LABOKLIN
LABORATORY FOR CLINICAL DIAGNOSTICS

The Experts for Equine Diagnostics

Your Colleagues from LABOKLIN

Information on equine patients

For veterinarians

2018 / 2019

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Haematology extra light for the equine practice

In general:

A detailed anamnesis is also of great importance for haematological evaluation. Starting with breed, age and sex, to the use, husbandry and feeding up to the actual symptoms, everything should be checked.

Not all horses are alike:

In order to interpret the haematological parameters, it is useful to subdivide them into thoroughbred breeds (including e. g. Appaloosa, Quarter Horse) and cold-blooded breeds (including ponies, donkeys), as the reference values differ. Thoroughbreds tend to have higher concentrations of erythrocytes, haematocrit, haemoglobin, leukocytes and thrombocytes than cold-blooded animals. At Laboklin, we indicate the reference values of warm-blooded animals in our findings.
Furthermore, the age of the animal should be taken into account when interpreting the results. For example, young horses have higher total lymphocyte counts in the peripheral blood.

Red blood count:

Erythrocytes are responsible for oxygen transport and have a lifespan of about 140 – 150 days in horses. Physiologically, they show an increased affinity towards rouleaux formation which must be differentiated from “true” agglutination. Different diseases associated with hyperproteinaemia or high concentrations of plasma proteins (fibrinogen, immunoglobulins) reinforce this physiological effect.
In equidae, the concentration of circulating erythrocytes in the peripheral blood strongly depends on the filling level of the spleen (storage-type spleen). Due to excitement (stress, blood withdrawal, handling), the spleen can release a large quantity of blood cells into the peripheral blood and can thus possibly mask anaemia.

Erythropoiesis:

In contrast to most other species, erythropoiesis in horses solely takes place in the bone marrow. Reticulocytes are generally not released into the peripheral blood, which makes it much more difficult to classify the anaemia into regenerative and aregenerative in this species. To be able to distinguish them, it is therefore only possible to monitor the course of the disease, focusing on an increase of the red blood count levels, or to carry out more invasive examinations such as bone marrow cytology.
Another particularity is the duration of the bone marrow response (regeneration) in horses. Erythropoiesis starts approximately 4 days after the onset of anaemia and complete regeneration can take between 1 – 3 months depending on the severity of the anaemia as well as the cause.

Anaemia:

Anaemia is defined as a reduction in erythrocyte count, haematocrit and haemoglobin concentration.
Depending on the cause, clinical symptoms include pale mucous membranes, loss of performance, weakness, lethargy, tachycardia, tachypnoea, possibly fever, bleeding, icterus, haematuria and melena.
A distinction can be made between relative anaemia (due to increased plasma volume during pregnancy, intravenous infusion) and true anaemia.
Reasons for true anaemia in horses are summarised in Figure 1.

Anaemia diagnostics:

The medical diagnosis is essential for therapy and prognosis.
A good diagnosis includes a complete blood count (erythrocyte count, haematocrit, haemoglobin concentration, erythrocyte indices (MCV, MCH, MCHC), leukocyte count

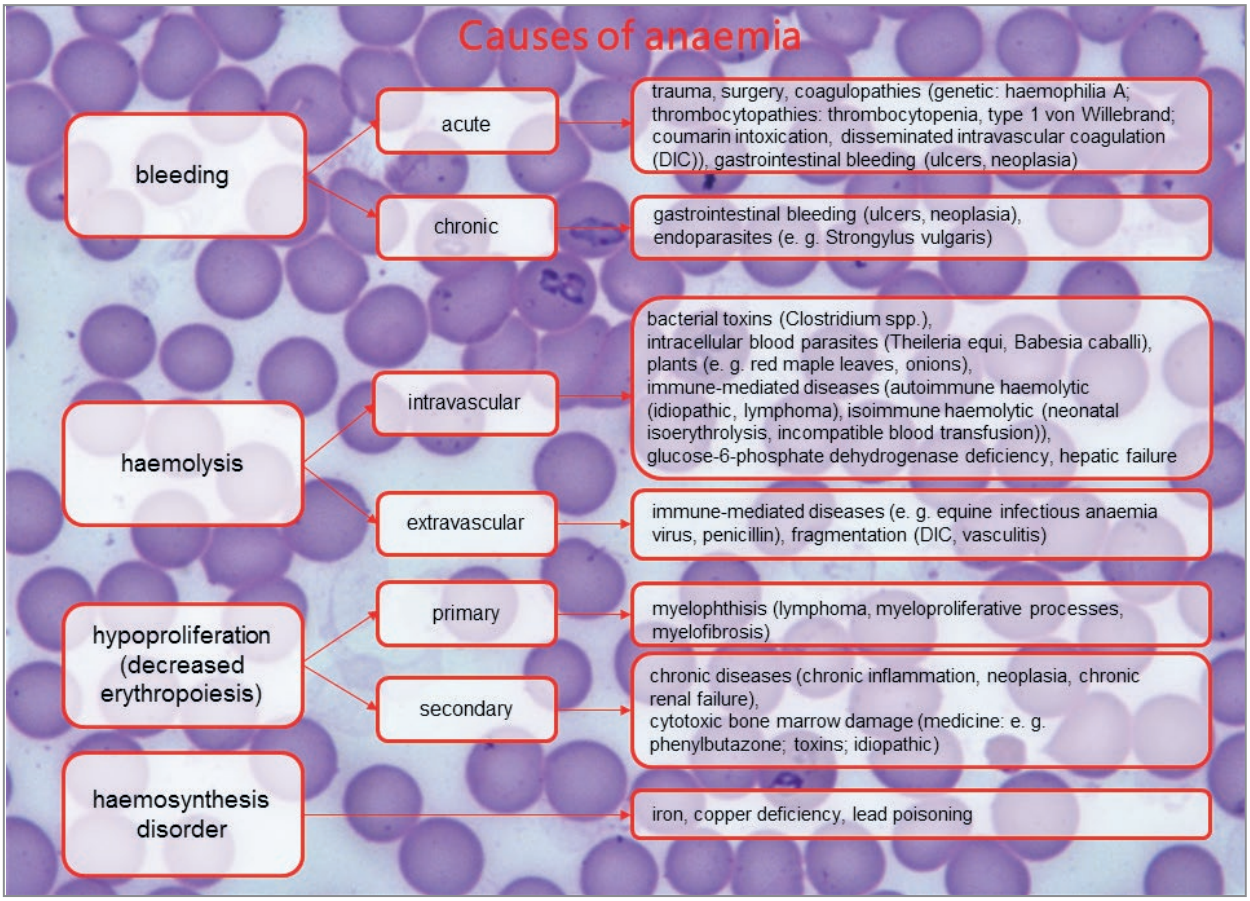

Fig.1 Causes of anaemia in horses

including differential blood count, platelet count), cytology of a fresh blood smear, clinical chemistry (with total protein and bilirubin) as well as urinalysis.
Depending on the clinical appearance and the preliminary report/cause (Fig. 1), further tests should be initiated, such as faecal analysis, faecal occult blood, Coombs test, coagulation tests, Coggins test, iron, bone marrow examination (of sternum or iliac crest) or blood group determination (can be ordered from Laboklin as a package consisting of 8 test units).

Erythrocytosis:

Erythrocytosis is defined as an increase in erythrocyte count, haematocrit and haemoglobin concentration.
Here, too, a distinction can be made between relative and absolute erythrocytosis. Relative erythrocytosis occurs in case of dehydration, haemoconcentration and splenic contraction. For differentiation, it is diagnostically helpful to determine the total protein as it increases during dehydration and does not increase during splenic contraction. Then again, in absolute erythrocytosis, a primary form (polycythaemia vera) and a secondary form (cardiac, pulmonary, renal diseases, EPO-producing tumour) can be differentiated.

White blood count:

Leukocytes are an essential component of the innate and acquired immune defence.
In contrast to other animal species, even minor changes in horses are considered to be pathologically significant. Measurement of total leukocyte count should always be complemented with a differential blood count to classify the leukocyte populations.

Leukopenia:

Neutropenia and lymphopenia are of particular pathological importance. Low numbers of other leukocyte subpopulations are not necessarily associated with a disease.

Neutropenia:

A decrease of neutrophil granulocytes usually results from reduced release of neutrophils from the bone marrow, increased migration from the blood, destruction in the blood or a shift from the circulating pool to the marginated pool.
Common reasons for this are hyperacute inflammatory reactions, endotoxaemia (colitis, ulcer), severe injuries or production disorders (radiation, medication, bone marrow diseases).

Lymphopenia:

Low lymphocyte counts are often the result of endogenous (stress, hyperadrenocorticism) or exogenous glucocorticoids (therapy). Other causes are endotoxaemias, bacterial and viral diseases, immunodeficiencies (e. g. severe combined immunodeficiency (SCID), foal immunodeficiency syndrome (FIS)) or therapies with immunosuppressive medication.
The two genetic diseases (SCID, FIS) can be tested at Laboklin.

