisciplinary research activities in this field has led to the immense application of this technology. Biosensors, as a method for detection of pathogenic microorganisms, have been applied in healthcare, detection of food and water-borne pathogens, in agriculture, and in defense.

2.3.5 Immunology-Based Methods

The field of immunology-based methods for bacteria detection provides very powerful analytical tools for a wide range of targets. Immunoassay technique is based on the interaction between an antigen and antibody, and the technology required for detecting or quantifying this interaction. There is a large variety of immunoassays, and these techniques are used broadly in many fields including toxicology and pathogen detection. Enzyme-linked immunosorbent assay (ELISA) (Herrmann, 1995) test is the most established technique nowadays as well as the source of inspiration for many biosensor applications. Another detection method based on immunological techniques is the fluorescent immunoassay. Both assays can be adapted to detect antigens that originate specifically from the targeted bacterial pathogen. Hence, these techniques are useful screening or identification tools in pathogen detection methods. Immunology-based methods require specific antibodies, which are among the most important molecules, with limitless applications in the field of biology, microbiology, medicine, and agriculture. For example, the most rapid diagnostic tests used in food or clinical laboratories are based on antigen-antibody reactions (Banada and Bhunia, 2008).

The antibody's ability to recognize and bind with high affinity to specific antigenic sites (epitopes), even in a complex mixture, is exploited for qualitative and quantitative measurement of the antigens. Thus, antibody application is broad – it is not only used for detection and classification of the antigens, but also for understanding the microheterogeneity among proteins resulting from recombinant or somatic mutations. The production and selection of a suitable antibody is imperative for the successful design of an immunoassay, which depends on the assay parameters: the choice of a polyclonal or monoclonal antibody; of purified or native sera; of fragmented, bispecific, or fusion proteins; and the relative cost (Herrmann, 1995).

Additionally, immunoassays can utilize immunomagnetic separation (Perez et al., 1998), a pre-treatment and/or pre-concentration step, that can be used to capture and extract the targeted pathogen from the bacterial suspension by introduction of antibody coated magnetic beads (Gu et al., 2006). Immunomagnetic separation can then be combined with almost any detection method; for example, optical, magnetic force microscopy, magnetoresistance (Baselt et al., 1998) and hall effect (Lazcka et al., 2007). Custom derivative magnetic beads are available from a number of companies. Beads of widely ranging sizes (from a few nanometers up to a few tens of microns) may be chosen depending on the application. While large beads may be used for the measurement of intermolecular forces, smaller particles are best for the detection of small analytes where high sensitivity is critical. In the case of whole bacteria, the use of beads in the low micrometer range may provide the right balance between time and sensitivity.

2.3.6 Molecular Detection and Identification of Microorganisms

The ultimate goal in microbial testing is to accurately and sensitively detect pathogens in real-time or as quickly as possible. Molecular diagnostics offer many advantages over traditional microbiological and immunological methods for the detection of pathogenic microorganisms. These include faster processing time as well as greater potential for intra-species identification and identification of antibiotic susceptibility and strain typing. Molecular diagnostics is revolutionizing the clinical management of infectious disease in a wide range of areas, including pathogen detection, evaluation of emerging novel infections, surveillance, early detection of bio threatening agents, and antimicrobial resistance profiling (Yang and Rothman, 2004).

Polymerase chain reaction is a molecular technique for in vitro amplification of a DNA fragment via enzymatic replication. Products of PCR amplification (amplicons) are separated on agarose gel, stained, and the resulting fluorescent DNA bands are detected. The original techniques of PCR are being superseded by real-time PCR technique that allows detection of PCR amplification products while they are formed (Heid et al., 1996). PCR is an extremely powerful, rapid method for diagnosis of microbial infections and genetic diseases, as well as for detecting microorganisms in environmental and food samples (Radstrom et al., 2004). PCR has advantages over conventional laboratory practices as it offers rapid and accurate detection of infectious agents, which is a crucial for the timely administration of appropriate treatments. PCR is particularly useful for the identification of organisms that cannot be cultured, or where culturing conditions are insensitive or require prolonged incubation times. Thus, PCR has opened up new possibilities for the detection of slow-growing pathogens, intracellular bacteria as well as viable, but non-cultivable, pathogens (Tenover et al., 1999, Traore et al., 2006, Glynn, 2008).

