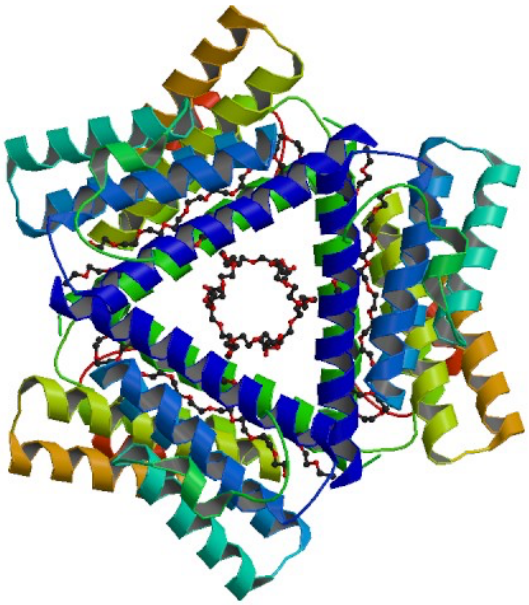
ISACP-ASVCP Pre-meeting Workshop
Oct 30, 2021

Understanding the Crucial Role of Acute Phase Proteins in Veterinary Clinical Pathology



Structure of native human serum amyloid A1. Authors: Lu, J, Sun PD
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PROCEEDINGS

Workshop Moderators

Prof Carolyn Cray, University of Miami

Prof David Eckersall, University of Glasgow

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Understanding the Crucial Role of Acute Phase Proteins in Veterinary Clinical Pathology

Welcome from the Organizers

A tremendous body of literature on acute phase proteins (APPs) in basic and clinical research in animal species shows utility in health assessments, diagnostics, and prognostics. Yet to date, APP testing has not been widely adopted by veterinary clinical pathologists and veterinarians. The primary goal of this workshop is to provide a robust foundation of information on APPs and specific applications in various animal species, led by world leaders in veterinary APP research and diagnostics.

Whether you already use APPs, are ready to implement APP testing, or are new to or just starting to think about APPs, this workshop will serve as an excellent primer and opportunity to learn about methods, reagents, and resources; to understand the challenges and pitfalls from those with firsthand experience in implementing, validating, and interpreting APP assays; and to discover how APPs form an essential part of routine clinical pathology testing.

Recorded presentations by leading APP investigators are available on the ASVCP website. Many thanks to our speakers for their valuable contributions to the proceedings, and to our sponsors for supporting this important educational event.

Please note: questions sent prior to and during the live event will form the basis of highly interactive sessions among attendees and workshop speakers.

To submit your questions prior to the workshop:
https://forms.gle/GCe24wnHGc5gEoup8

On behalf of the ISACP and ASVCP, we welcome you to this exciting and important workshop and hope you enjoy the learning experience and interactions with colleagues. We also hope this workshop will stimulate a new wave of diagnostic applications, collaborations, and research on APPs in veterinary clinical pathology and medicine.

Sincerely,

Carolyn Cray and David Eckersall

ISACP-ASVCP Pre-meeting Workshop, Oct 30, 2021

Understanding the Crucial Role of Acute Phase Proteins in Veterinary Clinical Pathology

Program

8:00-8:15 am – **OPENING REMARKS**—David Eckersall PhD, University of Glasgow, UK

8:15-8:45 am – **SESSION 1: EVIDENCE-BASED APP APPLICATIONS IN COMPANION ANIMALS**
Live Session Moderator Gabriele Rossi
Acute Phase Proteins in Dogs.....***Page 5***
Jose Ceron DVM, PhD, DECVCP, University of Murcia, Spain
Acute Phase Proteins in Cats.....***Page 8***
Gabriele Rossi DVM, PhD, DECVCP, Murdoch University, Australia

8:45-9:00 am — Break

9:00-9:30 am –**SESSION 2: EVIDENCE-BASED APP APPLICATIONS IN LARGE ANIMALS**
Live Session Moderator Stine Jacobsen
Acute Phase Proteins in Swine and Bovine.....***Page 16***
Anna Bassols PhD, Universitat Autonoma de Barcelona, Spain
Acute Phase Proteins in Equine Medicine and Surgery.....***Page 29***
Stine Jacobsen DVM, PhD, DECVS, University of Copenhagen, Denmark

9:30-9:45 am — Break

9:45-10:15 am—**SESSION 3: COMPARATIVE AND TRANSLATIONAL APPLICATIONS OF APPs**
Live Session Moderator Emma Hooijberg
Acute Phase Proteins in Wildlife and Zoo Animals.....***Page 38***
Emma Hooijberg BVSc, PhD, DECVCP, University of Pretoria, South Africa
Acute Phase Proteins in Laboratory Animals.....***Page 50***
Carolyn Cray PhD, University of Miami School of Medicine, USA

10:15-10:30 am—Break

10:30-11:00 am—**SESSION 4: APP R&D, IMPLEMENTATION IN THE LABORATORY AND FIELD**
Live Session Moderator Carolyn Cray
New Assay for Canine SAA & Results in Dogs with Sepsis (SIRS).....***Page 54***
Erica Behling-Kelly DVM, PhD, DACVP, Cornell University, USA
Haptoglobin Assay and Observations across Multiple Species.....***Page 57***
David Eckersall PhD, University of Glasgow, UK
Review of Methods and Reagents from Vendor Partners
Eiken Chemical and Life Diagnostics

11:00-11:15 am—Break

11:15 am-12:30 pm—**SESSION 5: LIVE ROUNDTABLE DISCUSSION AND Q&A**
Live Session Moderators Carolyn Cray & Stine Jacobsen
Voices from the Field—Veterinarians and researchers from across the world who currently use APPs in their practice and studies
Live Discussion Items from Speakers—Expert perspectives • Late-breaking work • Standardization • Future of APP – research & needs

Acute Phase Proteins in Dogs

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Learning Objectives

- Have a global idea of the possible applications of acute phase proteins in dogs
- Understand the importance of the use of a validated assay for the measurement of acute phase proteins
- Know the main acute phase proteins that can be used in the dogs
- Gain knowledge of the potential use that the acute phase proteins can have in the diagnosis and treatment monitoring in the dog

Abstract

In the last years there has been an increase in the practical use of acute phase proteins (APPs), especially the C-reactive protein (CRP) in the dogs. In this line, it is expected that in the next years possible the CRP will have a much wider use and will be consider as a routine analyte in many labs that still have not this analyte available. The main objective of this presentation will be to provide a global view about the possibilities that APPs have in canine practice and some basics about the methods and the way in which APPs can be interpreted and applied. It is expected that this abstract will be useful for those people that are already using APPs but also for those who are planning to use them in the near future.

Introduction

The use of acute phase proteins (APPs) in canine practice is having a major increase in the last years. There are many new companies producing new assays, central laboratories offering them and practitioners using APPs in their routine practice. In the following lines we will review the basis and possible applications of APPs in dogs updating and using as a basis the seven-point plan for APP interpretation that have been previously proposed[1,2]. These points are:

1. Use validated assays. An analytical validation of any assay should be performed, including at least analytical precision, accuracy and, in the case of assays using antibodies, a cross-reactivity test with the APP to be measured. In addition, an overlap performance test with healthy individuals and individuals with an inflammatory condition is recommended before the use of the assay.

The use of heterologous assays (assays originally developed for a different species) still constitutes a cheap and easily available alternative when they are appropriately validated. In particular in the case of canine CRP a commercially available human assay produces a high

precision and accuracy and this could be a cheap and easy way to get involved in the APPs measurements[3].

2. Use APP profiles. Ideally a profile should include at least one positive major, one positive moderate, and one negative APP. Major APPs show an early and high rise in concentration and a rapid decline, whereas moderate APPs require more time to increase and return to normal values. Negative APPs are those that decrease after an inflammatory stimulus. In the dog CRP and serum amyloid A (SAA) are major APPs, whereas haptoglobin (Hp), fibrinogen and ferritin are moderate APPs. In addition to albumin, which is considered a negative APP, other negative APPs such as paraoxonase-1, which is related to oxidative defense and decreases in conditions of oxidative stress, can be included in the profile[4]. A profile involving CRP or SAA, Hp and albumin can be very useful in canine practice.

The use of APPs profiles allows detecting divergences between APPs that can strengthen their use in diagnosis. For example, an increase in Hp concentration in dogs with normal CRP values can indicate the production of increased endogenous glucocorticoids, such as occurs in hyperadrenocorticism[5]. In addition, a decreased or normal Hp value together with an increase in major APPs may indicate hemolysis or hemorrhage[6].

3. The main use of APPs is for the detection of infectious–inflammatory diseases. These diseases produce increases in major and moderate APPs and decreases in negative APPs. Although APPs should be used together with white blood cell evaluation, they are more sensitive than leukocytes in detecting infection and inflammation. Furthermore, APPs have the advantage of being much more stable than cells.

In addition, APPs can detect the activation of inflammation in chronic infectious processes, such as canine leishmaniosis, that usually do not produce changes in white blood cell counts. A recently proposed classification of different clinical stages of canine leishmaniosis was based on APPs and could be applied to other infectious diseases. In this classification, individuals seropositive for the disease, but without clinical signs, can be considered to have active disease if positive APPs are increased[7].

As an additional reflection at this point, special care should be taken to consider whether an animal is really healthy if APPs are not evaluated, since APPs are the most sensitive markers of inflammation. This is especially important in clinical practice for routine check-ups and also for researchers who want to make sure that the animals they are going to use as controls or experimental subjects are healthy. In both of these situations, the measurement of APPs would be highly recommended.

4. APPs have application in clinical diagnosis. APPs are not considered the analytes of choice for making an etiologic diagnosis due to their low specificity. However the magnitude of their increase can be informative and add to their diagnostic usefulness, since very high concentrations of major APPs are usually associated with two main conditions: systemic bacterial disease and immune-mediated processes. In addition, as mentioned in point 2 above, divergences in the response between different APPs can be of clinical use.

Therefore. in some cases APPs help shorten the list of differential diagnoses. Especially when there are nonspecific clinical signs that can be produced by multiple diseases, the changes in APPs can raise the suspicion for an infectious–inflammatory etiology. For example, in cases of lameness, an APP profile can facilitate differential diagnosis between immune-mediated or septic polyarthritis and other conditions that do not produce changes in APPs, such as degenerative joint disease or intervertebral disk displacement[8].

5. APPs have an important application in monitoring treatment. When APPs are measured periodically during the course of a well-defined infectious–inflammatory disease, the return to values seen in healthy animals indicates that the patient is responding to treatment and usually implies a good prognosis. In infectious diseases, usually the return of APPs to low values is faster than for other classical markers used in monitoring treatment, such as globulins, serum protein electrophoresis, or specific antibody[9].

6. APPs have value for predicting the emergence of disease. Changes in APPs in an apparently healthy animal can indicate the presence of subclinical disease or predict the emergence of an active disease in the near future. This constitutes an important advantage to start early treatment. For example, in canine leishmaniosis increases in CRP occur two months before the appearance of external clinical signs[10].

7._____. This point it is left vacant because the future will produce novel findings to increase our knowledge and lead to new recommendations and applications of APPs that will fit perfectly in this space.

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Acute Phase Proteins in Cats

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Learning Objectives

- Understand how to use APPs in cats in clinical setting (e.g., what does it mean when APPs are increased)
- Be able to interpret APPs in cats with specific diseases, mainly FIP
- Use multiple and sequential measurement to objectively assess the efficacy of therapy
- Be able to consider if a higher APP at admission is a poor prognostic factor
- Guide the length of the antimicrobial therapy using the APPs for a shorten administration

Abstract

Despite clinical studies on acute phase proteins (APPs) has significantly increased in the last decade, with most commercial labs now offering major APPs in their biochemical profiles, APPs testing has not been widely adopted by veterinary clinical pathologists and veterinarians. Measurement of acute phase proteins (APPs) concentration is a useful marker for detecting the presence or absence of inflammation in cats with different diseases. APPs can also be reliably measured in different biological fluids (e.g., effusions and urine), to improve their diagnostic utility. Measurement of APPs can be extremely beneficial in cats with feline infectious peritonitis (FIP), to discriminate between FIP and non-FIP cats with similar clinical presentations. More benefits come from multiple and sequential measurements of APPs, particularly for the assessment of the efficacy of the therapy. The APPs are more sensitive than WBC counts for early detection of inflammation, to demonstrate an early remission as well as the recurrencies of the diseases. There is a huge potential for the use of APPs, and more studies are warranted with a particular focus on the applications of APPs to guide the length of antimicrobial therapies, as suggested by the antimicrobial stewardship policy. New inflammatory markers have been discovered in human medicine, with a higher specificity for the distinction between septic versus non-septic inflammatory diseases. It is desirable that these new markers will be investigated in veterinary medicine, to further test the power of APPs in diagnostic setting.