Leukocytosis:

Depending on the subpopulation affected, leukocytoses provide a good indication of the underlying cause.

Neutrophilia:

An increase in neutrophil granulocytes is often caused by inflammation (bacterial or viral infections, injuries), short-term or long-term stress or corticosteroids. However, immune disorders or bone marrow diseases can also cause neutrophilia.
In chronic inflammation, the initial neutrophilia is rapidly downregulated, so that often markers such as acute phase proteins (e. g. fibrinogen, globulins, serum amyloid A) have to be used for diagnosis.

Lymphocytosis:

An increased number of lymphocytes in the peripheral blood is mainly found during excitement, exertion and lymphoproliferative diseases (lymphoma stage V, lymphatic leukaemia) and rarely during immune stimulation. Young animals have physiological lymphocytosis.
Depending on the presumptive clinical diagnosis, in case of persistent lymphocytosis (>1 subsequent blood count) further tests such as cytology of a fresh blood smear, of enlarged lymph nodes or the bone marrow would be useful.

Monocytosis:

Causes of monocytosis are acute or chronic inflammation (e. g. chronic bacterial infections), stress, corticosteroids, autoimmune disorders and bone marrow diseases.

Eosinophilia:

Common causes for an increase in eosinophil granulocytes are parasites, hypersensitivity reactions or bone marrow diseases.

Basophilia:

A significant increase in basophil granulocytes is rare in horses.
Mostly, basophilia accompanies eosinophilia. Other very rare causes are bone marrow diseases and mast cell tumours.

Platelets:

Platelets are the smallest cells circulating in the blood with a survival time of 3 – 5 days. They play an important role in haemostasis and in mediating inflammatory and immune reactions.

Thrombocytopenia:

Pseudothrombocytopenia must be distinguished from true thrombocytopenia.
The former causes clotting of the platelets in the blood tube which causes the analyser to detect a lower platelet count.
The causes of true thrombocytopenia can be divided into four groups: reduced production in the bone marrow, excessive destruction of platelets, increased consumption/loss and increased platelet sequestration (splenomegaly). The destruction of thrombocytes is mostly immune-mediated (immune-mediated thrombocytopenia, IMT) and can be caused by primary autoimmune thrombocytopenia or secondarily by bacteria (especially Anaplasma phagocytophilum), viruses, neoplasia and drugs.
The symptoms are usually caused by the underlying disease. Profound thrombocytopenia, however, can cause coagulation disorders.

Thrombocytosis:

Thrombocytosis refers to an increased concentration of platelets.
The most common causes are inflammations or infections as well as, temporarily, splenic contractions and stress. This explains an increased occurrence with hyperfibrinogenaemia, leukocytosis, hyperproteinaemia and anaemia. Other causes include traumata, chronic bleeding, iron deficiency, hyperadrenocorticism and tumours.

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Serum amyloid A (SAA) - an update

SAA is a major acute phase protein (mAPP), which is primarily synthesized in the liver. There are also extrahepatic isoforms, e.g. an SAA in the synovial fluid in horses. It is an apolipoprotein, which, on the one hand, draws chemotactic inflammatory cells to inflamed areas but on the other hand can also inhibit lymphocyte proliferation. In healthy horses, SAA can only be found in very low concentrations in serum. Following an insult, the concentration can rise steeply (100 to 1000 fold) within a short period of time (6-12 hours). Once the insult has been removed or following successful therapy, the concentration falls again very quickly. Compared to haematology or fibrinogen, which has hitherto been most commonly measured, SAA is clearly better, since changes can be detected much sooner (for comparison, fibrinogen increases slightly within 24 hours, highest concentrations are detectable after about 48 hours, and the increase is often only 1 to 2 fold). SAA reacts not only to infectious, but also to inflammatory processes.

Indications for determining SAA in horses include the detection of subclinical diseases in which the clinical examination and haematology do not provide clear results as well as timely monitoring of therapeutic success or of post-operative healing. With more experience, it may be possible to estimate the extent of inflammatory processes based on SAA concentrations. In contrast to other inflammatory parameters, SAA increases very early during inflammation (“real-time” – within hours).

The following paragraph lists several areas in which SAA can be used as well as some in which SAA does not offer additional information:

Hooijberg et al. found significantly higher SAA values (mean 1583 µg/ml – range 688-4000 µg/ml) in patients with systemic inflammation than in those with local inflammation (mean 343 µg/ml – range 37-1609 µg/ml) or horses without inflammation (mean 5.6 µg/ml – range 1.8-14.5 µg/ml). In healthy foals, the SAA concentrations are similar to those in adults and have similar kinetics following insults. Significant SAA increases are found, for example, during local infections and omphalitis. An insufficient passive immune transfer (as a non-inflammatory deviation) does not lead to changes (Stoneham et al.).

A study on SAA concentrations in horses with colic showed no significant difference between surgical or conservative management. SAA values were only increased in those cases in which enteritis-like disease was diagnosed (e.g. enteritis, colitis, abdominal abscesses, peritonitis) (mean 65.5 µg/ml – range 3-500 µg/ml) (Vandenplas et al.). The SAA concentration is not a basis for a prognosis.

Surgical interventions, including minor uncomplicated procedures, provide sufficient stimulus for increased SAA concentrations: Concentrations between 100 and 400 µg/ml are found regularly 3 days after surgery (somewhat higher following larger procedures), which then – if healing is unproblematic – fall continuously over the

course of the following days. SAA values that remain elevated at the level they reach 3 days p.o. are an indication of problems with wound healing or infection (Jacobsen et al.).

Horses with bacterial pneumonias have SAA values in the thousands. SAA values that reach approximately 450 µg/ml can be measured in cases of acute equine influenza (Belgrave et al.; Hulten et al.). SAA is not useful for the detection of subclinical infections with *Rhodococcus equi* or for monitoring disease progression in horses with COPD. Vaccination (influenza and tetanus) leads to a slight increase in SAA on day 2 post vacc. (30-175 µg/ml; Andersen et al.).

An important use of SAA testing is the differentiation between septic and non-septic diseases of the joints and other synovial structures. While serum and synovial SAA is often <1 µg/ml in healthy horses, values >1000 µg/ml are often measured (in serum and synovial fluid) during septic processes (Jacobsen et al.). Laminitis, endocrinopathies, and racing did not have similar effects on serum SAA levels. Interesting aspects were only found in long distance endurance horses: although all of the starting animals had values within the reference interval, those horses that finished the race had much lower values (0.4 µg/ml) than horses that were disqualified from the race during the qualification period (5.8 µg/ml; Cywinska et al.).

Findings in peripartal mares were contradictory: In some cases, a clear elevation in SAA was detected up to day 3 p.p., in others, the measured values remained within normal limits. Very high values were measured in mares with placentitis (274 to 4385 µg/ml). The higher the SAA concentrations were, the more likely it was that the mare would abort (Canisso et al.).

Parasitosis or deworming of heavily parasitized horses did not have an effect on SAA concentrations.

In summary, SAA is a sensitive marker of early inflammation. Due to its rapid increase and short half-life, it can be used to monitor inflammatory processes and is also a stable parameter. In many studies, SAA has been shown to be better than traditional markers of inflammation (WBC, fibrinogen, etc.). It is also important to note that SAA does not respond to all forms of inflammation, so that it is not appropriate for everything. A careful clinical examination is the basis for a diagnosis. In cases in which the horse is reported to be “not doing well” (or similar), in which no abnormalities are found on clinical examination or routine blood tests, an increased SAA can provide evidence for subclinical changes and offer an indication for additional tests. In high performance horses, subclinical disease can have great effects: such animals are often presented with a history of “reduced performance”.

A large number of studies on SAA have been published in the past decade, and the number is increasing. We will keep you updated on developments!