Real-time PCR monitors the accumulation of PCR product in a reaction while it is taking place, compared to endpoint detection of the PCR product in conventional PCR. These technologies provide quick, sensitive, quantitative detection of PCR products in a closed-tube format, thereby significantly reducing the risk of contamination (Csordas et al., 2004, Raoult et al., 2004).

Fluorescent technologies employed are either nonspecific, using dyes such as SYBR Green I or SYBR Gold, which are minor groove-binding dyes and intercalate into the PCR product during amplification; or specific, using probes to detect specific sequence amplification in the PCR. A number of different fluorescent probe chemistries have been employed in real-time PCR assays, including hydrolysis probes (TaqMan, Beacons) and hybridization probes (FRET). While the mechanism of fluorescent signal generation is different for each of the probe chemistries, the fluorescent signal generated by the probes or minor groove-binding dyes is directly proportional to the amount of PCR product generated (Bustin, 2002, McKillip and Drake, 2004). Real-time PCR is quantitative, with a broader dynamic range than conventional PCR.

Clearly the method with the least risks of variability for genotypic identification would be *sequencing* the entire chromosome or a gene of the unknown organism and comparing the sequence to others in an identification database. The sequences are generated by a modification of the polymerase chain reaction, and the relatedness of the derived sequence to others in the proprietary database is determined as the basis for the identification. This technology has several advantages. It can be used to identify filamentous fungi, bacteria, and yeast. It can also be used to identify slow-growing organisms, or even those that cannot be cultured. The major disadvantage of the system is the high costs associated with it in terms of dedicated facilities, personnel training, time, and consumables.

2.4 Challenges to Current Detection Methods

Results of conventional detection methods are not very amenable to quantitative interpretations. Even the most practiced culture methods are not as quantitative as some analysts may have anticipated. Therefore, the accuracy required for microbiological criteria or specifications are not attainable by conventional detection methods. Real-time PCR is highly reproducible and allows for the *quantification* of microorganisms or physiological changes in gene expression. The dynamic range of the performed calibration curve can be up to nine orders of magnitude from $<10^1$ to $>10^{10}$ starting molecules, depending on the applied standard material.

Bacterial cell density differs from sample to sample, and from animal to animal, so the reliability of the used method depends on its *detection limit* in a quantitative sense.

This means a method with a detection limit of a single bacterium per certain amount of heterogeneous organic matter is likely acceptable. Some methods have smaller detection limits, which results in inability of detecting or quantifying small bacterial populations without cell propagation steps. Amplification of the bacterial cell population, or any cellular components targeted by the analysis, is a prerequisite for successful detection. Enrichment has been used reliably to augment the pathogen's population, but this technique is the most time-consuming step in the analysis (Pusterla et al., 2010).

Important questions when choosing a microbiologic detection method are: *which traits to analyze* and *how many tests are needed for identifying a bacterial pathogen?* Being prokaryotic single-celled organisms, bacteria have a simple morphology, which cannot be used as a basis for their classification or identification. However, microbiologists should carefully consider these morphological characteristics before they develop a battery of identification tests. Sometimes it is impossible to identify a bacterium reliably on the basis of a single test (Murray et al., 1995b, Holt et al., 1994). Serological tests are used extensively in pathogen identification, and some immunoassay techniques have been automated. It is difficult, however, to correlate serological properties of an isolate with its genotypic or other phenotypic traits. Therefore, it is unreliable to use only serological techniques to identify a causative agent or track diseases. Genetic techniques were introduced in pathogen detection methods by targeting characteristic sequences in bacterial genomes. There is no doubt that molecular techniques are valuable in modern detection methods, but several problems remain, such as inhibition of PCR or determination of viable and nonviable targets. It is generally agreed that the most acceptable approach for identifying an isolate is to integrate all available phenotypic and genotypic traits. Assembling and assimilating all data on an isolate's diverse traits should aid the analyst in making a sound judgment about its identity.