Introduction

The use of acute phase proteins (APPs) in clinical setting has significantly increased in the last decade, with most commercial labs now routinely including the measurement of APPs in their

biochemical profiles. Thanks to intensive research we have now a good understanding of the potential roles of APPs, from diagnosis to prognosis and treatment monitoring. Moreover, there are now multiple species-specific assays developed for veterinarians, including rapid point-of-care assays, which facilitate the measurement of APPs in any clinical setting. Nevertheless, APPs are still underused in veterinary medicine compared with human medicine; maybe because there is a lack of education on the multiple potential use of APPs in clinical setting.

Biology and Kinetics of APPs

The APPs are blood proteins, synthesised in the liver in response to release of pro-inflammatory cytokines as part of the acute phase reaction (APR).[1-3] The term acute phase reaction describes a series of pathophysiological events that occur in animal exposed to infection, inflammation, trauma or other stimuli. APR begins within inflammatory sites, where cells involved in the innate immune response (i.e. macrophages and, to a lesser extent, neutrophils) produce and release pro-inflammatory cytokines (mainly IL-6, IL-1 and TNF-α).[4] These cytokines influence organs involved in homeostasis, such as nervous system and endocrine glands to establish a rapid and intense protective and reactive response. Cytokines are also responsible for the common clinical signs observed during systemic inflammation, e.g. fever, lethargy and anorexia.[4] The acute phase response also includes changes in the concentrations of plasma APPs, some of which decrease in concentration (negative APPs; e.g. albumin, transferrin and PON-1 activity) and others increase in concentration (positive APPs; e.g. serum amyloid A, alpha-1-acid glycoprotein, haptoglobin, etc.).[1-3, 5] Therefore, the APPs can be used to assess the innate immune system's systemic response and to differentiate local and systemic inflammatory diseases. In any given species, particular APPs demonstrate 'major', 'moderate' or 'minor' responses.

A major APP 'responder' has a low serum concentration in healthy animals that rises dramatically >10-fold soon after the stimulation, peaking at 24-48h and then declining rapidly during the recovery phase.[2] Moderate responders increase 5-10-fold on activation, peak after 2-3 days and decrease more slowly than major responders.[2] The major APP in cats is serum amyloid A (SAA), moderate APPs are α1 acid glycoprotein (AGP) and C-reactive protein (CRP), while haptoglobin (Hp) and ceruloplasmin are minor APPs with only 50-100% increasing from the base value.[6]

APPs have different functions, not all clearly defined, but they shared an overall role in the modulation of the immune response. The biological functions of AGP include the inhibition of lymphocyte proliferation,[7] inhibition of platelet aggregation and inhibition of neutrophilic function (such as phagocytosis, chemotaxis, and superoxide generation).[8-10] Because AGP is a protein heavily glycosylated, it has been hypothesised a pathological role of the desialylation in the immunomodulatory function of AGP in cats with feline infectious peritonitis (FIP).[11] The biological function of the SAA is also not clearly defined; among his functions acts as a scavenger of oxidised metabolites, protecting tissues from excessive damage induced by inflammation.[12] It may also play a role in down-regulation of the inflammatory process by inhibiting myeloperoxidase release and direct migration of phagocytes.[13] Haptoglobin plays a critical role in tissue protection and prevention of oxidative damages binding the highly toxic

free haemoglobin.[14] Haptoglobin has also an inhibitory effect on granulocyte chemotaxis, phagocytosis and bacterial activity.[15]

The most investigated APPs in cats are SAA and AGP as they are commonly used by clinicians and researchers because SAA is the major APP in cats and there are multiple reliable species-specific assays that can be used. AGP is only a moderate APP in cats, but due to its higher specificity for diagnosis of FIP, it has been extensively used and investigated.

Kinetics of SAA and AGP has been investigated in cats subjected to two different experimental surgeries: ovariohysterectomy or gastrotomy.[16] Both AGP and SAA peak 1 to 2 days after the surgery (irrespective if spaying or gastrotomy), but SAA returned to normal values quicker (within 5 days) than AGP, which remained persistently above the reference interval for several weeks.[16] Another study demonstrated an earlier increasing in SAA concentration in cats after spay with a first increase within 3 to 6 hours after the surgery and the pick at 24 hour.[17]

As expected, the magnitude of increase is higher for SAA (major APP) than for AGP (only moderate); SAA increased up to 27-fold the pre-surgery concentration and the concentration is higher in cats after the most invasive surgery (gastrotomy) than in spayed cats.[16] Thus, the increase of SAA concentration is proportional to the severity of the inflammation. AGP increased only up to 4-fold the pre-surgery concentration in cats subjected to both surgeries. Haptoglobin is a minor APP in cats and its concentration decreases slowly in serum, compared with other major and moderate proteins.

Diagnostic Role of APPs

APPs are expected to increase in any pathological condition characterised by release of pro-inflammatory cytokines and systemic inflammation, such as infection, trauma, tumours, and surgery.[17-24] When the comparison is between healthy cats and cats with various pathological conditions, the SAA concentration is significantly higher in cats with inflammation than in the healthy cat group ($p < 0.001$).[17, 25] Specifically, SAA concentrations is higher in cats with confirmed diagnosis of inflammatory diseases such as upper respiratory tract infections, pneumonia, pyometra, and feline infectious peritonitis, than SAA concentration observed in healthy cats. Conversely, no increase was observed in cardiomyopathy, hyperthyroidism, and diabetes mellitus because systemic inflammation is not part of their pathogenesis.[25] Cats with inflammatory bowel disease (IBD) and small-cell gastrointestinal lymphoma had higher serum haptoglobin concentration than healthy cats. Nevertheless, in cats with lymphoma, haptoglobin concentration was similar to cats with IBD, but it could not differentiate between the two diseases.[22] Therefore, measurement of SAA concentration is a useful marker for detecting the presence or absence of inflammation in diseased cats.[25] Unfortunately, unlike in human patients,[26] APPs are not able to discriminate between septic versus non septic inflammation.[24] Serum AGP concentration is significantly higher in cats with tumours than in healthy cats, but there are no differences between different neoplasia (carcinoma, sarcoma, round cell tumours).[20] While APPs have high sensitivity and specificity for detection of systemic inflammation, they are poorly specific for differentiation between different inflammatory conditions.[25]

There are some exceptions: APPs play a crucial role in the diagnosis of feline infectious peritonitis (FIP) in cats. A definitive diagnosis of FIP is often challenging due to different clinical presentations (wet versus dry form) and most existing diagnostic tests cannot differentiate between feline enteric coronavirus and feline infectious peritonitis virus, and especially in cats without body cavity effusions, it is often difficult to reach a definitive diagnosis ante mortem.[27] AGP (but not HP) concentration is higher in feline serum from patients diagnosed with FIP than cats with clinical signs consistent with FIP (e.g. peritoneal effusion), but which were determined by histopathological examination not to be suffering from FIP and also higher than cats with other diseases characterised by systemic acute inflammation.[18] Specifically, a serum AGP concentration >1.5 g/L has 85% and 100% specificity in differentiating cats with FIP and clinically similar conditions and the overall efficiency is 90%.[18] Another study, confirmed that AGP is a powerful marker to discriminate between FIP and FIP-like conditions, but a higher cut-off is necessary when the pre-test probability of FIP is low. Specifically, when the pre-test probability of FIP is high, based on history and clinical signs, moderate serum AGP levels (1.5–2 mg/ml) can discriminate cats with FIP from other FIP-like conditions, while only high serum AGP levels (>3 mg/ml) can support a diagnosis of FIP in cats with a low pre-test probability of disease.[28]

APP can also be measured on different biological fluids; in cats with FIP the measurement of SAA and AGP can be measured in the peritoneal effusion. Similarly to serum, concentrations of the APPs (SAA, AGP and Hp) in effusion are significantly higher in cats with FIP than in cats without FIP but similar clinical presentation.[29] The best APP to distinguish between cats with and without FIP was AGP in the effusion; a cut-off value of 1550 µg/ml had a sensitivity and specificity of 93% each for diagnosing FIP.[29]

Prognostic Role of APPs and Evaluation of the Response to Treatments

Additional benefits from APPs could be gained from multiple, sequential measurements in sick animals as early markers for recurrences of diseases or as objective markers to drive the duration of the therapies. Because APPs are not stored and major APPs have a short half-life, as soon as the inflammatory stimulus ceases, it is expected a rapid decline in their serum concentration. A good example on how to use sequential measurements is shown in a cat with pancreatitis. SAA concentration was increased at the onset of the disease and gradually decreased over 5 days of treatment (with plasma transfusion, fluid therapy, prednisolone and antimicrobials). The decrease in SAA concentration paralleled the improvement of the clinical conditions, but other markers of pancreatitis (such as fTLI and WBC count) did not followed the same trend and remained increased for the whole time course examined (5 days). Therefore, SAA and other APPs could be used for early identification of treatment response. Moreover, the long-term monitoring (>2 years) of the same cat, revealed strong correlation between increased SAA concentration and the reoccurrence of clinical signs. On the contrary, WBC count did not increase with the reoccurrence of pancreatitis.[30] Another good example of a possible clinical use of APP in clinical setting is in cats treated with recombinant feline interferon-ω for retroviral infections (e.g. FIV or FeLV). Given the immunomodulatory functions of acute phase proteins, an increased in their concentration is expected if the therapy is able to stimulate the

immune system. SAA, AGP and CRP were significantly increased in cats undergoing interferon-ω therapy during and after the therapy.[31] A study on cats with various form of lymphoma showed a gradual decrease in serum AGP concentration after 4 weeks and in serum SAA concentration after 8 weeks of treatment, with values comparable to those of healthy cats by 12 weeks of treatment, by which all cats achieved complete remission of the disease.[32]

The role of APPs as prognostic markers is variable; in one study performed in cats with different diseases, the survival time at 180 days did not differ based on the SAA concentration at admission, suggesting that a single SAA measurement may not be useful as prognostic marker.[25] Similarly, in cats with lymphoma, serum AGP concentrations did not correlate with either remission duration or survival time.[21] In cats, congestive heart failure (CHF) is an inflammatory disorder and AGP, SAA and ceruloplasmin are higher in cats with CHF. While those APPs are not early markers (they cannot distinguish between pre-clinical cardiomyopathy and CHF), serum AGP concentration is an independent poor prognostic factor in CHF cats (hazard ratio=40.2).[33]

Analytical Aspects

With the exception of haptoglobin, which could be reliably measured using a colorimetric assay, the majority of APPs required immunoassay reagents for an accurate quantification. Although, some APP assays for humans have been automated also for veterinary medicine, species-specific tests are still limited. Interspecies APP variations and the limited availability of cross-reactive reagents could be partially responsible for the low routine determination of APP in veterinary labs, especially for cats.

While numerous ELISA assays are available, they are a good tool for research purposes, but are not compatible with auto-analysers, which are commonly used in clinical laboratories. Currently, other than with ELISA, APPs can be measured using radioimmunoassay, immunoturbidimetry and nephelometry. In recent years, due to increasing interest in APPs as part of the screening of animals' health, new species-specific assays have been developed with a special aim to adapt them to the common biochemical analysers.[34, 35] A particular effort is currently focused on developing point of care tests that can be easily used in clinical setting, providing quick and accurate measurements.

Future Perspectives

The future clinical applications of APPs in veterinary medicine most likely will mimic human medicine. In human, as well as in veterinary medicine, the measurement of a single APP (even when sequential measurements are performed) has not sufficient predictive accuracy to rule in or rule out sepsis in every clinical situation. Therefore, to improve the diagnostic capability of adjunctive diagnostic test, a number of authors have combined some of these tests into sepsis screens, with up to 5 different inflammatory markers.[36] A combination of multiple inflammatory markers will be the future in veterinary medicine, most likely with point-of-care assays able to provide simultaneous results.