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Laboratory testing: Equine (extract)

Equine profiles

PPID (Cushing) Profile +Complete Blood Count

Material: 2ml EDTA plasma
+2ml serum and NaFl blood (cooled!)
+1ml EDTA blood for CBC
Parameters: Insulin, ACTH, glucose, fructosamine, triglycerides, γ-GT, RISQI, MIRG, I/G-ratio

EMS Profile +Complete Blood Count

Material: 2ml serum and NaFl blood (cooled!)
+1ml EDTA blood for CBC
Parameters: Insulin, glucose, fructosamine, RISQI, MIRG, I/G-ratio

Foal Profile +Complete Blood Count

Material: 1ml serum
+1ml EDTA blood for CBC
Parameters: Triglycerides, urea, creatinine, protein, electrophoresis, γ-GT, GLDH, Na, Ca, Mg, PO_4

Senior Profile +Complete Blood Count

Material: 1ml serum and NaFl blood
+1ml EDTA blood for CBC
Parameters: Urea, creatinine, PO_4, Ca, bilirubin, γ-GT, GLDH, protein, albumin, globulin, glucose, triglycerides, zinc, selenium

Small Screening +Complete Blood Count

Material: 1ml serum
+1ml EDTA blood for CBC
Parameters: GLDH, γ-GT, AST, LDH, CK, urea, creatinine, protein, triglycerides

Large Screening +Complete Blood Count

Material: 1ml HP and NaFl blood or
1ml serum and NaFl blood
+1ml EDTA blood for CBC
Parameters: AP, γ-GT, bilirubin, cholesterol, glucose, AST, LDH, GLDH, CK, protein, albumin, globulin, urea, creatinine, Ca, Mg, K, Na, Fe, PO_4, Cu, Zn, selenium, triglycerides

Large Screening + SAA +Complete Blood Count

Material: 1ml serum + NaFl blood
+1ml EDTA blood for CBC
Parameters: see *Large Screening* + SAA

Performance Profile +Complete Blood Count

Material: 1ml serum and NaFl blood
+1ml EDTA blood for CBC
Parameters: AP, γ-GT, bilirubin, cholesterol, glucose, AST, GLDH, CK, albumin, globulin, urea, creatinine, protein, lactate, Ca, Mg, K, Na, Fe, PO_4, LDH, triglycerides

Strangles – an update

Streptococcus equi equi, the cause of strangles, is found throughout the world and can cause severe clinical disease and economic losses during outbreaks. Even a suspicion of this disease causes consternation among owners. Environmental factors such as cooler seasons, stocking density, frequent movements in the stables, and the stable climate can favour the spread of disease.

The pathogen

The gram-positive aerobic, mucoid, and slimily growing streptococci are easily inactivated in the environment and only survive for days in dirt, and for up to 8 weeks in moist environments. Sunlight shortens their survival time significantly. Horses with strangles can shed *Streptococcus equi equi* before developing clinical signs of disease. The pathogen is highly contagious. While the morbidity rate can be as high as 100%, the mortality rate is generally low (up to 2%). Mortality rates of up to 10% have been described, however, especially when the pathogen is newly introduced into a population.

Transmission

Transmission from horse to horse mostly occurs by direct contact, but aerosols as well as fomites and vectors, such as grooms or jointly used water buckets, feeding troughs, bits, etc., can also be responsible. The environment is not a source of infection, persistently infected asymptomatic carriers with abscesses of the retropharyngeal lymph nodes or with inspissated, hard abscess material are considered the true reservoir, and infectious material can be found in the sinuses for up to 5 years after a clinical disease outbreak. The bacteria can then be shed intermittently via the nose and can contaminate the environment. Horses that are kept together with such carriers often also develop inapparent infections. If one of these horses is introduced to another herd, bacterial shedding can lead to a new massive disease outbreak.

Pathogenesis

After introduction through the nose or mouth, *Strept. equi equi* reaches the tonsils and the retropharyngeal lymph nodes. The bacteria are protected by a capsule containing hyaluronic acid and by factors such as the antiphagocytic SeM protein and can resist phagocytosis by neutrophilic granulocytes. The streptococci are ordered in long chains and surrounded by hydropically degenerated neutrophils. Bacterial enzymes such as streptolysin and streptokinase contribute to abscess formation by destruction of cellular membranes and activation of plasminogen. Other lymph nodes in the thorax and abdomen can also be affected by haematogenous or lymphatic spread. Bacteraemia can occur between 6 and 12 days after intranasal infection.

The body temperature rises to 39.50 °C and higher 3 to 14 days after infection. During this time, the number of leukocytes increases due to neutrophilia. Abscess formation in the lymph nodes is accompanied by congestion of the lymphatic vessels. Shedding of *Strept. equi equi* begins about 4 to 14 days after initial infection. Shedding usually ends between 3 to 7 weeks after the end of the acute phase of the disease. It is therefore important to separate diseased and healthy horses in order to prevent

transmission of the disease. However, some horses continue to shed bacteria intermittently. These persistently infected carriers are then the reservoir for new outbreaks.

Immunity

Approximately 75% of the horses infected with *Strept. equi equi* develop a solid immunity after infection, the rest can become reinfected. Older horses with a residual immunity are again susceptible to a new infection, but the course of disease is milder. However, these horses can then be a source of infection for susceptible horses. In 2-10% of the affected animals, the immune system is unable to completely eliminate the pathogen. In these cases, the bacteria retreat to the air sacks and are occasionally shed from there in small amounts. They serve as reservoirs in populations. Through them, strangles can manifest within a group over an extended period of time. New additions to the group that have a low specific immunity are quickly infected. Milk from mares that have been infected with *Strept. equi equi* contains specific IgG and IgA antibodies which protect the nasopharyngeal mucous membranes of suckling foals that have received colostrum for the entire suckling period.

Clinical signs

Clinical signs develop after an incubation period of 3 to 14 days. These include green-yellowish nasal discharge, fever (up to 40.0-40.5 °C), loss of appetite, lethargy, coughing, and swelling of the cranial lymph nodes. Abscessed lymph nodes can break open and puss can drain out. Puss from the lymph nodes in the throat can, however, also get into the air sacks and cause a purulent nasal discharge. The puss and secretions from the nose contain massive numbers of bacteria, and the disease is easily transmitted to other horses during this period.

Complications

Strept. equi equi can spread to all organs by haematogenic or lymphatic means. This can lead to bronchopneumonia. Pneumonia and pleuropneumonia are also frequently found in horses in which *Strept. equi equi* infections have been fatal. Abscessed retropharyngeal lymph nodes can cause sinusitis. These horses are then often persistent shedders. Although the probability of metastasizing abscessation in other organs is generally considered low, abscess formation and the various consequences thereof in various organs, the central nervous system, or the musculoskeletal system can occur. Individual susceptibility as well as the virulence of the *Strept. equi equi* strain involved are responsible for this.

Immune mediated antigen- antibody complexes can lead to the development of purpura haemorrhagica, leading to an aseptic, necrotizing vasculitis. In addition to oedema of the head, the limbs, and the rump, petechia and ecchymosis may be found on the mucous membranes. Serous fluid can leak through the skin from the oedemas leading to sloughing. As a result of the vasculitis, animals may develop colic, muscular pain, or respiratory signs.

Myopathies caused by infarction and rhabdomyolysis with progressive atrophy have been described as a result of infection. Myocarditis and an immune mediated glomerulonephritis are also possible.

In breeding mares, strangles, like other infectious agents, can lead to milk stasis. The udder itself is generally not affected by abscessation.

Diagnosis

Strept. equi equi is detectable on mucous membranes 24 to 48 hours after the beginning of fever.

Culture: Nasal swabs, sinus washes as well as puss from abscessed lymph nodes can be used as samples. This method is still considered the gold standard. It is, however, important to note that the growth of other bacteria should be suppressed with the addition of nalidixic acid and colistin (CAN agar). ß-haemolytic streptococci must be differentiated in order to rule out other species such as *Strept. equi zooepidemicus*

PCR: This method, in which *Strept. equi equi* DNA is detected, was up to three times more sensitive than culture for samples submitted to Laboklin, although it is important to note that it also detects bacteria that are no longer able to replicate or that are dead (see Fig. 1).
This method (qPCR or real-time PCR) is becoming the new "gold standard" for diagnostics. The PCR can supplement bacterial culture and is also useful for the detection of inapparent carriers and shedders.

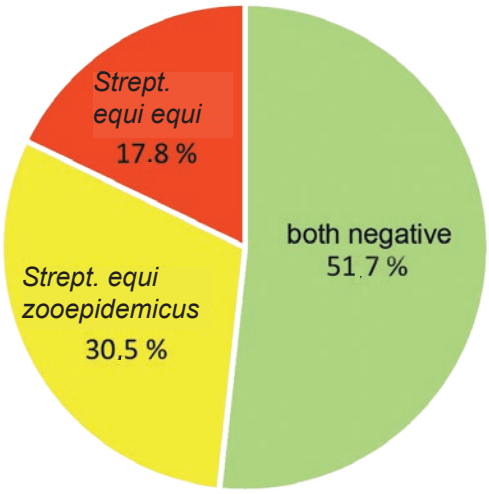

Fig. 1: Detection rate for *Strept. equi equi* and *S. equi zooepidemicus* by PCR in 2016 (n = 2352).

However, information on the disease status of the animals is not generally available when evaluating results.

Serology: Antibodies against several different surface antigens or other *Strept. equi equi* proteins are formed during infection and recovery. SeM is considered the main virulence factor. The ELISA detects antibodies against the SeM protein. It cannot differentiate whether these represent an immune response following vaccination or infection. It is therefore used both to determine the reaction to vaccination as well as to diagnose purpura haemorrhagica and metastasizing abscesses. The highest titres are measured 5 weeks post infection and can remain high for more than 6 months. ELISA results must be interpreted individually for each horse. Horses with very high results should not be vaccinated for strangles, since this can induce purpura haemorrhagica in these animals (see Table 1).