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3

Point-of-Care Testing

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3.1 Introduction

Point-of-care (POC) testing is the analysis of clinical specimens as close to the patient as possible. The testing may be stall-side or in the immediate vicinity at a nursing station. As computers and machines become smaller and more portable, nearly all testing could potentially be considered point-of-care. The readily available "hand-held" tests that equine veterinarians can perform stall-side are included next.

3.2 Advantages of POC Testing

Equine veterinarians with POC testing capabilities will have a unique advantage in rapid diagnosis, prognosis and treatment. It is important to determine which testing is cost effective for a given practice setting. POC analyzers can be separated into multi-test analyzers (MTA) versus single-test analyzers (STA). MTAs are a single machine that can be used to measure a variety of parameters, but are typically more expensive than STAs. STAs tend to be inexpensive and often easy to use. Ambulatory practitioners may find it more practical to own one MTA as opposed to multiple STAs.

3.3 Considerations for POC Testing

- Cost of the analyzer
- Cost per test
- Number of anticipated tests per year
- Ease of testing
  - If a given test is very challenging to perform, this will limit the use and increase the testing time.
  - If failure rate is high, then the additional costs associated with the extra time or supplies must be included.

3.4 Specific POC Tests to Consider

3.4.1 Blood Ammonia

Ammonia measured in the blood comes primarily from the gastrointestinal tract, however, it can also be produced in muscle and kidney. Ammonia is produced during exercise both from deamination of AMP, as well as catabolism of branched-chain amino acids. The deamination of AMP rises rapidly with increasingly intense exercise and is the primary route of ammonia production during high intensity work. Ammonia can be cleared by a variety of organs but liver and muscle are the major routes.

The measurement of ammonia is relevant in the fields of emergency and critical care, internal medicine, and sports medicine. Critical patients with gastrointestinal ileus, equine coronavirus infection, and hepatic failure can all experience life-threatening hyperammonemia (Hasel et al., 1999, McGorum et al., 1999). Sports medicine clinicians measure blood ammonia levels to help determine level of fitness and response to training (Lindner et al., 2006).

POC testing of ammonia allows immediate measurement after sample collection which negates the need for sample processing and freezing if measurement will be delayed. STA machine can measure blood ammonia concentration within minutes (PocketChem BA, Woodley Equipment Company, UK).

3.4.2 Creatinine

Creatinine is produced from the breakdown of creatine and creatine phosphate. Creatinine is freely filtered in the glomerulus but may undergo active secretion in horses (Bickhardt et al., 1996). The creatinine concentration is inversely related to GFR and is often used as an indirect measure of renal function. Even small increases

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(as little as 0.3 mg/dl) in creatinine in a normally hydrated person may indicate the presence of acute kidney injury (AKI) and similar changes in horses should be monitored carefully (Kellum and Lameire, 2013). Increased creatinine concentration can also be seen with post-renal problems including rupture of the urinary system (ureters, bladder, and urethra) or obstruction of the urinary system. In foals, creatinine can be significantly elevated at birth but may be related to placental dysfunction. In newborn foals with normal renal function, increased creatinine values typically return to normal within 48–72 h (Chaney et al., 2010). Creatinine is most commonly measured along with other parameters on a variety of MTAs.