Another future application for APPs is to guide the length of antimicrobial therapies, according to the antimicrobial stewardship core principles and policy; specifically, for a continuous evaluation of the outcomes of therapy.[37] A good example comes from a study in dogs with bacterial pneumonia: when normalization of serum CRP is used to guide the duration of antibiotic treatment, treatment duration is significantly decreased without increasing the number of relapses.[38]

Research of new inflammatory markers will also improve the diagnostic ability of clinicians. Recently, a new inflammatory marker has been investigated: paraoxonase-1 (PON-1) activity. An enzymatic test to quantify PON-1 activity in feline serum has been validated and it correlates with AGP (but not SAA).[5] PON-1 has a diagnostic role in cats with FIP and can accurately discriminated FIP from similar clinical conditions.[39] In neonates with sepsis, PON-1 activity measured at enrolment correlated significantly with serum Amyloid-A, CRP and IL-6 and could also discriminate septic than non-septic neonates. Therefore, PON-1 activity is a promising biomarker of neonatal sepsis.[40] Procalcitonin is a sepsis marker in human medicine and high procalcitonin levels in cats has been associated with bacterial infection. Hence, procalcitonin could be a valuable marker for diagnosing bacterial infections in cats.[41] Other new sepsis markers are currently under investigation in human medicine and most likely they will be tested in veterinary medicine, as well. A good example of clinical applications of new biomarkers in human medicine are the preterm infants because they are particularly susceptible to bacterial late-onset sepsis (LOS). Diagnosis by blood culture and inflammatory markers have sub-optimal sensitivity and specificity and prolonged reporting times. Secretory phospholipase A2 type IIA (sPLA2-IIA) plasma levels were elevated in infants with LOS compared to those without LOS with a sensitivity of 90.7 and specificity of 80.4. Thus, sPLA2-IIA may have clinical utility for the early diagnosis of LOS in very preterm infants, potentially informing clinical management and antibiotic stewardship.[42] Given that sPLA2-IIA is well conserved protein, it would be interesting to investigate this new marker in veterinary species.

Conclusions

Increased APPs concentration in feline serum are a useful diagnostic tool to demonstrate an ongoing acute systemic inflammation. Generally, APPs cannot discriminate between different pathological processes; however, FIP is an exception: particularly high AGP concentration in serum or effusion can discriminate between FIP and other conditions with similar clinical presentation. One of the main advantages of the APPs is their ‘real-time’ secretion due to cytokine-dependent pathways. Because APPs are not stored, as soon as the inflammatory process is in regression, APPs progressively decrease to baseline values. For that reason, APPs are extremely useful to assess the treatment response, bearing in mind that several treatments are symptomatic. Specifically, most of the clinical symptoms are mediated by prostaglandins and several therapies work interfering with phospholipase and COX pathways. For example, FANS or low dose of corticosteroids administration reduce prostaglandins release only, without any interference on the inflammatory pathway. A higher dosage of steroid will also reduce cytokine release, and thus decrease APP synthesis. Therefore, the improvement of clinical symptoms not necessary is related with the resolution of the inflammatory stimulus and APPs are the ideal marker to evaluate the real-time evolution of the inflammation.

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Acute Phase Proteins in Swine and Bovine

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Learning Objectives

- Main acute phase proteins (APP) in bovine and porcine
- Main methodological techniques for APP quantification in swine and cattle
    - The importance of harmonization
- Main applications of APP in bovine
    - The transition period
    - Metritis
    - Mastitis
    - Respiratory and gastrointestinal problems
    - Performance, Growth, Nutrition and Stress
- Main applications of APP in porcine
    - Acute phase proteins in inflammatory and infectious processes
    - Acute phase proteins as indicators of productive parameters
    - Usefulness of APP in nutritional studies
    - Acute phase proteins in slaughterhouse pigs as health status markers
    - Characterization of the APP response in stress situations
    - Other sample types: Determination of APP in saliva
- Reference intervals
- Conclusions

Abstract

The major acute phase proteins in bovine are Hp and SAA, and in porcine Hp, SAA, CRP and Pig-MAP. Many methodological assays are presently available for these parameters, which are still being improved to increase their specificity, sensitivity, user-friendliness and price. In bovine, the main applications are the diagnosis and monitoring of frequent pathologies as mastitis and metritis in dairy cows, and respiratory problems in young calves. In porcine, APP are useful in the control of bacterial and viral infections, and they may be used at the slaughterhouse to monitor subclinical pathologies and improve food safety. The utility of APP in animal production must not be forgotten: optimization of protocols to improve performance, welfare and nutrition may benefit of the use of APP. Other sample types besides serum or plasma have interesting qualities: determination of APP in milk is a powerful tool in the control of mastitis, saliva is a non-invasive sample type and meat juice is easily obtained at the slaughterhouse. Increasing our knowledge on reference intervals and the influence of variables such as age, breed, sex, season etc, is an important issue. Finally, worldwide harmonization and standardization of analytical procedures will help to expand the use of APP.

Main Acute Phase Proteins (APP) in Bovine and Porcine

APP are classified as positive (major, moderate or minor) and negative depending on either their concentration is increased or decreased in serum during a certain experimental condition. There is quite a consensus in which are the most relevant APP in cattle and swine[1–6].

Species	Major	Moderate	Minor	Negative
Cattle	Hp, SAA	AGP, ITIH4, CRP	Fb	Albumin, Apo-A1
Swine	Pig-MAP, Hp, SAA, CRP	AGP	Fb	Albumin Apo-A1

Main Methodological Techniques for APP Quantification in Swine and Cattle

The main characteristic for determination of APP and other analytical parameters in farm animals, and the difference with companion animals, is that usually a large number of samples has to be determined at the same time. This requires that techniques should be as automatable as possible.

Type of analysis	**APP**	**Advantages**	**Disadvantages**
Colorimetric	Haptoglobin	Simple, multispecies, basic equipment Easy to automatize	Some interferences Calibration unstable
ELISA	Haptoglobin, CRP, Pig-MAP, ITIH4, SAA	Species-specific	Species-specific Laborious High dilutions
Immunoturbidimetric	Haptoglobin, CRP, ITIH4, Pig-MAP	Species-specific Easy to automatize Good reproducibility	Species-specific No or low dilutions Lower CV than ELISA
Radial Immunodiffusion (RID)	SAA, Hp, ITIH4	Species-specific	Species-specific Manual Low number of samples
Point of care	SAA, CRP, Hp	Species-specific On-site testing Hp, MAA also for milk	Species-specific

More recently, other technologies and biosensors have been adapted, but they are outside the scope of this review.

The importance of harmonization

Harmonization and calibration to standardize different methodologies is one of the most important aspects to extend the use of APP and it is an indispensable requirement for the final transference of all the knowledge to the field, as well as the preparation of reference materials to be used for the calibration of the different reagents[7].

We have been involved throughout the years in the preparation, optimization and validation of several assays based on immunoturbidimetry, in collaboration with the Spanish company Acuvet[8–10].

Main Applications of APP in Bovine

APP have been studied in relation to many pathologies[11,12], but only some of them are going to be summarized here.

The transition period

In the dairy cow, the transition period is the period 3 weeks before through 3 weeks after calving. This period is associated with a high incidence of pathologies as ketosis, mastitis, hypocalcemia (milk fever) and reproductive problems. Risk of health problems increases during this time, and is one of the main reasons for involuntary culling. Proper management can significantly reduce incidence of disease, meaning that a control of the animals is required and the determination of APP can help in achieving it. When studying associated pathologies, it has to be taken into account that normal calving induces an acute phase reaction on the following days post partum[11].

Metritis

Metritis is produced because bacteria colonize the external membranes exposed to the external media during parturition. The incidence of metritis is high since between 5 and 20% of cows experience metritis, and consequently these conditions are costly for the affected cows and the farmer[13].

Recently, we have carried out an study on dairy cows in the transition period and the usefulness of Hp and ITIH4 determination in healthy cows and cows with mild, moderate and severe metritis. Cows with severe or moderate metritis had greater Hp concentrations than healthy cows between D0 and D+10 post partum. Clinical signs of pathological discharge for the mildly and severely metritic cows did not occur until later. These results indicate that an acute phase inflammatory response precedes clinical metritis. Similar results were reported in [14]. This association may occur also to chronic subclinical endometritis and decreased reproductive performance [15]. In conclusion, Hp screening may assist in the early detection of metritis, providing increased opportunities for early treatment and prevention.

Mastitis

Mastitis is recognized as being one of the most important reasons for culling dairy cows. Up to now the evaluation of somatic cell count (SCC) has remained as the gold standard for determining udder health. However, APP are useful as either a valuable supplement or even as an alternative to SCC.

In mastitis, there is a general increase in serum APP. The most relevant are Hp[11] and SAA[16], although CRP, ITIH4 and LBP have been also identified[17]. APP can also be quantified in milk, which could be advantageous. The SAA isoform present in milk is SAA3 (or milk amyloid A,

MAA). SAA3 and Hp are synthesized locally in the mammary gland. No association exists between APP in serum and milk, especially for SAA[18]. It has to be taken into account that APP are physiologically present in colostrum and milk during the first days of lactation[19].

In comparison to SCC, the values of the three APP (Hp, SAA3 or CRP) in serum are able to distinguish subclinical mastitis samples from healthy samples (SCC below 200,000 cells/ml). In milk, SAA3 and Hp allow to differentiate samples from healthy quarters from those with mastitis which are not showing clinical signs of intramammary inflammation[19]. Thresholds for these proteins have been published[20].

The etiological pathogen of mastitis influences the clinical form of the disease, its severity and its treatment. For example, *S. aureus* is particularly problematic due to its resistance to antibiotic treatments and ability to reside within mammary tissue in a subclinical state. Likewise, the secretion patterns of APP (Hp, SAA3 and CRP) into milk ultimately reflects the kind of pathogen[21–24].

Respiratory and gastrointestinal problems

This is especially important related to young calves. Normally, these animals are subjected to marketing situations where calves of different origins and health status are mixed, and where provision of milk or solid feed is not always suitable, followed by long transportation hours until arrival to the rearing facilities. At arrival, calves are not able to consume the amount of milk and solid feed to cover their requirements, and the incidence of disease spikes. The most common disease is the bovine respiratory syndrome (BRD) aggravated by the stress suffered during transportation[25]. The most common agents found in association with BRD are viruses such as bovine respiratory syncytial virus (BRS), parainfluenza-3 virus, adenovirus and bovine coronavirus. However, secondary bacterial infections are common[26]. Besides the welfare and economic problems associated to this, transport and commingling is one of the most important causes for the use of antibiotics in cattle.

Measurement of Hp, LBP and SAA have been studied as tools to detect BRD in feedlot conditions and the combination of high values together with fever was found to be a very good indicator of disease[26–29].

In conclusion, APPs (SAA, LBP and Hp) are sensitive markers of respiratory infection, and they may be useful to explore host response to the respiratory infections in clinical research. Concomitantly, determination of APP at feedlot arrival may be also a tool to evaluate the subsequent performance and mortality rates of calves since high Hp concentration inversely correlated with dry matter intake, BRD rate and body weight. Furthermore, calves with elevated serum Hp concentrations had higher odds of being treated with antibiotic, and thus Hp could be useful for making decisions regarding targeted prophylactic treatment[30].

Performance, growth, nutrition and stress

In large animals, there is an intense investigation on the acute phase response triggered off by the stress related to several conditions, such as transport, feeding or housing conditions. These

studies are valuable in order to determine the animal welfare status of the herds in order to both improve the production and obtain products of higher quality.

The effect of weaning, transportation and commingling on measures of the APP response has been studied and Hp has been proposed as the most sensitive APP to monitor body weight increase in order to improve the management procedures of young calves. Thus some authors have suggested that early weaning in the adequate management conditions may more tolerant to the stressors associated with transportation and feed yard entry[31–33].

In our laboratory, we have studied how APP can reflect the overall welfare status of cows living in different conditions. Thus, we have analyzed the serum biochemical profile of Bruna dels Pirineus and Alberes breeds living under different systems of housing and feeding, and SAA and Hp were increased in groups living in hard conditions[34,35]. APP have been also used to study the immune system activation in nutritional studies, as in supplementation with amino acids[36].