Evaluation of SeM specific ELISA results:

Negative	• No exposure /vaccination • Incubation period
Weak positive	• Acute infection/incubation period • Vaccination a long time ago
Moderately positive	• 2-3 weeks after exposure • Infection 6 months to 2 years ago
Strong positive	• 4-12 weeks p. inf. • 2-4 weeks p. vacc.
Very strong positive	• Metastasizing abscessation • Purpura haemorrhagica

Table 1

Treatment

Treatment of horses with strangles depends on the phase and severity of the disease. Antibiotic therapy is generally not considered warranted if the animal is not severely ill, the airways are not constricted and abscesses have already formed. Bacteria in existing abscesses are not affected by antibiotics due

to the abscess capsule and puss. In the early stages of disease, in horses with fever and lethargy, if the lymph nodes are not yet abscessed, antibiotic therapy over the course of 7-10 days can counteract the infection and the spread of the pathogen. However, these animals do not develop a strong immunity. Penicillin is still the antibiotic of choice, but cephalosporins or macrolid antibiotics are also effective. Although sulfonamide/trimethoprim are effective in vitro, their effect in vivo is often suboptimal.

As soon as the lymph nodes abscess, antibiotics are generally contraindicated. Treatment must, however, also depend on other clinical signs. Symptomatic treatment with NSAIDS can help alleviate signs such as fever and pain caused by enlarged lymph nodes, so that animals can resume eating and drinking. If complications such as purpura haemorrhagica occur, immuno-suppressive treatment should be initiated despite the bacterial infection. In addition to administration of glucocorticoids in conjunction with antibiotics, the use of NSAIDs, fluid therapy, hydrotherapy, and bandaging may also be appropriate in treating complications.

<u>Important points in summary</u>:

- **Suspected strangles: culture and/or PCR of a nasal swab or lavage sample**
- **Antibiogram not mandatory since *Strept. equi equi* has no resistance to ß-lactam antibiotics.**
- **Serology: Sensitive and specific, only detects exposure, not carrier status, therefore not useful for diagnosis**
- **Sick horses should be isolated**
- **Disinfection and hygiene**

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Respiratory diseases of horses: Cytology as a diagnostic tool

Respiratory diseases are an important disease complex both in sport and in leisure horses. A wide variety of clinical signs can be associated with these diseases and many differential diagnoses must be taken into account in conjunction with the age and use of the horse.

Infectious bronchopneumonias can be divided into viral (especially influenza and equine herpesvirus), bacterial (e.g. streptococci), and fungal (e.g. *Aspergillus* spp.) disease. Parasitic diseases (lung worms) are rare. Identification of the cause can be done e.g. by molecular or microbiological methods.

Non-infectious causes must be differentiated from the infectious causes. These can lead to general signs of disease and reduced performance. In older horses especially, recurrent airway obstruction (RAO) can cause respiratory signs such as coughing, nasal discharge, or dyspnea. Animals can also have reduced performance and weight loss. If disease persists over a longer period of time, the animals can become “broken winded”. Various allergies (e.g. moldy hay) or endotoxins have been discussed as possible causes.

This should be differentiated from inflammatory airway disease (IAD), which is also associated with respiratory signs (coughing, nasal discharge) and reduced performance. In contrast to the infectious diseases, the causes of these non-infectious diseases have not yet been fully elucidated.

Endoscopic examination allows for the collection of samples for various additional diagnostic tests. Samples for bacteriological testing can be collected during endoscopic exams. Swabs with medium are appropriate for this. Material for molecular or virological assays should be submitted in sterile containers. Collection of tracheal secretions (TBL) is a relatively easy method of obtaining cells from the entire tracheoalveolar region. Broncheoalveolar lavages (BAL) are somewhat more difficult to obtain, but do result in a better defined spectrum of cells. For both methods, macroscopic examination can already be diagnostically helpful. Viscous or lumpy samples are an indication of slime components. Purulent elements are an indication of bacterial infection.

In order to preserve the cells as much as possible, the material (TBL and BAL) should be submitted as an air-dried slide smear.

Cytology can offer indications of various aetiologies and disease complexes: In infections, inflammation, or insufficient ciliar clearance, slime components are often found in the material. So-called Curschmann spirals are an indication of bronchial obstruction.

Alveolar macrophages are also found in healthy animals, but their number increases in disease processes that persist for extended periods of time. They can have phagocytic activity and ingest e.g. bacteria, cellular detritus, or fungal structures.

Neutrophils are found in non infectious processes as well as in bacterial infections. An increased number of eosinophils may be a sign of a hypersensitivity reaction (allergy).

Erythrocytes can occur as contaminants, but are also typically found in exercise induced pulmonary haemorrhage (EIPH). In these

cases, haemosiderin and erythrophagocytosis are important features for differentiation from contamination.

It is important to differentiate between an aspiration pneumonia and a contamination when evaluating foreign material in a sample. If fungal structures are found in a sample, correlation with the quality of the hay can be evaluated. In addition, these can be an indication of inadequate ciliar clearance.

The clinical relevance of bacteria in a sample must also be evaluated in conjunction with the clinical relevance and differentiated from contamination.

For diagnosticians, an extensive clinical history is particularly important for the interpretation of all findings, since cytological presentations can overlap. This should include both clinical findings (age, fitness, type and duration of respiratory signs) and past treatments (secretolytics or antibiotics).

In conclusion, cytology of a broncheoalveolar lavage or tracheobronchial secretions can be an informative tool in determining the cause of respiratory disease. The interpretation must, however, always be done in a clinical context.

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EHV-1 and EHV-4

The equine herpesviruses types 1 and 4 are among the most economically important viral pathogens in horses. Both virus types have high prevalences within the horse population and, like all herpesviruses, they are able to cause life-long latent infections (>80% of adult horses are latently infected). A reactivation and renewed virus shedding are possible following endogenous or exogenous stress with or without clinical signs.

EHV-1 and EHV-4 are highly contagious and are shed mostly via nasal secretions. Transmission is by droplet infection by direct contact or via fomites and human vectors.
Infections with EHV-1 can occur as more or less severe respiratory disease, but late abortions and neurologic disease are especially feared. In recent years, the often fatal CNS form (myeloencephalopathy) appears to have become more common. This was also true in 2016, when 25 horses in a stable in Hessia were infected with EHV-1 and at least 10 of them had to be euthanized (source: pferaktuell).

EHV-4 associated disease is generally limited to the respiratory tract (rhinopneumonitis), especially in young horses. Infections are often subclinical or mild, but can be complicated by bacterial secondary infections.

There are three recommendations for the control of EHV-1 and EHV-4 infections: hygienic measures, avoidance of stress, and consistent vaccination of the entire stock.

Sample material, time for testing

For PCR detection, sample materials should always be those in which virus shedding is suspected. For EHV-1 especially, nasal swabs and EDTA blood should be submitted, since only one of these may be positive depending on the stage of infection. Swabs with transport medium (for bacteriology) are unsuitable for PCR detection, since the medium can inhibit the PCR. Transport time and temperature, on the other hand, are not critical factors for the PCR. Testing is generally carried out on the day the sample reaches the lab and the results are sent out the same day.

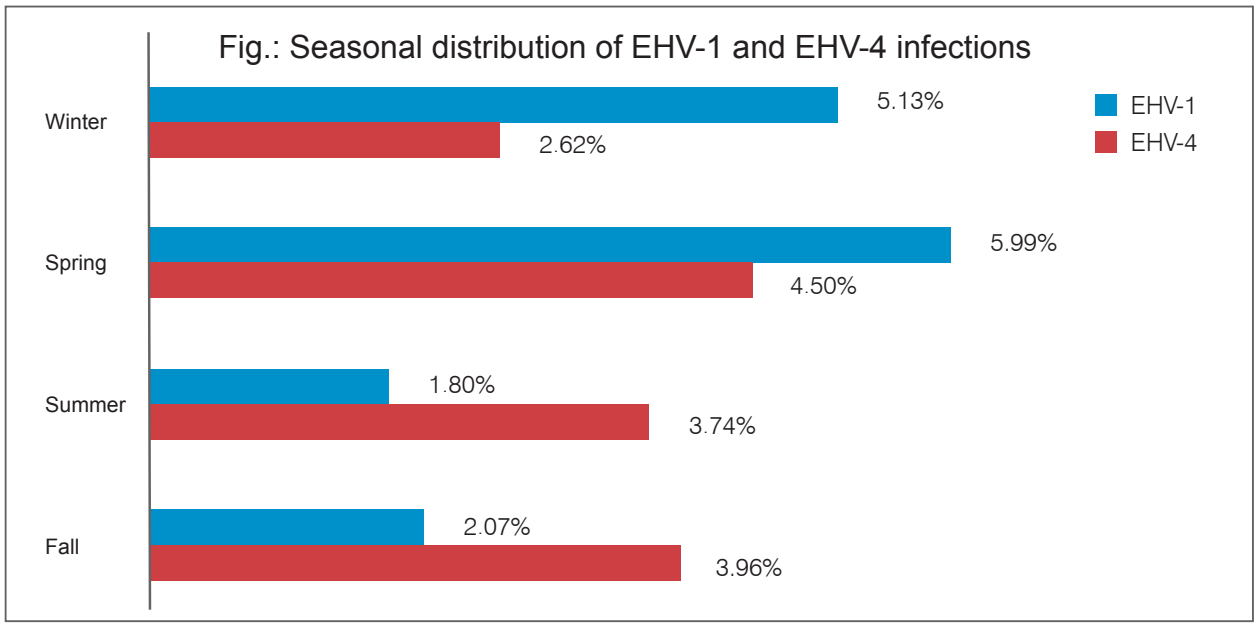
Frequency of detection in samples submitted to our lab:

Since January 2014 (n = 2914) EHV-1 has been detected in 3.91% (n = 114) of the samples and EHV-4 has been detected in 3.64% (n = 106) of the samples. Double infections were only found in three horses.