Uses in equine practice: The measurement of creatinine is relevant in the fields of general equine practice, emergency and critical care, internal medicine and anesthesia. The POC measurement of creatinine is useful for field practitioners as many medications used in equine practice have some degree of nephrotoxic potential (NSAIDs, aminoglycosides, tetracycline, etc.). Ideally renal function should be tested before starting treatment with nephrotoxic medications, and during the course of therapy.

3.4.3 Electrolytes

Sodium (Na^+), potassium (K^+), and chloride (Cl^-) are the most commonly measured electrolytes. The measurement of the ionized forms of calcium (Ca^{++}) and magnesium (Mg^{++}) has applications in equine practice as well.

Sodium is the predominant extracellular cation. The sodium concentration represents the balance between the total amount of sodium in the body (and indirectly potassium as well) and the total amount of water. Determination of the sodium concentration is important for evaluating fluid balance and managing renal failure cases.

Potassium is the predominant intracellular cation. Increased extracellular concentrations of potassium can be life-threatening. Hyperkalemia is frequently seen with renal failure and hyperkalemic periodic paralysis (HYPP). Hypokalemia is commonly encountered in anorexic horses receiving large volumes of intravenous fluids containing dextrose.

Chloride is the predominant extracellular anion. The chloride concentration frequently changes in conjunction with the sodium concentration. Chloride has a significant role in acid-base balance particularly when the difference between the sodium and chloride concentration changes dramatically. Treatment of acid-base disturbances without knowledge of the chloride concentration can be challenging.

Most analyzers determine a group of electrolyte concentrations which typically include a minimum of sodium, potassium, and chloride. Calcium, blood gases, and other acid-base variables are often included in combination with the basic electrolytes.

The measurement of electrolytes is relevant in the fields of general equine practice, emergency and critical care, internal medicine, and anesthesia.

3.4.4 Blood Gases/Acid-Base Parameters

Arterial blood gases are used to evaluate the respiratory system, as well as acid-base balance. The arterial partial pressure of oxygen (PaO_2) generally represents lung function and more specifically the ability of the lungs to transfer oxygen from inhaled air into the arterial blood. The arterial partial pressure of carbon dioxide ($PaCO_2$) generally represents the adequacy of ventilation. The measurement of pH and bicarbonate (HCO_3^-) concentration is important for understanding and treating acid-base disorders.

Analyzers that measure blood gas or acid/base variables will commonly measure these parameters as a group and are often combined with electrolytes and measured on MTAs. The measurement of blood gases/acid-base parameters is relevant in the fields of anesthesia, critical care, and internal medicine. Blood gas measurement is not typically performed in a field situation in general equine practice.

3.4.5 Lactate

L-Lactate is produced during anaerobic metabolism and primarily cleared by the liver and kidneys. Increased concentrations of lactate are commonly associated with increased production or decreased clearance. Lactate has been shown to have prognostic value in a variety of equine conditions and is commonly measured in emergency situations on admission, as well as for serial monitoring during treatment (Hashimoto-Hill et al., 2011, Tennent Brown et al., 2010).

The measurement of L-lactate is relevant in the fields of emergency and critical care, internal medicine, and sports medicine. Lactate determination can be performed on both STAs and MTAs. The low analyzer cost for lactate testing combined with a low per test cost makes it ideal even for ambulatory practitioners that have a moderate emergency caseload.

3.4.6 Cardiac Troponin I

Cardiac troponin I (cTnI) is a specific marker of myocardial damage. It can be increased in a variety of circumstances

including heart failure, toxic injury to the myocardium, or myocardial injury secondary to systemic inflammation/disease (Díaz et al., 2014, Davis et al., 2013). Uses in equine practice are generally restricted to emergency and critical care or internal medicine cases. Testing for cTnI is currently available for POC testing on an MTA (i-STAT Handheld analyzer, Abaxis, Union City, CA).