Main Applications of APP in Swine

Acute phase proteins in inflammatory and infectious processes

In pigs, acute inflammation has been induced in non-infectious experimental models, using the subcutaneous application of turpentine[37]. This model has made it possible to characterize the acute phase response in this species and to describe that the proteins major APP in swine are Hp, CRP and Pig-MAP. In the swine species there is a large number of experimental studies with bacterial or viral infections. Besides the diagnostic, prognostic or etiopathogenesis purposes, APPs have been also studied in experimental studies as tools for the evaluation of antimicrobial and anti-inflammatory agents, as well as the efficacy of vaccines. In the farm control programs aimed to improve the quality of pig production, some studies have supported the idea that the analysis of Pig-MAP in one sub-sample of a group of pigs might be a useful tool in routine health and welfare monitoring[38].

In natural infections, the response of APP is, in general, greater in animals that present clinical symptoms and usually of greater magnitude in bacterial diseases or viral infections concurrent with bacterial infections[39].

Bacterial infections

In general, the Hp and Pig-MAP plasma concentration increase rapidly and for a long time, whereas CRP and SAA show very rapid increases but normal values are restored earlier, especially in the case of SAA. The kinetics of the response have been analyzed in experimental infections with *S. suis*, *A. pleuropneumoniae*[40–42], *Y. enterocolitica*, *P. multocida*[43], and *H. pararasuis*[44]. APP concentrations correlate with the degree of clinical affectation of the animals and in some cases they are detected before the specific antibodies against the bacteria[40].

Viral infections

The APP response in case of viral diseases is more heterogeneous and it would depend on the innate response the virus is able to arise.

Two of the diseases that currently cause a significant economic impact on pig farms are Porcine Reproductive and Respiratory Syndrome (PRRS) and Postweaning Multisystemic Wasting Syndrome (PMWS), caused respectively by PRRS virus (PRRSV) and porcine circovirus type 2 (PCV2).

PRRSV is characterized by presenting a weak innate immune response with low production of pro-inflammatory cytokines and, above all, a very low production of interferon gamma (IFN-γ). Therefore an efficient acute phase response is not induced[5]. CRP and Hp are the most sensitive APP[45,46]. The importance of the viral strain is clearly demonstrated in our analysis of the response to several isolates of PRRSV, which demonstrated that the APP response (Hp, CRP and at a lower degree Pig-MAP) was heterogeneous and dependent on the strain, but correlated to the severity of the clinical signs[47]. In PCV2-infected pigs, Hp and CRP have been found to correlate to PCV2 viremia and the clinical course of the disease[48].

Swine influenza virus (SIV) is one of the main etiological agents that produces respiratory diseases in pigs. Experimental studies designed to evaluate the acute phase response have showed increases in the serum concentrations of CRP, SAA and Hp, being Pig-MAP less sensitive. The increase correlated to the severity of clinical signs and lung lesions[49].

In infections caused by the African swine fever virus (ASFV), Aujeszky's disease virus (ADV) or classical swine fever virus (CSFV), different response patterns were observed. In general, Hp, SAA and Pig-MAP increase more than CRP in these conditions, and changes evolved in parallel to the viremia levels[50].

A lot of information exists also for coinfection with more than one pathogen and, in general, APP increased correlated to the severity of the clinical signs, which were more severe than in single-pathogen infections[51].

Vaccination

In general APP are not useful indicators of the efficacy of a vaccine, but can be used as biomarkers in vaccine nonclinical safety studies and help to choose the vaccination protocol that produced a lower inflammatory reaction in the individual. Our own results indicate that a vaccine against PCV2 that only caused a very mild and temporal increase in rectal temperature did not induce an increase in Hp either[52]. In a study on vaccination for *L. intracellularis*, a reduced Hp response was observed in vaccinated pigs after challenge with the bacterium, indicating that the vaccinated pigs were less affected than the non-vaccinated pigs[53].

Parasites

The APP response in case of parasites is heterogeneous. As example, in the case of experimental infection with three species of *Trichinella* the increase in Pig-MAP, CRP, and Hp was mild and transitory, and none of these proteins was increased in all experimental groups of pigs at the same time point after infection[54].

Gastrointestinal diseases

More recently, we have analyzed whether the acute phase protein Pig-MAP correlates with the gold-standard measure for intestinal wall integrity, i.e., the ratio between villi and crypt (V:C

ratio) and with other inflammatory markers (TNF-α) or markers for mucosa integrity (serum i-FABP). Our results showed that indeed Pig-MAP and TNF-α inversely correlated to V:C ratio[55].

Acute phase proteins as indicators of productive parameters

APPs have been frequently proposed as biomarkers to assess zootechnical performance, i.e., there is an inverse correlation between serum levels of APP and productive parameters (the better the productive parameters, the lower APP serum concentrations). The reason is that, where there is an stimulation of the immune system, there is an increased production of pro-inflammatory cytokines and APP. This is accompanied by reduced growth performance due to anorexia as well as the partitioning of nutrients away from growth to support the immune system.

As early as in 1992, it was proposed that the determination of the Hp concentration in serum of pigs at 7 weeks of age can be an indicator of the future weight gain[56] Similar results were described in 24-week-old animals[57] and in animals in the post-weaning period[58]. Thus, Hp may be a surrogate marker for reduced pig growth in a study comparing several nursery feeding programs in pigs raised in commercial farms[59]. In our laboratory, low serum Hp was an indicator of ADWG (average daily weight gain) throughout the growing and fattening periods of the pigs[52].

More recently, it has been proposed that α-1-acid glycoprotein (AGP) can be used as an indicator of growth potential in newborn pigs, which have a very high AGP compared to older pigs[60].

APPs may be very useful as non-specific biomarkers to detect disturbances in critical phases of life, as for example the influence of suboptimal outside climate[61].

Usefulness of APP in nutritional studies

Sub-therapeutic doses of antibiotics have been used to prevent infectious diseases, improve pig performance and reduce medication costs. However, due to the association of the use of sub-therapeutic doses of antibiotics in feed with the increasing emergence of antibiotic-resistant bacteria, the use of antibiotics as feed additives has been banned in the European Union. In this scenario, the latest research indicates probiotic supplementation in pigs to be a better alternative to infeed antibiotics. In this context, APP have been used to control the status of the immune system in efficacy studies of probiotics and immunomodulators[62–64].

Likewise, serum Hp has been used to evaluate the effects of diet on the immune status in studies about supplementation with amino acids during disease challenge[65] and in diets supplemented with essential amino acids[66].

Acute phase proteins in slaughterhouse pigs as health status markers

APP have been also used as a complement to ante-mortem and post-mortem inspection of animals. This could offer information on the degree of well-being and health of the farms from which the pigs come, taking into account that apparently healthy animals that arrive at the slaughterhouse may present a chronic state of disease. It has been seen that the Hp

concentration in pigs from farms with different health states show different mean values[67]. Therefore, it is considered that the determination of Hp in the slaughterhouse can be of potential use to identify problems on farms[68].

Cranio-ventral pulmonary consolidation (CVPC) and pleuritis are the most common pathological lesions found in the lungs of pigs at the slaughterhouse, with *M. hyopneumoniae* and *A. pleuropneumoniae* being the most important primary respiratory pathogens, respectively. These pathologies are associated with significant economic losses. At the slaughterhouse, Hp concentration was significantly higher in animals with CVPC lesions compared to animals without these types of lesions[69]. Significant differences in CRP and Hp concentrations have also been described in animals without clinical symptoms but with pulmonary lesions[70]. Our own study on 24 farms with a register of CVPC and pleuritia indicated that Pig-MAP and possibly Hp are potential markers to characterize and discriminate respiratory lesions at slaughter [71]. Elevated Pig-MAP was found in blood samples obtained at slaughter in association with the carcass inspection[72]. An extended study indicated that APP could also be valuable as a predictive indicator for risk assessment in meat inspection, as increased Hp and Pig-MAP concentrations in slaughter blood indicate a 16 and 10 times higher risk for organ abnormalities, respectively[72].

APP in the meat juice

In addition to APP measurements in blood serum or plasma, other fluids can be interesting for practical applications. Meat juice might be an adequate matrix in which the health status of the animal is reflected and can be easily obtained at slaughter for end-point analysis. The concentrations of Pig-MAP and Hp in meat juice are closely correlated to those in plasma[73]. Later work supports the usefulness of CRP and Hp in identifying carcasses with organ alterations with high sensitivity and specificity[74]. These results open new possibilities for assessing animal health in pig production, with implications for food safety and meat quality.

Characterization of the APP response in stress situations

Swine species are generally exposed to stressful environmental conditions that can affect the immune response and increase the risk of disease. Transportation, social stress, heat and food changes are some of the main causes that produce stress. In response to stress, the hypothalamic-pituitary-adrenal (HPA) axis is activated, stimulating glucocorticoid production in the adrenal gland. Although the traditional view is that glucocorticoids are homogenously anti-inflammatory, there is an increase in proinflammatory cytokines when there is a cross activation of the immune system and glucocorticoids[75].

The welfare of the animals during transport and lairage in the slaughterhouse is closely related to the quality of the meat. The increase in APP depends on the duration of transport since Pig-MAP and Hp increased in longer transports whereas CRP and SAA are increased after shorter transport, probably because they are faster responding proteins[76–79]. Alterations in the feeding pattern can also affect animal welfare and can cause stress and induce higher levels of Pig-MAP, Hp and CRP[80].

Stress in pigs has been widely studied in our laboratory. Pigs that are housed in high-density pens have higher Pig-MAP concentrations than those that are in larger spaces[81]. Sows have commonly been housed under field conditions in individual stalls throughout pregnancy because it eases animal handling, reduces social stress and allows appropriate feeding. This individual housing system has been considered to be stressful and harmful for animals consequently, this practice has been banned by the European Union (CD 2001/88/EC). Our work in gilts demonstrated that Hp and Pig-Map were good markers for this kind of stress[82]. In our work on mixing stress and human-animal relationship we did find a very interesting correlation of Hp, Pig-MAP and CRP with the stress status [83]

Other sample types: Determination of APP in saliva

Saliva has been used to measure APP such as Hp and SAA to detect pathological and stress situations in pigs. They were useful as non-invasive indicators of the health status of farms[84]. Regarding stress, results are variable, but Hp and SAA have been proposed to be increased in some stress conditions as transport, housing, isolation and restraint[85,86]. It has to be taken into account that they are affected by circadian patterns and can be influenced by sex [87].

Reference Intervals

A last comment on the importance of having accurate reference intervals, which take into account the influence of variables such as age, sex, breed or housing. In our laboratory, we have determined reference intervals for young calves and post-weaning piglets and found significant differences[88]. Without being comprehensive, information for calves and pigs is available[89 90,91].

Conclusions

The main limitation of the use of APP is their lack of specificity. Nevertheless, this is also their main strength. Acute phase proteins have been extensively investigated in pathologies, but their use as monitoring tools for productive and welfare purposes deserves to be credited and further explored. A long time has passed for APP research and one important bottleneck, identified longtime ago, still is the standardization and harmonization of the diagnostic tests, and the establishment of reference intervals.

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Use of Acute Phase Proteins in Patient Assessment and Management in Equine Medicine and Surgery – with Emphasis on Serum Amyloid A

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Learning Objectives

Listeners will learn:

- In which situations assessment of serum amyloid A (SAA) will be of value in patient assessment and management
- How the choice of SAA assay affects results of studies and clinical use of the analyte
- When other acute phase proteins are more suitable

Learning objectives will be obtained through presentation of literature and highlighted by brief presentation of cases from The Large Animal Teaching Hospital at University of Copenhagen, Denmark.

Abstract

Acute phase proteins (APPs), especially the major APP serum amyloid A (SAA), has become an indispensable part of appraisal, management, and monitoring of equine patients in general practice as well as in specialized hospital settings. While several proteins possess acute phase properties in horses, usefulness of SAA exceeds that of the other APPs greatly. This is due to the highly desirable kinetics of the equine SAA response, with low/undetectable concentrations in healthy horses, a fast rise-and-fall pattern, and a great magnitude of response (serum concentrations increase from < 0.5 mg/kg to > 10.000 mg/kg in response to severe inflammation). Taken together, these properties makes it easy to discriminate healthy from inflamed and facilitate real-time monitoring of inflammatory activity. SAA may be used at several stages of patient management: 1. Before diagnosis (to rule in/rule out inflammation), 2. At the time of diagnosis (to assess severity of the condition and assist in prognostication), 3. After diagnosis, for monitoring changes in inflammatory activity, as may occur when the patient is responding to therapy, with relapse of disease, or for detecting occurrence of infectious/inflammatory complications.