There was a clear seasonal distribution of infections since 2014 (see figure): EHV-1

was detected twice as often in the winter (5.13%, n = 45) and spring (5.99%, n = 44) as in the summer (1.80%, n = 13) and fall (2.07%, n = 12).

EHV-4 had a similar seasonality with a peak in spring (4.50%, n = 33) compared to winter (2.62%, n = 23), summer (3.74%, n = 27), and fall (3.96%, n = 23).



Laboratory testing: Equine (extract)

Specific Profiles

Mineral Profile

Material: 3ml HP or serum
Parameters: Mn, Zn, Se, Cu, Na, K, Ca, Mg, PO_4, Cl, Fe

Muscle Screening

Material: 1ml serum
Parameters: CK, a-HBDH, AST, LDH, Na, K, Ca, PO_4, Mg, Fe

Ext. Muscle Screening

Material: 3ml serum
Parameters: CK, a-HBDH, AST, LDH, vitamin E, selenium, K, Ca, PO_4, Mg

Eye (PCR)

Material: Swab
Parameters: EHV2, EHV5

Uveitis (PCR)

Material: Inocular fluid
Parameters: Bornavirus, leptospira, EHV1, EHV4

Respiratory PCR-Profiles

Respiratory (small)

Material: Swab
Parameters: EHV1, EHV4, Influenza A virus

Respiratory (large)

Material: Swab
Parameters: EHV1, EHV4, EVA, Influenza A virus, Streptococcus equi ssp. equi / zoo-epidemicus

Respiratory (foal)

Material: Swab
Parameters: EHV1, EHV4, Influenza A virus, Rhodococcus equi

Breeding management and CEM diagnostics

Before a mare is bred, whether naturally or by artificial insemination, a cervical swab should be tested for bacteria. This is necessary to evaluate the mare's health and to prevent the spread of possible genital infections.

When planning to breed a mare, it is advisable to do the sampling early in the year. That way, if potential pathogens are detected, it is still possible to treat the animal before breeding her. The success of the treatment is determined with a repeat bacterial culture at least 10 days after the end of treatment. The next heat can then be used for breeding. Our data show that this is necessary in a significant percentage of the samples we test.

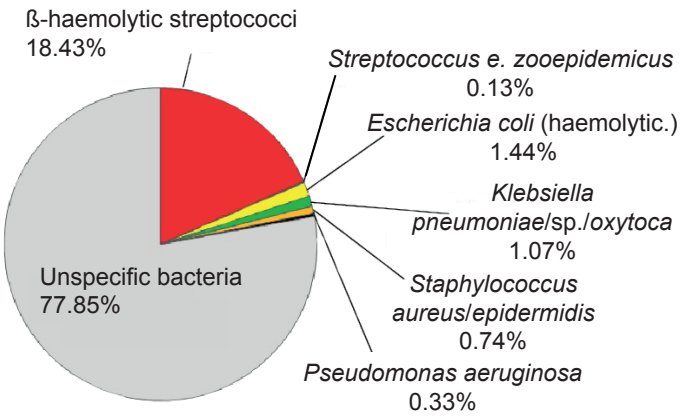
It is important to observe certain hygienic criteria during sampling in order to obtain meaningful bacteriological results. Swabbing should be done with a sterilized speculum, so that the sample is collected under visual control. This also allows for evaluation of the vaginal mucosa and the cervix. Swabs are generally collected from the cervix and sometimes also from the uterus.

The bacteriological exam is done on several media in order to ensure optimal growth conditions for the bacteria of interest. In our laboratory, differentiation of isolates is carried out by MALDI-TOF, which is the fastest and most accurate method currently available. The antibiogram is done by microdilution. Results are usually available within 48 hours.

Evaluation

Pathogens include predominantly ß-haemolytic streptococci (Lancefield group C streptococci, e.g. *Streptococcus equi* ssp. *equi*, *equisimilis*, and *zooepidemicus*). *Staphylococcus aureus*, *Escherichia coli* var. *haemolytica*, *Pseudomonas aeruginosa*, *Klebsiella* spp., *Actinobacillus equuli*, *Bordetella bronchiseptica*, and *Rhodococcus equi* are also all obligate pathogens – with or without clinical signs of disease. If any of these are detected, an antibiogram is automatically carried out to determine a specific antibiotic therapy.

Figure: Breeding hygiene in horses, bacterial pathogens (n = 4568)



Results of breeding hygiene exams in mares 2016

Detections in samples submitted to our lab

Bacterial pathogens of one of the species listed above were detected in one fourth of the swabs submitted for bacteriological testing. Of these, ß-haemolytic streptococci were found most often (see Figure).

CEM

A lot of progress has been made in the detection of contagious equine metritis (CEM). CEM is caused by the bacteria *Taylorella equigenitalis*. This highly infectious venereal disease causes inflammation of the endometrium and reduced fertility in mares. Infected stallions are generally clinically inapparent and are therefore a bacterial reservoir. Mares may also be inapparently infected.

Exportation exams of sexually mature stallions and mares must include testing for CEM. According to directive 92/65/EEC, testing should be carried out on three swabs from the following sites in stallions:

- the penile sheath (prepuce)
- the urethra
- the fossa glandis

In mares, testing must be carried out on at least two swabs from the following sites:

- the mucosa of the clitoral fossa
- the clitoral sinuses

In addition to culture methods, PCR is also considered a suitable method for detection. Based on this directive, we offer a 3-fold CEM PCR profile for stallions. It includes three individual tests for *Taylorella equigenitalis* by PCR from the three prescribed sites. For mares, we offer a corresponding 2-fold PCR profile. Bacterial culture for the diagnosis of CEM is also still available.

Swabs must be placed in Amies transport medium for submission (this is also true for the PCR). Testing via culture should begin no more than 24 hours after sample collection (48 hours if the sample is cooled during transportation). For the PCR, the maximum time between collection and testing is 48 hours. This should be considered during sampling and shipping.

A combination of testing for general breeding hygiene and cultural detection of *Taylorella equigenitalis* is also possible.

The advantage of the PCR is the shorter duration, with test results available within 24 hours after arrival at the lab, as opposed to the mandatory 7 days necessary for microaerophilic culture. Laboklin has the necessary accreditation. Bacteriology and PCRs are carried out from Monday to Saturday.

Practical aspects of laboratory testing for equine reproductive hormones

The clinical exam, in particular rectal examination of the genital tract of mares, possibly supplemented by ultrasound, are the most important tools in routine breeding exams (breeding and insemination management, pregnancy diagnostics). Stallions are also evaluated principally by clinical exam supplemented by examination of the ejaculate. In specific cases, an evaluation of various hormones may also be necessary. The interpretation of laboratory results should always be carried out in conjunction with clinical findings and the patient history.

Testing is available for a number of hormones for both female and male horses, depending on the case. Some of these hormones are independent of the sex and should then be interpreted based on the case.

Gonadal steroids

Oestradiol:
Produced during heat – this is produced in the ovarian follicle during the cycle. During pregnancy, the feto-maternal system biosynthesizes large amounts of oestrogen.

Progesterone:
Produced in the corpus luteum – in the lutein cells of the corpora lutea (c.l.). Normal c.l. function is associated with >1 ng/ml. Laboratory testing cannot differentiate between progesterone from a normal cycle and pregnancy.

Testosterone:
Synthesized in the Leydig interstitial cells in the testes, and to a lesser degree in the adrenal cortex. Values are low in the early morning and highest in the afternoon. Mares produce low amounts of this hormone in their ovaries and adrenal cortex.

Note: The interpretation of gonadal steroid levels can only be done in the context of a clinical exam. In some cases, follow up tests may be necessary.

Hormonal pregnancy diagnostics

Pregnancy diagnosis by rectal palpation is not always possible in practice, e.g. in small horses or miniature breeds, ornery or wild and zoo animals, animals with rectal lesions, etc. In such cases, testing for pregnancy specific hormones can be helpful.