3.4.7 Glucose

Blood glucose monitoring has many applications in equine practice, including in the management of sick neonatal foals and horses with insulin resistance. It also has prognostic value in critically ill horses (Johnson et al., 2012, Hassel et al., 2009, Hollis et al., 2008). The measurement of glucose is relevant in the fields of emergency and critical care, internal medicine, anesthesia, and general equine practice. There are numerous analyzers (both STA and MTA) that are available, but many STAs are inexpensive. The cost per test is extremely low making this another ideal entry level POC diagnostic test.

3.4.8 Clotting Times (PT and PTT)

The coagulation system is complex and closely linked to the inflammatory cascade. PT, or prothrombin time, was originally considered to evaluate extrinsic clotting cascade. PTT, or partial thromboplastin time, was originally considered to evaluate intrinsic clotting cascade. PT and PTT are typically measured in critically ill horses, cases of unexplained bleeding, or prior to a surgical/invasive procedure. Uses in equine practice are generally restricted in the fields of emergency and critical care, internal medicine, and surgery. The rapid determination of PT and PTT are an essential test for most equine ICUs

3.4.9 Serum Amyloid A

Serum Amyloid A (SAA) is a positive major acute phase protein that increases with inflammatory diseases. In equine practice, SAA testing is often used as a screening tool to identify occult or early infection that may not be otherwise evident from the physical examination or other blood testing. SAA can also be used to monitor the response to treatment (i.e., antimicrobials, post-surgery) (Daniel et al., 2015, Belgrave et al., 2013). The testing of SAA has only recently become available for POC testing and is measured on an STA. The measurement of SAA is relevant in the fields of internal medicine, emergency and critical care, and general equine practice.

3.4.10 Triglycerides

Triglycerides are a type of lipid that circulates in the blood. Measurement is particularly important in ponies and miniature horses that are in a negative energy balance, especially during lactation and pregnancy (McKenzie, 2011). If these animals become anorexic, they can develop hyperlipidemia or hyperlipemia with significant morbidity and mortality in severe cases. Even in full-sized breeds of horses, triglyceride concentrations can increase with anorexia and disease. Hypertriglyceridemia may be present with pars pituitary intermedia dysfunction (PPID) or equine metabolic syndrome. The measurement of triglycerides is relevant in the fields of internal medicine, emergency and critical care, and general equine practice.

3.5 Conclusion

The addition of POC testing is likely to raise the level of care that an equine veterinarian can provide. It is important to evaluate the costs and benefits of this type of investment. In the author's experience, many veterinarians enthusiastically enter the world of equine POC testing only to be plagued by expired cartridges and a poor return on investment.

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4

Test Performance

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4.1 Introduction

Diagnostic test performance is characterized by its diagnostic accuracy, which relates to the ability of a test to discriminate between the anticipated target condition and health. Measures of diagnostic accuracy are determined in validation experiments and are quantitatively described by a test's sensitivity and specificity, predictive values, area under a receiver-operating-characteristic (ROC) curve, Youden's index, and diagnostic odds and likelihood ratios.

The validation phase itself can be split into an analytical and clinical part; the analytical validation determines particular test characteristics such as precision, reproducibility, spiking, recovery and linearity checks, and analytical limit of detection (Box 4.1). The clinical validation is run with defined samples from clinically affected or healthy individuals and aims to fully answer the discriminative power of the test between the target condition and health by defining diagnostic sensitivity and specificity, predictive values, ROC curves, likelihood ratios, and Youden's index (Box 4.2).

4.2 Analytical Validation

Analytical performance of a diagnostic test requires the use of the target analyte in a stable sample matrix environment in order to determine the quality of the diagnostic test itself. The stability of the target analyte by itself and mixed into the sample matrix are important variables to determine and are somewhat different depending on the diagnostic test use.

4.3 Minimum Information for Diagnostic Tests

Every new diagnostic entering a validation protocol should be accompanied by a diagnostic test manual that includes some basic information: intended use of the test (disease diagnostics, monitoring, screening, wellness testing); description of the analytical test principle; specification of instrumentation and equipment; reagent and disposable list; standard operating procedure (SOP); sample type requirements; description and definition of calibrators and control material; safety procedures; waste management; time to result calculations; and approximate costs.