To exploit the full potential of SAA, assays with extensive measurement range are needed. In this respect, horse-side assay systems often fall short. These may be used for basic detection of inflammation, but for assessment of severity and monitoring purposes (where information on absolute concentrations are required), automated assays with integrated dilution steps are needed.

A large body of research on the equine SAA response and clinical use has been conducted in the last decade. This is the prerequisite for evidence-based used of this analyte. However, many of the studies involve a fairly low number of horses, and future studies should thus strive to have bigger sample sizes.

The Equine Acute Phase Response and Acute Phase Proteins

Several proteins are positive acute phase proteins (APPs) in horses, i.e. proteins that are *de novo* synthesized in response to inflammation and released to the circulation. Serum amyloid A (SAA) is a major APP in horses, and fibrinogen a moderate APP. Several APPs that are very useful in other species have been shown to be of limited value in horses, e.g. haptoglobin [1-3] and C-reactive protein [1,4], with modest or no differences in plasma concentrations in healthy horses and horses suffering from inflammatory/infectious conditions. Recent studies have shown that also other proteins may in the future prove to be useful APPs in the horse, e.g. procalcitonin [5-7] and neutrophil gelatinase-associated lipocalin (NGAL) [8,9]. We recently demonstrated a very fast and pronounced NGAL response in serum and synovial fluid of horses with experimentally induced arthritis, and higher NGAL concentrations in horses with septic arthritis than in healthy horses [8]. NGAL thus seems to be a promising biomarker with a response pattern that is similar to that of SAA. Currently there is very limited knowledge about this protein in horses, and more research is needed to support use of NGAL in evidence-based equine medicine.

The response patterns of the APPs differ quite substantially, with some having a fast and others a slower response to an inflammatory stimulus. SAA is fast reacting, with plasma concentrations increasing within 12 hours after induction of experimental inflammation [10,11] and peaking after 48-72 hours [10-12]. The amplitude of the SAA response is impressive: plasma concentrations increase from healthy levels of approximately 0.5 mg/L (Table 1) to 5000 mg/L or more in horses with severe inflammation [13]. With its concentration changes paralleling changes in inflammatory activity, SAA is very useful for real-time monitoring of inflammation [14]. For screening purposes, an APP with a more protracted response may be better suited. This has been shown in foals with *Rhodococcus equi* pneumonia, where SAA was not useful, while fibrinogen could be used for detection of disease [15,16]. Plasma fibrinogen concentrations remain elevated for days or weeks after inflammation has subsided [10,12].

A clinically useful approach to use of APPs is to measure both some with fast and some with slower response pattern[17]. This ensures that peracute, acute and subacute inflammation is detected and can be monitored. It is very important to keep in mind that, despite their name, APPs are produced and can be measured as long as there is active/ongoing inflammation, also if this has been of several days' (or even weeks') duration and thus may be considered to subacute. At The Large Animal Teaching Hospital (LATH), University of Copenhagen, a panel of inflammatory markers is used for assessing inflammation in horses: white blood cell counts (WBC, total and differential counts), iron, SAA and fibrinogen. WBC and iron concentrations change within very few (approx. 2) hours of an inflammatory stimulus[10], and this panel thus combines very fast, fast (SAA) and slower reacting (fibrinogen) markers of inflammation. Hereby, a very robust characterization of the horse's inflammatory status is achieved.

In addition to the hepatic production of SAA and release of the protein to the systemic circulation, SAA is also synthesized in extrahepatic tissues[18-21], and SAA release to local compartments has been shown to occur under healthy and pathological conditions. SAA has thus been found in normal colostrum[20,22], in saliva [23] and in inflamed synovial fluid[10,24,25] and peritoneal fluid[26]. Measuring SAA in these biological fluids may provide information on compartment-specific inflammatory activity.

The reference intervals for acute phase reactants used at The LATH are shown in Table 1.

Table 1. Acute phase reactant reference intervals used at The Large Animal Teaching Hospital, University of Copenhagen

Acute Phase Reactant	Reference Interval	Assay
White blood cell count (x 10^9/L)	5.45-12.65	Automated counting
Iron (μmol/L)	13.10-43.00	Colorimetric spectrophotometry
Serum amyloid A (mg/L)	< 0.5	VET-SAA, Eiken Chemical Co., Japan
Fibrinogen (g/L)	1-4	HemosIL RecombiPlastin (PT-based assay)
Haptoglobin (mg/L)	728–4265	Phase Range Hp Assay; Tridelta Development Ltd., Ireland

Use of SAA in Equine Medicine and Surgery

Assessment of SAA (and other APPs) may be valuable in all stages of patient management[14]:

- Before the diagnosis has been made: SAA is used to rule in/rule out inflammation and hence prioritize differential diagnoses with and without inflammation.
- At the time of diagnosis: SAA has been suggested to parallel intensity of the inflammatory response and as such serve as a prognostic indicator.
- After the diagnosis has been made: SAA is highly suited for monitoring changes in inflammatory activity, and repeated measurements are thus useful for assessment of response to therapy or detection of relapse or occurrence of infectious/inflammatory complications. SAA can also be used to support decision to terminate antimicrobial therapy.

Several comprehensive reviews on the equine acute phase response and, more specifically, SAA are available[14,27-29].

Before diagnosis

SAA (and other APPs) have been shown repeatedly to reliably indicate presence of systemic inflammation in horses[30-33]. It has been suggested that local inflammation gives rise to lower plasma SAA concentrations than systemic inflammation[31]. Certain specific conditions do not seem to elicit an SAA response, including gastric ulcer syndrome[34], intestinal cyathostominosis[35], inflammatory airway disease[36], and ulcerative keratitis and anterior uveitis[37]. More-over, it has been suggested that SAA concentrations in horses with abscesses (i.e. walled off

inflammation) are low/normal[15,38]. Clinicians using SAA thus need to have fairly detailed insight into the equine SAA response to be able to interpret measurement results in their patients.

For certain patient groups, it may be necessary to rule in or rule out inflammation using SAA and/or other APPs to properly prioritize differential diagnoses. In horses with severe acute abdominal pain (colic), it is crucial for outcome to quickly categorize the underlying condition as either surgical or medical in nature. Horses with inflammatory abdominal conditions most often treated medically (duodenitis-proximal jejunitis, acute typhlocolitis, peritonitis) may be clinically indistinguishable from horses with strangulations, displacements, or severe impactions that require surgical intervention. Both groups often present with the same severe signs of shock, pain, positive gastric reflux, and/or changes in peritoneal fluid, and Pihl et al. (2016) [2] therefore investigated whether SAA, haptoglobin and fibrinogen in blood and peritoneal fluid could assist in differentiating horses with inflammatory colic from those with surgical colic. Assessment of SAA concentration in serum resulted in significantly more horses (4 %) to be correctly classified as inflammatory (requiring medical therapy) or surgical cases.

Also in neonatology, assessment of SAA is useful for patient management. Diagnosing infections in neonatal foals can be a challenge, because the clinical signs are nonspecific, and diseases with a noninfectious cause, such as prematurity, failure of passive transfer, neonatal maladjustment syndrome, and isoerythrolysis, can manifest similarly to infectious diseases such as sepsis[14]. SAA concentrations are higher in foals with infectious diseases, particularly sepsis, than in foals suffering from non-infectious disease[38-41], and measurements may thus aid the clinician in his/her decision to instigate antimicrobial therapy or not while waiting for bacteriology results.

At the time of diagnosis

To evaluate ability of SAA and haptoglobin to predict survival in horses admitted with a variety of inflammatory conditions (peritonitis, colitis, trauma, renal insufficiency and cystitis, pneumonia, cellulitis, fever of unknown origin, and miscellaneous inflammatory conditions), Westerman *et al.* (2015)[42] measured protein concentrations in plasma at the time of admission in 53 horses (36 survivors and 17 non-survivors). Single-point assessment of the concentrations of the two proteins was not significantly associated with survival outcome, and authors suggested that serial analysis during treatment may be more likely to be a prognostic tool for horses with an inflammatory condition. This notion was corrob-orated by a study using repeated perioperative assessment of SAA and fibrinogen in horses undergoing exploratory laparotomy to predict post-operative complications and survival to discharge. Preoperative concentrations did not differ between groups, but SAA concen-trations day 1-5 after surgery differed significantly between horses that did and did not survive to discharge [43]. In contrast to these findings, daily measurements of SAA in horses being treated for synovial sepsis were unable to predict outcome (survival/non-survival)[44].

Using admission concentrations of SAA for predicting outcome in horses admitted with colic has been investigated in three studies [3,45]. Vandenplas *et al.* (2005)[45] retrospectively investigated SAA concentration at admission in 718 horses with acute abdominal pain admitted to two centers. In survivors, serum SAA concentration was significantly lower (median 1.4 mg/L) than

in horses that died or were euthanized (median 10.8 mg/L), and when a cutoff value of 50 mg/L was applied, a significantly larger proportion of non-survivors than survivors had serum SAA concentrations greater than the cutoff. Westerman *et al.* (2016)[3] conducted a small study involving 42 horses admitted with colic and found that horses with SAA > 5 mg/L at admission were more likely to develop thrombophlebitis or be euthanized due to a poor prognosis (OR 7.6, 95% confidence interval 1.1-52.4) than horses with normal SAA. In contrast, Dondi *et al.* (2015)[46] found no difference in admission SAA concentrations between colic survivors and non-survivors.

Several factors apart from disease severity may influence measured concentrations of SAA, e.g. timing of sampling (where a horse with peracute disease may be presented to the hospital before SAA concentrations have increased), volume and maybe also type of tissue affected (at The LATH, peritonitis, colitis, cellulitis and septic arthritis are the diseases causing the highest serum SAA concentrations), and disease etiology (it has been suggested that bacterial infections give rise to more pronounced APP responses than viral infections[47]). These factors may explain why many studies fail to demonstrate a strong association between admission SAA concentration and outcome.

In horses undergoing castration, postoperative infectious complications occurred more frequently in horses with preoperatively increased serum SAA levels than in horses with normal SAA levels[48], and SAA may thus be a predictor of surgical risk in horses undergoing elective surgery. At The LATH, all horses undergoing elective surgical procedures have a full panel of inflammatory markers assessed preoperatively, and if any of these are abnormal, the owner is advised to postpone surgery to mitigate the increased surgical risk in patients with preexisting inflammation/infection.

After diagnosis

SAA is very useful for patient monitoring. This could be for example in patents being treated for infectious conditions, where measurements every 2-4 days helps clinicians ascertain that infection is being eradicated, resulting in subsiding inflammation and a decline in plasma concentrations of SAA. This is particularly useful in cases awaiting culture results, where choice of antibiotics is based on the clinician's best guess. Decline in SAA concentration parallel to successful treatment of synovial sepsis has been demonstrated[44,49].

In horses undergoing exploratory laparotomy, SAA first increase in response to the surgically induced inflammation and then decline towards normal levels within 4-6[50,51] and 11 days[12]. Fibrinogen also increase in response to lapatoromy, but due to the long half-life of the protein, plasma concentrations stay elevated for longer periods, making fibrinogen of limited value for real-time monitoring of inflammatory activity[12,50,51]. When APPs are used for monitoring occurrence of infectious complications, the expected APP response for the existing or underlying disease must be characterized before deviations (sustained increases or unexpectedly high APP concentrations), can be identified[14]. In horses that developed complications after exploratory lapatotomy (e.g. diarrhea, ileus, thrombophlebitis, and fever) postoperative SAA[50,51] and fibrinogen[50] concentrations were significantly higher than those found in horses without complications. In horses undergoing castration, sustained high SAA[52] or

haptoglobin and fibrinogen concentrations[53] were identified in horses classified clinically as having excessive inflammation.

The effect of several inflammatory conditions may be additive and result in higher than expected SAA concentrations. This can be exploited for identifying bacterial superinfection in horses with viral infections, as demonstrated in horses with experimental influenza infection, where a group that developed clinical signs of secondary bacterial infection had persistently high plasma SAA concentrations [54].