PMSG/eCG:
Is synthesized in the endometrial cups between the 35th and 120th day of pregnancy (and later in individual cases). The highest values are found between days 60 and 75. During the “marginal periods” of the temporal window, the results may be borderline, and follow up testing may be necessary. We recommend sample collection between days 45 to 100 post ov. It is also important to remember that the endometrial cups can continue to produce PMSG for several weeks after foetal resorption, so that PMSG can be false positive, even though no live foetus is present. Affected mares also do not go into heat for an extended period of time. In mares that have been determined to be pregnant based on PMSG, it is therefore advisable to confirm the pregnancy by testing for oestrone sulphate later in pregnancy (after day 110).

Oestrone sulphate:
Is synthesized by the intact foeto-maternal unit and is therefore an indicator for a live foetus. This hormone is detectable in

increasing amounts beginning around day 40 of pregnancy. It is, however, not possible to clearly distinguish it from cyclic oestrogen secretion in these early stages. We recommend determination of oestrone sulphate after the 110th day post ov., since mares in this stage of pregnancy have higher concentrations.

Oestrone sulphate determination can also be done from urine.

Caution: Not all mares have a typical pattern of secretion. In borderline or unclear cases, repeat testing should be carried out 3-4 weeks later.

A negative test in a mare that is known to be >120 days pregnant can indicate damage to the foetus/placenta. In this case, it is necessary to carry out a rectal or ultrasound exam.

Several comments on progesterone:
Progesterone is not pregnancy specific. If mares are tested 18-21 days post ov. and corpora lutea are detected, that only indicates that the mare will not go into heat at the expected time. Laboratory testing cannot differentiate between cycle specific and pregnancy specific corpora lutea. Progesterone values are often higher in pregnant mares, but double or multiple ovulations can also be the cause. In later stages of pregnancy (>50 days), the hormone concentration increases again, but pregnancy specific hormones (see above) are available for this time-period.

Diagnosing ovarian tumours

In mares with abnormal cycles or behaviour that also have remarkable findings on rectal or sonographic examination of the ovaries, the suspected diagnosis is often an ovarian tumour. Ovarian tumours are indeed one of the most common neoplasms in horses. By far the most common are granulosa cell tumours (GCT). This tumour is able to produce oestradiol and testosterone, but does not always do so. Approximately 50% of the affected mares have increased testosterone levels. These hormones can be used to aid diagnosis. It must, however, be taken into account that mares with tumours may have normal hormone levels and that increased hormone levels can be found in mares with other cycle anomalies and in pregnant mares.

Progesterone is almost always low in mares with GCT, since these mares can have a prolonged anoestrus, persistent oestrus behaviour, or stallion-like behaviour, but rarely cycle and without ovulation no corpora lutei are formed.

Anti-Müllerian hormone (AMH):
Is a glycoprotein that plays an important role in sexual differentiation during embryonal development. In female animals, AMH is secreted by the granulosa cells of preantral and small antral follicles. Since granulosa cell tumours are the most commonly diagnosed tumours of the genital tract in mares, it would be logical to use this for diagnosis – similar to what is done in human medicine. Mares with GCT have much higher AMH concentrations than normal mares. AMH has a sensitivity of 95% for the diagnosis of GCT.

In human medicine, AMH detection also provides information on the antral follicle reserve of a woman. This use is not yet applicable in horses, since mares have

individual AMH levels and would have to be monitored over years in order to correlate declining AMH levels and associated reductions in fertility.

Inhibin:
Is also produced in the granulosa cells of the ovaries. The serum inhibin B level is therefore significantly increased in mares with GCT and can be used for diagnostic purposes.

Inhibin also always leads to suppression and loss of function of the contralateral ovary.

Since samples for inhibin testing are shipped to the United States, the turnaround time for testing can unfortunately be up to 4 weeks. Testing is also quite expensive.

Since AMH testing is at least as good as inhibin B and provides much faster results, **AMH should be specifically tested in cases in which GCT is suspected**. Borderline results are rare (e.g. in early stages of neoplasia). In these cases the gonadal steroids can also be determined, in order to approach a diagnosis. However, repeat testing of AMH after 1 or 2 months is generally recommended.

Diagnosis of cryptorchidism
This includes detection of cryptorchidic and incompletely castrated male horses.

Individual testosterone determinations are often not conclusive due to pronounced circadian and seasonal fluctuations.

hCG stimulation test / "Cox test":
This test was long considered the gold standard for the diagnosis of cryptorchidism. It is based on the principle that hCG has LH effects.

Procedure:
- Blood collection (in the morning if possible) = testosterone baseline
- Immediately following: i.v. injection of 5000-10000 I.U. hCG/horse
- +60 min.: blood collection = stimulation value

Interpretation: completely castrated horses have minimal testosterone levels and low to no stimulation. A clear increase in testosterone, on the other hand, is proof of the presence of testosterone producing tissue. At the same time, it is important to look at the absolute values:

Testosterone concentrations of 0.05 to 0.1 ng/ml after stimulation are borderline and require further testing, e.g. for AMH. Higher values are an indication of the presence of testicular tissue.

Limitation: In horses up to 18 months of age, the testicles may only have a limited reaction to hCG injection.

Anti-Müllerian hormone (AMH):
This is expressed by the Sertoli cells. It is high until puberty and then declines with increasing testosterone production. Stallions and cryptorchids can nevertheless be clearly differentiated from castrated animals. The AMH concentration is therefore a helpful biomarker for the presence of testicular tissue in male animals. AMH can also be used for diagnosis in young animals – in contrast e.g. to oestrone sulphate assays.
Borderline results are rare. In very old

stallions, the AMH concentration can, however, be severely decreased.

In male animals, the AMH concentration can also be used as a marker for rare Sertoli cell tumours.

Oestrone sulphate:
Can also be used for the diagnosis of cryptorchidism. The test is not, however, informative in horses up to 3 years of age or in donkeys.

Because of this limitation, oestrone sulphate testing has been increasingly replaced by AMH in the diagnosis of cryporochidism.

GnRH stimulation test:
The principle is the same as for the hCG stimulation test: the use of a releasing hormone also allows testing of the hypoathalamic-pituitary axis. This is not necessary for the diagnosis of cryptorchidism, but may be of interest for sub-/fertility questions

Procedure:
- Blood collection (in the morning if possible) = testosterone baseline
- Immediately following: i.v. injection of 0.04 mg GnRH/horse
- +60 min.: blood collection = stimulation value

Interpretation:
Depending on the problem. For cryptorchidism as in the hCG stimulation test. In order to evaluate the pituitary gland, LH/FSH must also be measured. There are no validated routine methods available for this.

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Endometrial biopsies in mares: A safe and simple diagnostic tool

Clinical context
Venereal diseases of mares are important for both professional and enthusiast horse breeding. Comprehensive diagnostic testing is important especially in mares with fertility issues in order to realistically assess the potential breeding success. In addition, e.g. for embryo transfers, only sexually healthy mares can provide an adequate milieu for the conceptus.

Fertility diagnostics are always carried out in a clinical and gynaecological context and can be supplemented by bacteriological, endocrinological, and histopathological testing.

It is important to note that although individual results can provide important indications of possible pathologies, a final reliable prognosis of fertility should ideally be based on the sum of the examination results.

Relevant information on the patient history includes general (e.g. age, use) and specific (e.g. maiden, multi-parous, infertile, number of previous births, abortions, cycle sequence) data.

A clinical **gynaecological** exam includes examination of the internal and external genitalia by adspection, palpation, sonography, and endoscopy. Changes in the external genitals can include e.g. changes in the position of the labia (inadequate closure of the vulvar commissure), which can lead to inflammation. Mares with pneumo- or urovagina often have a chronic vaginitis or cervicitis which can reduce fertility.

The internal gynaecological exam includes e.g. size ratios, symmetry of the uterus and ovaries as well as condition of the uterine wall and contents of the uterus. The exam serves, among other things, to determine the state of the oestrus cycle as well as the diagnosis of potentially endocrinally active ovarian masses.

Common causes of megaovaries are primarily tumours and persistent anovulatory follicles. Granulosa cell tumours are the most common type of ovarian tumour in mares. It is important to note that granulosa cell tumours potentially have endocrine activity, which is often associated with increased **AMH serum** levels.

The significance of endometrial biopsies
Although the diagnosis of gynaecological pathologies in mares has significantly advanced in the past years, many lesions are not detectable by palpation, endoscopy or sonography, and can only be detected by histopathology.

The examination of endometrial biopsies is a reliable and low-risk method which can be used to complement clinical examinations, since it provides information on the current functional status of the endometrium and possible pathological changes.

Depending on the case history, a uterine swab can be collected before the biopsy for microbiological testing for breeding hygiene.