4.4 Precision

Precision of a test measures the closeness of a series of repeat measurements of the same material and expressed as a coefficient of variation (CV) in percent. CV is calculated using the standard deviation (SD) and mean (X_{mean}) of all measurements in the formula $CV = (SD \times 100\%)/X_{mean}$. Precision should be analyzed over the intended dynamic range of the diagnostic test using a dilution series of the target analyte in the stable sample matrix. For virtually all diagnostic procedures, precision varies with target analyte concentration: CVs are usually higher at the end of the standard curve with very low target analyte concentrations and have to be defined to determine acceptance of the test.

Interpretation of Equine Laboratory Diagnostics, First Edition. Edited by Nicola Pusterla and Jill Higgins.

Box 4.1 Analytical validation.

Minimum Information for diagnostic tests
Precision (within-run and between-run)
Accuracy
Spiking recovery and linearity checks
Control material
Method comparison
Analytical sensitivity and specificity
Additional analytical performance tests

Box 4.2 Clinical validation.

Overlap tests
Sample selection process for clinical validation
Diagnostic sensitivity and specificity
Predictive values
ROC curve
Likelihood ratios

Within-run precision can be assessed in different ways. A practical approach is to use a representative number of patient specimens containing different target analyte concentrations and analyze in duplicate. Then, the determined quantitative target analyte concentrations are grouped into three groups (low, medium, high concentration), SD and CVs calculated for each group, and statistically tested for significant differences using modified F-tests. Results can be graphed as a precision profile by plotting CVs (on the *y*-axis) versus analyte concentration (*x*-axis).

4.5 Accuracy

An alternative term for accuracy is bias or systematic error and is defined as agreement between the mean of repeat measurements on the same sample against the true value. Because naturally occurring samples with known concentrations are difficult to obtain, an appropriate sample matrix is normally spiked with known concentrations of the target analyte. Alternatively, accuracy can be determined by comparing a new diagnostic test to an existing test. In that case, additional parameters such as constant systematic error (consistent differences between the two methods indicating method dependent error) and proportional systematic errors (inconsistent differences between the two methods, related to additional variables than the test methods themselves) can be determined.

4.6 Spiking Recovery and Linearity Checks

Spiking recovery experiments can be carried out in different ways. One method requires the existence of the target analyte in pure form and known concentration, to be spiked into the sample matrix with known concentration of the target analyte. The difference between those concentrations gives information about how much of the analyte can be recovered in the spiked sample. Recovery is normally expressed as percentage of recovery compared to the known concentration and should be around 100%, with acceptance criteria to be defined specifically for each diagnostic test.

A different test characteristic is assessed by using spiked sample matrix diluted in sample matrix without presence of measurable target analyte (zero calibrator). It is important to use a sample matrix that is identical or as close as possible to the sample type intended to be used for the diagnostic test as a zero calibrator to prevent a bias resulting in a proportional systemic error. The difference between expected and measured analyte concentration is then investigated in a linear regression analysis. The confidence interval of the slope should be close to 1, indicating 100% recovery. It is also important to test for linearity by using a Runs-test, also called Wald–Wolfowitz test. This is a non-parametric statistical test that checks for randomness in a two-valued data set.

4.7 Control Material

Defined and stable control material is produced in order to test multiple lab locations for accuracy in tests called external quality assurance, ring trials or proficiency testing. The control material, coded with different concentrations of analyte mixed with zero calibrator samples are tested in replicates and analyzed using a Wilcoxon Signed Rank test, depending on sample distribution. Means and or medians are compared to the expected concentrations of the target analyte to determine the bias of the analytical method, expressed in percentages.