Methods for Measuring SAA

An analyte such as SAA, whose response pattern is characterized by very pronounced concentration changes from essentially unmeasurable in the healthy horse to several thousands of mg/L in response to inflammation, is very difficult to quantify reliably in the entire concentration range. SAA in healthy horses is < 0.5 mg/L, and levels of 4000-5000 mg/L are not unusual in our clinic, where concentrations as high as 12,000-15,000 mg/L are occasionally observed in horses with typhlocolitis or peritonitis. We recently validated an assay (VET-SAA, Eiken Chemical Co., Japan) with a very broad working range[13], which measured SAA with acceptable reliability in the concentration range of 0 to > 6000 mg/L. The assay is set up to perform a 1:14 reflex dilutions at an SAA concentration > 200 mg/L, and it can thus measure SAA in the entire attainable concentration range (for samples with very high SAA concentrations, repeated dilutions are necessary). When using SAA measurements for monitoring purposes (e.g. response to treatment) in horses with severe inflammation, it is important to choose an assay that can reliably detect concentration changes in the high concentration range.

While horse-side test systems and assays developed for smaller laboratories have performed reasonably well in validation studies[55-57], it is our impression that those, we have used in our clinic, do not work very reliably in daily use, where we frequently encounter unexpected results that cannot later be confirmed with the VET-SAA assay. These assays are marketed for measuring SAA concentrations up to 3000 mg/L (e.g. the StableLab assay, https://www.stablelab.com/pages/serum-amyloid-a-blood-test [56] and VMRD assay system, https://vmrd.com/core/files/vmrd/uploads/files/SAA%20circular%20version%202.pdf [57]) or less (e.g. up to 180 mg/L for the EquiCheck system, https://www.targetvet.com/equine-progesterone-igg-and-saa-testing/visual-equichek-saa-test-or-quantitative-saa-chek/, up to 500 mg/L for the Eurolyser, https://www.eurolyser.com/veterinary-diagnostics/poc-test-parameters/saa-test/, and up to 1000 mg/L for the LifeAssays test system, https://www.lifeassays.com/equine-serum-amyloid-a-saa-test/). Considering the limited working range and inferior reliability, these assays are mainly useful for basic detection of inflammation in the field or out-of-hours, where fast results are needed and/or samples cannot be shipped to larger reference laboratories.

Concluding Remarks

There is no doubt that the use of SAA (and other APPs/inflammatory markers) have revolutionized patient assessment in equine medicine and surgery. To obtain a reliable SAA measurement result, it is recommended to perform measurements in a larger reference lab rather than with the smaller handheld devices. The body of literature on the equine SAA response has increased over the years, with less than 5 annual publications on equine SAA in the 1990s to 67 publications in 2020 (Web of Science). It is, however, unfortunate that even very recent studies have very small sample sizes of 5-15 horses per group [44,49,58], which makes them prone to type II errors. Small proof-of-concept studies are of less relevance at this stage, where it is well established that SAA is indeed a useful marker of inflammation. Researchers should thus strive to design studies with much larger sample sizes, potentially with patients from multiple centers, to provide increasingly robust evidence for use of SAA in equine medical and surgical patients.

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Acute Phase Proteins in Wildlife and Zoo Animals

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Learning Outcomes

- List the acute phase proteins most measured in wildlife and zoo animals, describe the assays most commonly used to detect and quantify them, and explain the analytical limitations of using these assays in a range of species.
- Discuss the applications of acute phase proteins in wildlife and zoo animals, with particular focus on their use in detecting and monitoring disease in individual animals and in monitoring population health.
- Identify areas in the field of acute phase protein research as it relates to conser-vation medicine, where veterinary clinical pathologists can play an important role.

Abstract

Acute phase proteins have been measured in a range of wildlife and zoo animals. Several assays have been identified, and some have been analytically and clinically validated in a few species. Acute phase proteins have utility in identifying inflammatory disease and monitoring the course of specific diseases in individual animals, and also show promise as bioindicators for monitoring health of free-living populations. Future directions include further research into the utility of acute phase proteins in the veterinary care of captive animals, but possibly more importantly, in monitoring and surveillance of free-living populations. Clinical pathologists have a vital role to play alongside clinicians, ecologists and physiologists in these types of studies, in terms of assay selection, validation and interpretation of results.

Introduction

The acute phase response involves increases and decreases in a multitude of proteins. Only the most researched and analytically available have been reviewed here. Albumin is well documented to be a negative acute phase protein (APP) in most species and is not reviewed here. Cytokines and other less useful/ available inflammatory proteins are also not discussed.

Acute Phase Protein Expression in Wildlife Species

Mammals

Most studies have evaluated concentrations of APPs in healthy animals, and then additionally at one point in time in a group of unhealthy animals, with a variety of inflammatory diseases. There are often only one or two studies available for a species. It is therefore not always possible to classify an APP as major, moderate or minor, as not enough information is available.

Table 1 includes a non-exhaustive list of APP studies in mammalian wildlife. Acute phase proteins have been denoted as major, moderate or minor if enough information exists to make that classification. Serum amyloid A (SAA) appears to be a major APP in many species, including cheetah, elephants, Iberian ibex, Florida manatees and northern elephant seals. As in most domestic mammals, haptoglobin is a moderate APP in bongo, cheetah, capybara, elephants, ibex and Stellar sea lions. Although less data is available, C-reactive protein (CRP) is often a moderate or minor APP and fibrinogen is a minor APP in species where they have been investigated.

Birds

Serum amyloid A, alpha-1 acid glycoprotein (AGP), ceruloplasmin and fibrinogen have been identified in birds and unlike in mammals, (ovo)transferrin is a positive APP.[1] Serum amyloid A was found to be a moderate APP in chickens, measured using SDS-PAGE and immunoblotting.[2] Other methods used for quantification of APPs in birds include species-specific ELISAs and radial immunodiffusion assays.[3]

Reptiles

Serum amyloid A and fibrinogen have been identified as positive APPs in reptiles.[1] For example, SAA expression was increased in Chinese soft-shelled turtles with bacterial infections.[4]

Amphibians

Very little has been published.[1]

Fish

Acute phase proteins identified in fish include fibrinogen, haptoglobin, α-2 macroglobulin, ceruloplasmin, hepcidin, warm acclimation protein 65 kDa, ntelectin, SAA, CRP and serum transferrin, which is a positive APP as in birds.[1] C-reactive protein and SAA have been identified in shark serum. Serum amyloid A levels were increased in Russian sturgeon challenged with *Aeromonas hydrophila*, measured using an in-house ELISA.[5] Serum amyloid A expression was increased >3000 fold in rainbow trout infected with *Yersinia ruckeri.*[6]. Serum amyloid A could therefore be a potentially valuable biomarker of bacterial infection in fish, but methods of quantitation of the protein in serum need to be developed. Hapto-globin and CRP have shown promise as markers of inflammation in some shark species.[7]

Methods of Quantitation

The most used commercially available assays are turbidometric immunoassays (TIA) for SAA (Eiken LZ-SAA) and CRP (Randox CRP) and a colorimetric assay for haptoglobin determination (Tridelta PHASE haptoglobin). All three of these assays can be run on most automated wet-chemistry analyzers. Fibrinogen is usually measured by heat precipitation or by the modified Clauss method on a semi-automated or automated coagulation analyzer. Of these assays, the Eiken LZ-SAA assay has probably been used most frequently for use in clinical cases, in species where the assay is known to show cross-reactivity.

The LZ-SAA assay will be discontinued at the end of 2021 and will be replaced by the new Eiken VET-SAA assay, which utilizes monoclonal antibodies. This assay has already been used successfully in rhesus macaques and northern elephant seals.[8,9] This assay also shows promising potential cross-reactivity for wild felids, some species of antelope, some species of monkeys and non-human primates, otters and tapirs, but more research is necessary. The new VET-SAA unfortunately does not show cross-reactivity for elephants, rhinoceros, white tail deer, moose, caribou and auklets (author's experience and personal communication from Prof C Cray, Acute Phase Laboratory, University of Miami).

ELISA assays for some acute phase proteins are also available and are either intended for research use across multiple species (for example Tridelta Development Ltd. PHASE Multispecies ELISA) or in single species (for example Acute Phase Protein ELISA kits from Life Diagnostics Inc.).

In research settings, investigation of APP gene sequences or mRNA expression is sometimes performed. Other techniques used to identify APPs in non-domestic species include SDS-PAGE and immunoblotting. Based on these results, research groups may construct in-house ELISAs.

Assays used in novel species should undergo method validation.[10] This is particularly important when using immunoassays as antibody cross-reactivity is initially unknown and will vary from species to species. Many studies measuring APPs in wildlife do not mention even the most basic method validation experiments (See Table 1). Blood samples from wildlife species are often low in volume or difficult to acquire and carrying out a full method validation can be challenging. However, at a minimum, determination of intra- and interassay imprecision and linearity, using species pools, should be carried out. Recovery and limit of detection experiments are also recommended, if resources allow.

Applications of Acute Phase Proteins in Wildlife and Zoo Animals

Most studies have been exploratory in nature, often with the ultimate aim of generating information that will contribute towards conservation of a particular species. Specific objectives of published studies usually include the identification of assays that can be used to measure APPs, generation of reference intervals, and documentation of increases in APPs in animals with clinical evidence of inflammatory disease. Fewer studies have explored the changes in APPs in specific conditions, elucidated their role as prognostic markers, or explored the use of APPs as bioindicators and markers of population health.

Detection of inflammatory disease

Serum amyloid A is a major acute phase protein in both African and Asian elephants. Serum amyloid A is useful for tracking the progress of EEHV infection, and concentrations reflect the degree of viremia.[11,12] Serum amyloid A also increases in other inflammatory conditions, for example pneumonia, pododermatitis and peritonitis.[13] Given that the large size of these animals prohibits the effective use of diagnostic imaging to investigate the peritoneal and pleural cavities, SAA could be a very useful screening test. Unfortunately, the new Eiken VET-SAA TIA and the Tridelta Multispecies SAA ELISA do not show cross-reactivity for elephant SAA, and studies are underway to validate new assays.

Given the ongoing decimation of wild rhinoceros populations due to poaching, it is vital to ensure the health of captive animals. Captive black rhinoceros are prone to diseases such as hemolytic anemia, rhabdomyolysis and iron overload.[14] Serum amyloid A increases have been documented in black rhinoceros with clinically evident inflammatory disease (dermatitis, colic, dental abscess, pneumonia). Increases in SAA, haptoglobin and fibrinogen have been noted in free-living white rhinoceros with traumatic tissue injuries, and in captive white rhinoceros with colic, foot abscesses and hypophosphatemia. Haptoglobin and fibrinogen may have a better diagnostic performance than SAA in this species, due to the poor sensitivity of the latter.[15] An obstacle to using SAA in these animals for routine clinical use is that so far, no automated assay has been identified that shows cross-reactivity and all measurements have been performed using the Tridelta SAA Multispecies ELISA.