Of 4568 swabs submitted in 2016, unspecific bacteria were detected in 78%, and pathogenic bacteria were found in 22%: ß-haemolytic streptococci (18.4%), *Sc. equi* subsp. *zooepidemicus* (0.1%), haemolytic *E. coli* (1.5%), *Klebsiella* sp. (1.1%), *Staphylococcus aureus*

and *Staph. epidermidis* (0.7%), as well as *Pseudomonas aeruginosa* (0.3%).
Because of the seasonal cycle of mares, there are **two times at which collection of endometrial biopsies is recommended**:

- At the beginning of the season in March/ April (evaluation of the functional state of the endometrium after the winter anoestrus).
- At the end of the season in September (following failure to conceive or early resorption after breeding).

Biopsies can, however, be taken at any point in the cycle and at any time of year. The only contraindication is pregnancy, since sampling can cause an abortion.

Fig. 1: Biopsy forceps (Fa. Eickemeyer)

Sample collection is done with the help of uterine biopsy forceps (see Fig. 1). Depending on the results of the clinical gynaecological exam, samples are either collected from

- at least one location on the dorsal corpus uteri on the border to the cornua uteri

or

- multiple areas with notable endometrial abnormalities.

The biopsies (see Fig. 2) should be at least 1.5x1.0x1.0 cm in size , go deep enough, and should not be from the cervix, since this area is not representative for the entire endometrium. The biopsies should be fixed in 10% buffered formalin directly after collection.

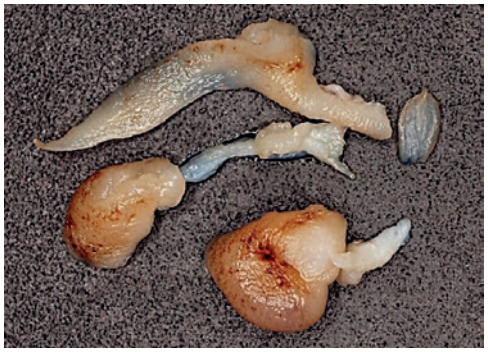

Fig. 2: Endometrial biopsies (formalin fixed)

In addition to the tissue samples, it is important to include a **submission form** with data on the case history and important results of the clinical gynaecological exam (see above), in order to evaluate the histopathological results in a clinical context.

Histopathology of the endometrium and categorization of results
All layers of the endometrium are examined for inflammatory and degenerative lesions as well as abnormal differentiation.

Endometritis
An acute catarrhal endometritis can usually be diagnosed directly during clinical examination based on the secretions, but can be more exactly characterized histologically.

In contrast, subacute or chronic predominantly lymphoplasmacytic inflammation (so called endometritis sicca) can only be detected by histopathology. In nonpurulent endometritis, bacteriological testing is often negative. In infertility with negative clinical and bacteriological results, endometritis is therefore still a possible differential diagnosis and should be ruled out by endometrial biopsy.

While acute catarrhal endometritis – depending on the degree and extent – is generally easily

treatable , endometritis sicca is not always reversible, so the prognosis is more guarded.
Some mares (susceptible mares, as opposed to non-susceptible mares) develop chronic recurring endometritis despite adequate therapy (especially following breeding/insemination), which also causes reduced fertility.

Endometrosis

Endometrosis is a periglandular and/or stromal fibrosis which can gradually affect both individual and multiple glands (nests) (see Fig. 3). These glands have a morphologically divergent epithelium, which is accompanied by a modified pattern of secretion of endometrial proteins, leading to an inadequate supply of the embryo.

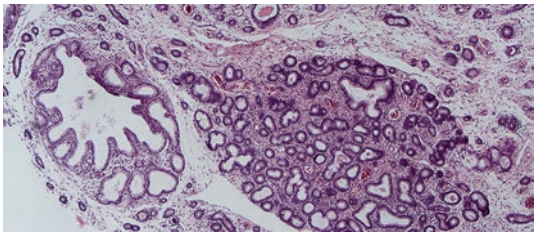

Fig. 3: Nest forming endometrosis

Endometrosis is a progressive disease which increases with the age of the mare – regardless of her previous breeding use. There is currently no treatment available for endometrosis. The case history can provide important indications for diagnosis (see above): In older mares that have remained infertile for two years or more, the probability that they have endometrosis is high.

Abnormal endometrial differentiation

During the physiological breeding season it is possible to differentiate three different forms of abnormal endometrial differentiation:

1) Atrophy

2) Irregular differentiation

3) Uneven differentiation

Pathological changes should be differentiated from temporary irregular endometrial morphologies during the transitional cycle in the fall and spring.
All three pathological forms of endometrial differentiation are a reflection of a dysfunction of the hormonal pituitary-ovarian-endometrial axis, although the exact cause is only found in a minority of cases (e.g. endocrinally active granulosa cell tumours, hormone therapy). The changes are only partially reversible (e.g. by ovarectomy, discontinuation of hormone therapy) and have a variable prognosis depending on their severity as well as treatment options.

Angiopathies

Degenerative and inflammatory diseases of the endometrial blood and lymph vessels can reduce fertility. Degenerative vascular lesions are a common finding, especially in older mares that have had multiple foals (see Fig. 4). They are caused mostly by progressive modification of the vessels during pregnancy.

The result can be reduced perfusion of the endometrium, lymphangiectasia, and lymph lacuna as well as the occurrence of vascular endometrial cysts. Depending on their size, uterine cysts may be detectable by palpation, hysteroscopy, or ultrasonography. Since they inhibit the preimplantation movement of the embryo, they can have an effect on fertility. Although they are not included in the current categorization, they are critically discussed. It is sometimes possible and useful to remove them with the help of a hysteroscope.

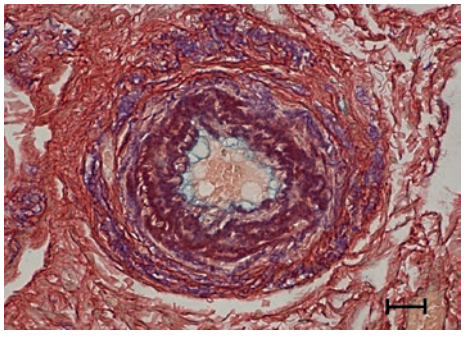

Fig. 4: Severe angiosclerosis

Other findings
Additional and rare findings in endometrial biopsies include e.g. delayed postpartal endometrial involution or hydromucometra, but these are not included in the categorization according to Kenney and Doig (1986).

Categorization of biopsy results
Kenney and Doig (1986) developed a prognostically relevant categorization scheme. This was modified by Schoon and coworkers (Schoon et al., Pferdeheilkunde 8, 1992, pp. 355-362). The factors foaling interval, gradually variable endometritis, endometrosis, and lymph lacuna as well as endometrial atrophy during the physiological breeding season are the histological basis for a statistical evaluation of the fertility prognosis. Depending on the number of relevant diagnoses, the results can be divided into the following categories:

Category I: -Birth rate 80-90%
Category IIa: -Birth rate 50-80%
Category IIb: -Birth rate 10-50%
Category III: -Birth rate <10%

The percentages represent the statistical probability that the mare will conceive and give birth to a live foal.

Additional fertility relevant diagnoses are then evaluated and discussed separately.

Differentiating between reversible and irreversible lesions is also important for the interpretation of the fertility prognosis. Lesions of the endometrium that are generally reversible include e.g. abnormal endometrial differentiation and endometritis. The prognosis improves significantly after successful treatment. It can be helpful to collect a follow-up biopsy in order to determine the success of treatment. In contrast, endometrosis is an irreversible lesion that increases with age and cannot be treated.

It is important to note that other causes of infertility (e.g. salpingitis, husbandry related, endocrinopathies) are not detected by biopsy and cannot be ruled out.

Summary
In summary, it can be said that histopathological examination of endometrial biopsies from mares combined with complementary clinical gynaecological exam is a safe, simple, and reliable diagnostic tool for a multifaceted fertility prognosis.