4.8 Method Comparison

The systemic error is assessed by analyzing the same sample set with the new diagnostic test and comparing the results to a gold standard test. The goal is to prove that the new diagnostic test compares to the gold standard test within the inherent precision ranges of both methods, based on acceptance criteria.

4.9 Analytical Sensitivity and Specificity

Analytical sensitivity and specificity are distinctly different terms than diagnostic or clinical sensitivity and specificity and produce confusion in the diagnostic use of laboratory test results.

Analytical sensitivity describes the limit of detection of a particular diagnostic test at the molecule level, meaning what the smallest amount of target analyte is which can be measured in the appropriate sample matrix in a reliable fashion. For DNA testing, for example, it is important to determine how many nucleic acid equivalents can be detected within a single PCR reaction. For that purpose, dilution series of known concentrations of target DNA are analyzed in replicates. If very low analytical sensitivity has to be obtained for a particular test, a larger number of replicates are necessary to obtain single digit analytical sensitivity, due to the randomness of molecular distribution within a highly dilute sample.

Specificity at the analytical level refers to the ability of a diagnostic test to measure the particular target analyte and not a closely related analyte. Again, for molecular diagnostic tests, this is confirmed by sequencing the PCR product using outside primers in order to confirm the recovered nucleotide sequence is identical to the targeted nucleic acid sequence of the PCR test.

Diagnostic sensitivity and specificity will be further specified in the next section.

4.10 Additional Analytical Performance Tests

Parameters associated with the sample matrix which influence the test characteristics have to be analyzed before a test enters the clinical validation phase. For example, high contents of lipids, bilirubin, hemoglobin, and glucose in blood samples, mucus on swabs or nasal washes, soil or litter contaminants in fecal material can all influence the analytical behavior of a diagnostic test, and influence diagnostic performance indices. Depending on the target analyte it may be useful to examine the effects of these substances in detail. To that effect, many of these interfering substances can be obtained in pure form, such as unconjugated bilirubin and glucose, hemoglobin, lipids, and so on. For molecular tests, the influence of PCR inhibition is of particular importance. Humic acid for example, a well-known soil substance to cause complete inhibition of the DNA polymerase enzyme, can be purchased in pure form. It can be used to (1) test the nucleic acid extraction method and its ability to remove humic acid and (2) test the reverse transcription reagents and the PCR mastermix for its susceptibility to humic acid inhibition. In order to test for absence of inhibition, molecular diagnostic laboratories are using specialized and dedicated quality controls such as a spike-in Inhibition Positive Control (IPC), which is analyzed with a specific PCR test: by comparing the known concentration with the recovered concentration of the IPC analyte, the inhibitory component in the nucleic acid eluate can be assessed. Particular acceptance criteria have to be worked out for different sample types and diagnostic tests.

Other than sample matrix components, there are additional variables which have to be assessed separately, such as medication and interfering diseases. The presence of excess amounts of antibodies in the blood stream has the potential to complex out a target antigen and render it undetectable. In such instances, protocols have to be validated to release the target protein from its masking antibody.

Related to sample matrix conditions are considerations of collection protocols, storage containers, handling and shipping conditions, type of anticoagulants, venous site of blood sampling, and so on. These factors can affect test performance significantly and have to be considered when random test result deviations occur.

4.11 Clinical Validation

Once analytical variables of a diagnostic test procedures are defined, clinical sample analysis becomes the focus of the validation. In order to prevent a bias in the determination of the clinical usefulness of a new procedure, a blind, prospective validation adhering to certain criteria should be utilized.

4.12 Study Design in the Clinical Validation Phase

Definition of the appropriate clinical target population is instrumental in the determination of the clinical utility of a diagnostic test. For example, patients with illnesses on the list of differential diagnosis should be included into the test sample set. The total number of test subjects in general should be in the 50–100 range. Patient selection and proper definition of inclusion criteria are essential for this clinical validation phase and depend on the