Atlantic bottle-nosed dolphins show steadily increasing SAA concentrations from the first trimester of pregnancy through to shortly after parturition and fibrinogen is also increased in the second half of gestation.[16,17] This indicates that Atlantic bottlenose dolphins also show a significant inflammatory response during pregnancy, with tissue damage during parturition being the assumed cause of the increased levels of SAA observed postpartum.[18] Median haptoglobin showed a 3-fold increase in bottle-nose dolphins with varying inflammatory disorders.[19]

Serum amyloid A has a high sensitivity (93%) and specificity (98%) for detecting inflammation in Florida manatees, and is useful for monitoring clinical progress during rehabilitation of these animals. Concentrations of SAA were increased by more than 30 times in manatees that were taken into care after suffering from cold stress or trauma caused by boating accidents, and decreasing concentrations were consistent with clinical improvement.[20] SAA is also a useful marker of successful rehabilitation in northern elephant seals.[9]

Acute phase proteins have been described to have potential as indicators for subclinical disease in several scenarios. Increased SAA was observed in juvenile northern elephant seals pre-clinically infected with *Otostrongylus circumlitis* (lungworm), and elevated SAA had a reported specificity of 100% for the presence of infection.[9,21] In red deer infected naturally infected with *Mycobacterium bovis*, several animals showed increased concentrations of haptoglobin before *M. bovis* infection was detected with the cervical comparative skin test, indicating that haptoglobin has potential as a complementary tool for the diagnosis of tuberculosis in this species.[22] Although African buffalo are asymptomatic carriers of Foot and Mouth Disease Virus, sustained elevated haptoglobin concentrations predict the presence of the virus and could be used as an adjunctive measure for disease surveillance.[23]

Acute phase proteins as measures of animal welfare

Acute phase proteins have also been shown to be indicators of stress, and can therefore be used as markers for animal welfare. Haptoglobin levels in newly captured juvenile Stellar sea lions increased by more than three times from their reference values when these animals were handled in captivity, indicating the negative effect of human interaction with wild marine mammals.[24] Black rhinoceros transported by road for translocation purposes had a four-fold increase in SAA over the 20 hour duration of transport, indicating that the stressors involved in capture and translocation have at least short-term effects on innate immunity in these animals.[25]

Acute phase proteins as a measure of population health

Comparison of APPs between free-living and captive populations may serve as a marker of subclinical disease in either population. Captive black rhinoceros have higher concentrations of SAA and tumour necrosis factor than their free-living counterparts, suggesting that husbandry measures involved in keeping black rhinoceros predispose them to a pro-inflammatory state.[14] Both SAA and haptoglobin were higher in wild-caught Florida manatees, possibly due to subclinical viral or bacterial disease secondary to hypothermia-related immunosuppression.[26] Both SAA and Hp were found to be different in free-living Atlantic bottlenose dolphins compared to those under human care.[27] Haptoglobin is consistently higher in free-living dolphins, while SAA has been found to be both higher and lower.[27,28] One of the populations of free-living dolphins under investigation was found to have a subclinical morbillivirus infection and gastritis, two potential causes for increased values of haptoglobin the free-living animals.[27]

Acute phase proteins may also be used as indicators of health within free-living populations. This has been particularly well-researched in marine mammals. This group of animals is exposed to a progressive bioaccumulation of environmental toxins, and work has been done to investigate the physiology of their innate immune system and how it responds to marine pollutants. Increased serum levels of haptoglobin in declining populations of Stellar sea lions and harbour seals in the Gulf of Alaska and the Aleutian Islands, compared to haptoglobin levels in stable populations, may have been an indicator that the former population was faced with stressors not present in the latter.[29] A regional difference in serum haptoglobin concentrations in Stellar sea lions was found in a second study conducted in a similar geographical area, and was hypothesized to reflect regional differences in circulating mercury concentrations in the water, a factor potentially affecting the health status of these animals.[30] Two studies conducted on harbour seals from the Wadden Sea off the east coast of Germany and Denmark between 2002 and 2007 found that haptoglobin levels were higher in 2002, probably due to a phocine distemper virus epidemic that was coming to an end in this population in 2002.[31] Based on these studies, haptoglobin appears to be a particularly promising marker for monitoring health of free-living populations.

Conclusion

Acute phase proteins play a useful role in conservation medicine and can be used for detection and monitoring of disease in individual animals, as well as for monitoring wild animal welfare and population health. The lack of species-specific antibodies and standards is a challenge for the measurement of APPs in wildlife, and more attention needs to be focused on ensuring adequate analytical performance of APP assays, before assessing concentrations in healthy and diseased animals. Differences in APPs between free-living and captive animal populations indicate the need for reference intervals specific to these different populations, which should be generated according to published guidelines. The field of acute phase proteins in wildlife offers veterinary clinical pathologists an opportunity to become involved in conservation medicine. We can make important contributions in the areas of assay validation, investigation of novel APPs, and development of more convenient automated APP assays.

Table 1. Non-exhaustive list of acute phase protein (APP) investigations in mammalian wildlife. APPs have been denoted as major, moderate or minor where enough evidence exists to classify them in this manner. Reference intervals noted if these were generated according to ASVCP guidelines.

Species	Acute phase protein	Analytical method	Analytical validation	Clinical and research applications
Arabian oryx (*Oryx leucoryx*)[32]	SAA	Eiken LZ-SAA	None	Results from healthy animals
	Fibrinogen	Clauss method	None	Results from healthy animals
Blackbuck (*Antilope cervicapra*)[32]	SAA	Eiken LZ-SAA	None	Results from healthy animals
	Fibrinogen	Clauss method	None	Results from healthy animals
Bongo *(Tragelaphys eurycerus)*[33]	Haptoglobin	Tridelta PHASE haptoglobin	Linearity	RIs Minor APP
	Fibrinogen	Unknown	None	RIs Minor APP
Buffalo, African (*Syncerus caffer*)[23]	SAA	Life Diagnostics anti-bovine SAA ELISA	None	Elevated post-infection with Foot and Mouth Disease Virus
	Haptoglobin	Life Diagnostics Haptoglobin ELISA (antibody not specified)	None	Elevated post-infection with Foot and Mouth Disease Virus and *Mycobacterium bovis*
Capybara (*Hydrochoerus hydrochaeris*)[34]	Haptoglobin	Tridelta PHASE haptoglobin	Linearity Imprecision Limit of detection	Results from healthy animals Moderate APP Useful for monitoring response to treatment for sarcoptic mange
	Acid-soluble glycoprotein	Modified Winzler method	Linearity Imprecision Limit of detection	Results from healthy animals Moderate APP
Cheetah (*Acinonyx jubatus*)[35]	SAA	Eiken LZ-SAA	Western blot	RIs Major APP Higher in animals with chronic kidney disease
	Haptoglobin	Capillary zone electrophoresis	None	RIs Moderate APP Higher in animals with chronic kidney disease
	CRP	Modified Tugirimana method	None	RIs
Deer, red (*Cervas elaphus*)[22]	Haptoglobin	Tridelta PHASE haptoglobin	Imprecision Linearity Limit of detection	Results from healthy animals Higher in animals infected with *Mycobacterium bovis*
Deer, white-tailed (*Odocoileus virginianus*)[36]	SAA	Eiken LZ-SAA	Linearity Imprecision Recovery	RIs Increased in animals with inflammation

Dolphin, bottlenose (*Turciops truncatus*)[16,18,19,27,28,37]	SAA	BioSource Multispecies SAA ELISA	No cross-reactivity	
		Eiken LZ-SAA	Imprecision	RIs Lower in free-living than managed Increased in last third of gestation
		Life Diagnostics dolphin specific ELISA	None	Results from healthy animals Major APP Higher in free-living than captive animals
	CRP	Randox anti-human TIA CRP	Imprecision	RIs
	Haptoglobin	Tridelta PHASE haptoglobin	Imprecision Limit of detection	RIs Higher in free-living than managed Increased in animals with inflammation
		Immunology Consultants Lab pig haptoglobin ELISA	Western blot Imprecision Limit of detection	Increased in animals with inflammation
	Fibrinogen	Sysmex thrombin method	None	Results from healthy animals Increased in second half of gestation
Elephant, Asian (*Elaphas maximus*)[12,13,38,39]	SAA	Eiken LZ-SAA	Imprecision Linearity Recovery	RIs Major APP Increased in animals with EEHV viremia
		Tridelta multispecies SAA ELISA	No cross-reactivity	
	CRP	Bayer anti-human TIA CRP	Imprecision	RIs
	Haptoglobin	Tridelta PHASE haptoglobin	Imprecision Linearity Recovery	RIs
Elephant, African (*Loxodonta africana*)[11,13,39]	SAA	Eiken LZ-SAA	None	RIs Major APP Increased in animals with EEHV viremia
	Haptoglobin	Tridelta PHASE haptoglobin	None	RIs Moderate APP
Ibex, Iberian (*Capra pyrenaica*)[40,41]	SAA	Tridelta multispecies SAA ELISA	Linearity Imprecision Limit of detection	Results from healthy animals Major APP Increased in animals with sarcoptic mange
	Haptoglobin	Tridelta PHASE haptoglobin	Linearity Imprecision Limit of detection	Results from healthy animals Moderate APP

Impala (*Aepyceros melampus*)[32,39]	SAA	Eiken LZ-SAA	None	Results from healthy animals Increases in inflammation
		Tridelta multispecies SAA ELISA		
	Fibrinogen	Clauss method	None	Results from healthy animals
	CRP	Bayer anti-human TIA CRP	None	Results from healthy animals Not increased in inflammation
		In-house ELISA		
	Haptoglobin	Tridelta PHASE haptoglobin	None	Results from healthy animals Increases in inflammation
		In-house ELISA		
Manatee, Florida (*Trichecus manatus latirostris*)[20,26]	SAA	Eiken LZ-SAA	Linearity Imprecision Recovery	RIs Major APP Increased in cold stress and trauma
		Tridelta anti-bovine SAA ELISA	None	Results from healthy animals Increased in injured animals Higher in wild-caught versus captive animals
	CRP	Tridelta antiporcine CRP ELISA	No cross-reactivity	
		Roche Tina-quant CRP	No cross-reactivity	
	Haptoglobin	Tridelta PHASE haptoglobin	None	Results from healthy animals
		Roche Tina-quant haptoglobin ELISA	No cross-reactivity	
	Fibrinogen	Heat precipitation	None	Results from healthy animals
Musk ox (*Ovibos moschatus)*[39]	SAA	Eiken LZ-SAA	None	Results from healthy animals Increased in inflammation
		Tridelta multispecies SAA ELISA		
	CRP	Bayer anti-human TIA CRP	None	Results from healthy animals Increased in inflammation
		In-house ELISA		
	Haptoglobin	Tridelta PHASE haptoglobin	None	Results from healthy animals Increased in inflammation
		In-house ELISA		
Reindeer (*Rangifer tarandus*)[39]	SAA	Eiken LZ-SAA	None	Results from healthy animals
		Tridelta multispecies SAA ELISA		
	CRP	Bayer anti-human TIA CRP	None	Results from healthy animals
	Haptoglobin	Tridelta PHASE haptoglobin	None	Results from healthy animals
Rhesus macaque (*Macaca mulatta*)[8]	SAA	Eiken SAA-1*	Imprecision	RIs Increased in chronic inflammation
	CRP	Randox anti-human TIA CRP	Imprecision	RIs Major APP Increased in acute and chronic inflammation
	Haptoglobin	Tridelta PHASE haptoglobin	Imprecision	RIs Moderate APP

				Increased in chronic inflammation
Rhinoceros, black (*Diceros bicornis*)[14,25]	SAA	Tridelta multispecies SAA ELISA	Linearity Imprecision Recovery	RIs Increased in inflammation Higher in captive versus free-living animals Increased after long transport
Rhinoceros, white (*Ceratotherium simum*)[15]	SAA	Tridelta multispecies SAA ELISA	Linearity Imprecision	RIs Increased in injured animals
	Haptoglobin	Tridelta PHASE haptoglobin	Linearity Imprecision	RIs Increased in injured animals
	Fibrinogen	Modified Clauss method	None	RIs Increased in injured animals
River otter (*Lutra canadensis*)[42]	Haptoglobin	Agarose gel electrophoresis		Higher in animals from oiled versus non-oiled environments
Seal, harbor (*Phoca vitulina*)[29,43-47]	Haptoglobin	Tridelta PHASE haptoglobin	None	Results from healthy animals Elevated during phocine distemper virus epidemic
		Agarose gel electrophoresis	None	Higher in endangered than non-endangered population
	CRP	In-house ELISA	None	Increased in seal pups with inflammation
		Olympus anti-human TIA CRP	None	Results from healthy animals Higher in animals with higher haptoglobin Increased in some animals with inflammation
Seal, northern elephant (*Mirounga angustirostris*)[9,21]	SAA	Eiken SAA-1*	Linearity	Results from healthy animals Major APP Increased in malnourished animals and those with pre-clinical and clinical lungworm infection
	CRP	Randox anti-human TIA CRP	Linearity	Results from healthy animals Increased in animals with clinical lungworm infection
Seal, ringed (*Pusa hispida*)[48]	Haptoglobin	Tridelta PHASE haptoglobin	None	Results from healthy animals Higher in mature males, possibly due to fighting injuries
Sea lion, Stellar (*Eumetopias jubatus*)[24,29,49]	Haptoglobin	Tridelta PHASE haptoglobin	None	Results from healthy animals Three-fold increase during first month of captivity
		Agarose gel electrophoresis	None	Higher in endangered than non-endangered population

Sitatunga (*Tragelaphus spekii*)[39]	SAA	Eiken LZ-SAA	No cross-reactivity	
		Tridelta multispecies SAA ELISA		
	CRP	Bayer anti-human TIA CRP	None	Results from healthy animals
		In-house ELISA	No cross-reactivity	
	Haptoglobin	Tridelta PHASE haptoglobin	None	Results from healthy animals
		In-house ELISA		Increases in inflammation
Wallaby[39]	SAA	Eiken LZ-SAA	None	Results from healthy animals
		Tridelta multispecies SAA ELISA		Increased in inflammation
	CRP	Bayer anti-human TIA CRP	None	Results from healthy animals
		In-house ELISA	No cross-reactivity	
	Haptoglobin	Tridelta PHASE haptoglobin	No reaction	
		In-house ELISA	None	Results from healthy animals
Zebra, Grant's *(Equus burchelli)*[50]	Haptoglobin	Tridelta PHASE haptoglobin	None	Results from healthy animals
		In-house ELISA	No cross-reactivity	

CRP, C-reactive protein; EEHV, Elephant Endotheliotropic Herpesvirus; RIs, reference intervals; SAA, serum amyloid A; TIA, turbidometric immunoassay.