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Laboratory testing: Equine (extract)

Endocrinology

Anti-Müllerian Hormone

Material: 0.5ml serum (cooled!)
Time frame: Any time (independent of cycle and season)

Oestrone sulphate

Material: 0.5ml serum
Time frame: Beginning 110 days after breeding to 1 week before foaling

PMSG

Material: 0.5ml serum
Time frame: 45-100 days after breeding

Progesterone

Material: 0.5ml serum
Time frame: 18th/19th day after breeding

Fertility

Abortion Profile (PCR)

Material: Abortion material
Parameters: EHV1, EHV4, EVA, leptospira

CEM PCR Profile (stallion) *(Taylorella equigenitalis)*

Material: 3x swab with medium (Amies)
Sampling: 3 locations (penile sheath, urethra, fossa glandis)

CEM PCR Profile (mare) *(Taylorella equigenitalis)*

Material: 2x swab with medium (Amies)
Sampling: 2 locations (fossa clitoridis, sinus clitoridis)

Reproductive Fitness

Material: Swab with medium
Method: Aerobic culture

Endometrial Biopsy +Reproductive Fitness +Mycology

Material: Tissue sample (formalin), swab with medium
Methods: Histology, aerobic culture

Laboratory testing: Equine (extract)

Pathology

Cytology

Material: Swabs, punctate
Method: Microscopic evaluation

Pathohistology

Material: Tissue samples (formalin)
Parameters: Tumour diagnostics, dermatopathology, organ pathology, endoscopic biopsies

Endometrial Biopsies

Material: Tissue (formalin)
Method: Histological examination

Genetics

Warmblood Fragile Foal Syndrome (WFFS)

Heredity: autosomal recessive

Polysaccharid Storage Myopathy Type 1 (PSSM)

Heredity: autosomal dominant

Cerebellar Abiotrophy (CA)

Heredity: autosomal recessive

5-Panel-Test

PSSM (Polysaccharid Storage Myopathy Type 1), GBED (Glycogen Branching Enzyme Deficiency), HERDA (Hereditary Equine Regional Dermal Asthenia), HYPP (Hyperkalaemic Periodic Paralysis), EMH (Equine Malignant Hyperthermia)

Combination Arabian

CA (Cerebellar Abiotrophy), LFS (Lavender Foal Syndrome), SCID (Severe Combined Immunodeficiency)

Material (for all genetic tests): 0.5- 1 ml EDTA blood or mane or tail hairs (with hair roots)

WFFS (Warmblood Fragile Foal Syndrome)

Symptoms and prognosis

WFFS affected foals show signs of an inherited systemic disorder immediately after birth. Clinical signs are comparable to those found in Ehlers Danlos syndrome in humans. The skin lacks tensile strength (extreme skin fragility characterized by tearing, ulceration, etc. after contact with normal surroundings). Lesions occur anywhere on the body, but are most frequently noted at pressure points. In addition to skin wounds, lesions are found on the gums and other mucous membranes of the oral cavity and the perineum. The limb joints are loose and hyperextensible, the fetlocks are especially affected. Affected foals can not stand normally and must be euthanized soon after birth. Premature birth and abortion have also been linked to WFFS.

WFFS affects all breeds of warmblood horses and is an autosomal – recessive disease. This means that foals suffer from WFFS only if they received a copy of the mutated gene from both mother and father.

Study in 2012

LABOKLIN published an initial study on WFFS in 2012. Among 500 randomly chosen Warmblood horses across all Warmblood breeding associations, 47 horses were identified as being carriers of the WFFS gene mutation (N/WFFS). This corresponds to a carrier rate of 9.4 percent. In a horse population in which homozygous animals are not viable, such a carrier rate statistically leads to a risk mating, in which the genotype N/WWFS is paired with the genotype N/WFF, in one out of every 100 cases. Every forth foal from such a risk mating is statistically affected by WFFS. This means for a random mating, that one out of 400 foals will be homozygous (WFFS/WFFS) and will either suffer from the gene defect after birth, which will lead to euthanasia, or will have died prenatally.

Statistics January to June 2018

The most recent figures indicate an even higher carrier rate. Results from all samples analysed in our lab from the beginning of January to the end of June 2018 were used to calculate the prevalence of WFFS. Among the horses tested, 84.8 percent were free of the WFFS mutation (N/N) whereas 15.2 percent of the tested horses were carriers (N/WFFS). Genotype WFFS/WFFS was also found in isolated cases. These cases were not, however, included in the following calculations, because homozygous WFFS/WFFS horses are not viable and therefore are not capable of reproducing. These figures indicate that statistically, 848 animals out of 1000 are free of the WFFS mutation (N/N), whereas 152 out of 1000 horses are carriers (N/WFFS) of the causative mutation for WFFS. A carrier rate of 15.2 percent results in a statistical probability regarding a carrier x carrier pairing of 2.3 percent. 23 pairings out of 1000 are thus risk pairings, from which WFFS/WFFS foals could develop.

Numbers of affected foals from random matings (genotype of parents unknown)

The ratio of free, carrier, and affected animals arrising from a mating of carriers N/WFFS x N/WFFS is 1:2:1. This means that 25 percent of the descendants of such a risk mating are free of the WFFS mutation (N/N), 50 percent of the progeny are carriers (WFFS/WFFS) and 25 percent of the offspring are homozygous (WFFS/WFFS).

One quarter of 23 risk pairings corresponds to 6 affected foals per 1000 random pairings. These foals with the genotype WFFS/WFFS are not viable. If 6 foals out of 1000 pairings are affected, this means that every 170th mating results in a WFFS/WFFS affected foal.

Numbers of affected foals from matings with a carrier (N/WFFS)

If a breeder uses a broodmare with the genotype N/WFFS for mating with a sire of unknown WFFS genotype, the probability for a risk pairing is no longer 2.3 percent, but 15.2 percent. One quarter of the offspring from these risk parings will suffer from WFFS (3.8 percent or 38 out of 1000 foals or every 26th foal of the offspring from a carrier (N/WFFS) broodmare).

This is also true for a stallion with the genotype N/WFFS. Statistically, every 26th foal from such a stallion will be affected by the WFFS/WFFS genotype. It is more than unlikely that a broodmare will carry out 26 foals. Therefore, a carrier genotype N/WFFS can be statistically be overlooked. However, statistically, a remarkable number of descendants of a top stallion will be affected by the WFFS/WFFS genotype, resulting in affected foals or abortion, if the genotype of the mare is unknown.

Knowledge of the genotype avoids WFFS affected foals

If for at least one of the mating partners the genotype N/N is known, all of the descendants will be healthy with respect to WFFS. Pairing of a horse with an N/N genotype randomly with a partner with an unknown WFFS genotype will result in 84.8 percent of pairings in a mating with a partner with the genotype N/N. All foals from such a mating will have the genotype N/N. In 15.2 percent of random matings of an N/N horse, the mating partner will have the genotype N/WFFS. 50 percent of the progeny of such a mating will have the genotype N/N (7.6 percent) whereas the other half of such pairings will result in a carrier genotype N/WFFS (7.6 percent).

Conlcusions:

1) If one of the mating partners is known to have the genotype N/N, a random mating with a partner of unknown genotype in a population with a carrier rate of 15.2 percent will statistically result in progeny in which 92.4 percent of the foals have the genotype N/N and 7.6 percent are genotype N/WFFS.

2) If one of the mating partners is a carrier (N/WFFS), the genotype of the other partner must be N/N to ensure that only healthy foals areborn from the pairing. Assuming a carrier rate of 15.2 percent, 84.8 percent of all potential partners are still available for mating in this case. 50 percent of the progeny will be genotype N/N. The other 50 percent will be carriers (N/WFFS).

3) A pairing of carriers N/WFFS x N/WFFS should be avoided. One quarter of such pairings statistically results in affected foals (WFFS/WFFS).

Limitations of the statistical analysis

These calculations are based on the results of the samples analysed by LABOKLIN from January to June 2018. At this point in time, it is possible that an increased number of suspected cases was submitted to our laboratory for analysis, especially when WFFS was first broadly discussed. If this is the case, the number of WFFS carriers postulated in these analyses would be slightly overestimated. This could explain the discrepancy between the calculated carrier rate of 15.2 percent compared

N/WFFS x N/WFFS	N	WFFS
N	N/N	N/WFFS
WFFS	N/WFFS	WFFS/WFFS

Table 1

to the carrier rate of 9.4 percent determined in the 2012 study. However, an increase in the carrier rate could also be explained if one or more top stallions were carriers who then transmitted the mutations to their offspring. The current statistical analysis is based on the assumption that all animals are equally and randomly paired. That is not, however, usually the case, and breeding partners are selected, resulting in a restriction of the breeding partners used. Due to this selection, the genetic material of some animals is passed to the next generation more often than the genetic material of others, while some horses are excluded from breeding. This selection process has an impact on the outcome of the statistical analysis.

Genetic testing by LABOGEN - Many years' experience with highest quality standards

LABOGEN has many years' experience in developing and conducting genetic tests, especially in the areas of hereditary diseases, colour analyses, DNA profiles and parentage analysis. A distinguishing feature of our high standard of quality is the fact that all of the results of our genetic tests are checked and verified by two experts before being sent out. Our workflows are accredited according to DIN EN ISO/IEC 17025:2005.

This means that our tests are done within a reliable and constantly verifiable framework. As such, we not only undergo a stringent sequence of daily internal checks, but also regular external inspections in the form of cooperative testing.

We store DNA that has been prepared for a genetic test for up to five years. This DNA can thus be used for new genetic tests that become available in future or for parentage testing. In most cases the sample therefore does not need to be resent.

Licenses/patents

Patents exist for both the WFFS and the PSSM (polysaccharid storage myopathy type 1) gene tests. Laboklin is licence holder for both. By paying license fees, we honour the scientific work and the intellectual property of the scientists and support further research.

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