*Eiken SAA-1 now available as Eiken VET-SAA

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Acute Phase Proteins in Laboratory Animals

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Learning Objectives

- Recognize the roles of acute phase proteins in innate and cell mediated immunity.
- Describe the major and minor APP in laboratory animals and recognize the types of experiments which can lead to these definitions.
- Formulate potential experiments to use APP to aid research investigations and the practice of laboratory animal medicine.

Abstract

It can be argued that the roles and functions of acute phase proteins (APP) are best studied using laboratory animal models. Research has revealed that APP are not only major elements of innate immunity, but they also can have a broader reach in cell mediated immunity. The key tenets of the applications of acute phase proteins are health assessment and prognostication. These hold true for applications in laboratory animal medicine where testing may be applied to assess the health of animals prior to and during experimental protocols as well as potential applications to animal welfare. In addition, APP quantitation can be used to aid in the characterization of experimental models. As with other aspects in the adoption of APP in veterinary medicine, these biomarkers are still underutilized. Part of this is likely due to the ready ability to analyze cytokines in rodents in research laboratories. However, as access to APP testing increases, it would be expected that these tools could be applied to health assessments of rabbits, cats, dogs, pigs, and non-human primates to ensure health status prior to the start of experiments as well as aid in monitoring the welfare of the animals during experiments.

Knowledge Base of Acute Phase Response and Acute Phase Proteins Derived from Laboratory Animal Studies

- Laboratory animal studies assisted in the identification of the first described APP. After an initial study of humans with pneumonia, C-reactive protein (CRP) was described in the serum of cynomolgus monkeys with experimental pneumococcus infection in 1937. CRP was named for is reaction with the “c” polysaccharide on the capsule of the bacteria.[1] The reactivity was described to be present during acute illness with recovery.

- APP like serum amyloid A (SAA) have several roles in innate immunity including chemotaxis of macrophages, induction of cytokine expression, inhibition of apoptosis, increased phagocytosis, protease inhibition, and opsonization.[2,3] CRP binds Fc receptors allowing for opsonization and complement activation.[4] Serum amyloid P (SAP) is essential to the response to *Aspergillus*.[5] Much of this understanding has been solidified through the use of transgenic mice.
- APP also have roles in cell mediated immunity. Local and systemic production of SAA has been demonstrated in the pathogenesis of psoriasis, colitis, allergic airway inflammation, and experimental autoimmune encephalitis among many others via its action primarily on Th17 cell differentiation.[6-8] The use of HP (haptoglobin) knockout mice has demonstrated this APP does aid in the response to antigen and T and B cell differentiation.[9]
- Understanding that APP can regulate many aspects of the immune response, there is an increased interest in modulating these responses through the removal or addition of APP. SAP has been examined in animal models and clinical trials for its effect in reducing the decline in lung function in pulmonary fibrosis, decreasing acute lung injury, decreasing experimental autoimmune encephalitis, decreasing wound healing among others.[10-12]

Special Applications of Acute Phase Proteins in Laboratory Animal Medicine

- Stress
    - Hyperthermia resulted in increased α-1 acid glycoprotein (AGP) and CRP in mice.[13]
    - Inescapable tail shock resulted in increased AGP and HP 24 hours post insult.[14]
- Welfare
    - Lateral tail incision, retrobulbar sinus puncture, and saphenous vein puncture but not tail tip amputation, sublingual puncture, and submandibular puncture resulted in elevated levels of HP in mice.[15]
    - CRP expression in rabbits was examined as a possible tool for the assessment of inflammation in vaccine safety testing.[16] As a major APP, the magnitude of increase as consistently high and correlative with the circulating heterophils which marked improvements over fibrinogen which is routinely used in these types of studies. CRP was postulated as allowing for the refinement of experimental design thus limiting the number of animals and experiment repetitions.
- Health Assessment and Laboratory Animal Care
    - In mice, LPS and CFA resulted in changes in the protein electrophoretogram and SAA, CRP, and HP.[17] Mice experimentally infected with Sendai virus and mouse parvovirus as well as sentinel animals from colonies with endemic mouse hepatitis virus and mouse parvovirus infection showed no changes in APP values although albumin and globulin fractions were significantly different.
    - Higher SAP and HP are observed in wild mice vs. laboratory animal mice.[18] APP in dogs kept as laboratory animals are similarly lower than those kept as pets.[19]

This supports that good husbandry and lack of enzootic infection minimizes inflammation.
  - CRP levels were observed to decrease in rhesus macaques with experimental treatment for diarrhea which is a common gastrointestinal disease in captive primates.[20]
  - SAA levels were found to be a possible tool in the assessment of captive rhesus and pig-tailed macaques for amyloidosis.[21]

APP and Methods to Quantitate APP by Species of Laboratory Animal

- In the mouse, HP, SAA, and SAP are considered major APP and CRP is considered a minor/moderate APP.[22] APP can be measured by ELISA and multiplex platforms.
- In the rat, AGP and α2-macroglobulin are considered major APP and CRP and HP are considered moderate APP.[22] APP can be measured by ELISA and multiplex platforms.
- Guinea pig APP have not been well studied. SAP and CRP were described to not be major APP in this species after experimental induction of inflammation.[23] A protein called female protein (FP) is an analog of CRP in the hamster which is under hormonal control. It appears to be a minor APP.[24,25]
- In the rabbit, SAA and CRP are major APP. It should be noted that there may be different pathways for the induction of these APP as differential expression has been observed to vary by type of stimulus.[26,27] APP can be measured by ELISA, VET-SAA (Eiken), lateral flow device, and some human CRP reagents.[28-30]
- In the pig, cat, and dog, refer to other workshop presentations.
- In non-human primates (NHP), CRP and SAA are major acute phase proteins.[31,32] There may be differential sensitivity of these two APP in differentiating enterocolitis from amyloidosis in captive macaques.[20,21,33] Human reagents for CRP have been found to cross react with NHP CRP and SAA-LZ and VET-SAA (Eiken Chemical, Japan) are both viable reagents (C. Cray, personal observation).[32] Many commercially available ELISA are also available.

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Acute Phase Protein Response and Changes in Lipoprotein Particle Size in Dogs with Systemic Inflammatory Response Syndrome

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Learning Objectives

- Compare and contrast the biological functions of CRP and SAA.
- Design a method validation study appropriate for evaluation of a new (or modified) assay.
- Evaluate the diagnostic utility of APP in veterinary patients.
- Explore the interconnections between the acute phase response (APR) and lipid metabolism.
- Propose future studies that could inform diagnosticians and clinicians on the use of APP and lipoproteins in monitoring patient health.

Abstract

Improved methodology to measure acute phase proteins (APP) and lipoprotein particle-size distribution (PSD) could be clinically useful in dogs with systemic inflammatory response syndrome (SIRS). This study had three objectives. First, to evaluate the performance characteristics of a new immunoturbidometric assay for SAA (Eiken Chemical Co LTD) in the dog. Second, to compare the clinical utility of CRP and SAA in dogs with systemic inflammatory response syndrome (SIRS). Finally, to characterize the particle-size distribution (PSD) of both major lipoproteins in dogs with SIRS and correlate the changes with other biomarkers of inflammation. Sixty-three dogs (25 dogs with septic inflammation (SI), 15 dogs with non-septic inflammation (NSI), and 22 healthy controls) were enrolled in a case-controlled study. Parameters measured included SAA, CRP, full biochemical panels including cholesterol and triglyceride concentrations, and electrophoretic measure of total and subfractionated high-density and low-density lipoproteins (HDL and LDL, respectively). The SAA assay performed well and was useful in discriminating SI from NSI, a property not demonstrated for the CRP assay. Specific subfractions of both LDL and HDL differed between all groups. Detailed lipoprotein profiling in dogs with inflammation may hold value in separating categories of inflammation and identifying novel therapeutic interventions.

Introduction

C-reactive protein (CRP), originally named for its ability to bind the pneumococcal somatic C-polysaccharide, and serum amyloid A (SAA) are both members of the pentraxin family and major positive acute phase proteins in the dog. These proteins are divergent in a number of diagnostically and clinically important ways. Early studies investigating the inflammatory actions of CRP used recombinant proteins derived from bacteria, thus contamination with toxins was a concern. Recent studies using proteins produced in mammalian cells have confirmed CRP-mediated complement activation in humans and opsonization of pathogens in multiple species.[1] CRP is highly water soluble[2] whereas SAA is lipophilic and predominantly found bound to high-density lipoprotein (HDL).[3,4] This property complicates routine measure of SAA and has functional implications due to the impact SAA has on serum lipoprotein function. HDL particles that are loaded with SAA rather than apolipoprotein A1 are less efficient at cholesterol transport.[5] SAA can also displace antioxidant proteins such as paraxonase-1 from HDL, reducing the anti-oxidant capacity of the HDL. Changes in HDL composition are often reflected in a change in the distribution of particle sizes and this can be measured electrophoretically. We hypothesized that a more detailed investigation into the particle sizes of lipoprotein in dogs with SIRs would identify subfractions of major lipoprotein with significant changes in these dogs as compared to healthy dogs.

Objectives

This study had three objectives:

1. Evaluate the performance characteristics of a new immunoturbidometric assay for SAA in the dog.
2. Compare the clinical utility of CRP and SAA in dogs with systemic inflammatory response syndrome (SIRS).
3. Characterize the particle-size distribution (PSD) of both LDL and HDL in dogs with SIRS, and correlate the changes with other biomarkers of inflammation.

Methods

Brief method validation study of the SAA assay included: determination of inter- and intra-assay precision,linearity on dilution, recovery, limit of quantification and limit of detection, and routine stability studies.

Case controlled study evaluating APP protein response and lipoprotein PSD in dogs with SIRS (both SI and NSI) and healthy controls.

Analysis of biochemical changes including SAA, CRP, and lipoprotein PSD for clinical utility in separating SI from NSI and relation to disease severity scoring (APPLE).

Major Findings

1. CRP and SAA are both increased in dogs with SIRS
2. SAA is diagnostically useful in separating SI from NSI
3. LDL and HDL subfractions are different between dogs with SIRS and healthy dogs, and also different between dogs with SI and NSI
4. Specific subfractions of HDL are different depending on the anatomical location of the inflammation

Future Directions

Decline in SAA is proposed to be part of a feedback loop using the proresolving (M2) macrophage phenotype and facilitate tissue repair,[6] supporting longitudinal studies investigating the use of SAA in monitoring disease resolution.

Additional studies using the VET-SAA assay to more extensively gauge the diagnostic utility of the assay in identifying septic inflammation.

Determining the ratio of apoA1 to SAA could be informative, and help account for the overall decline inHDL particles that occurs in many inflammatory diseases.

Additional studies aimed at finding interventions to improve the function of HDL (perhaps targeting the ability to ward off SAA displacement of anti-oxidant proteins by altering the phospholipid composition of the membrane).

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Haptoglobin Assay and Observations Across Multiple Species

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Learning Objectives

- Understand the improvements to the haptoglobin assay by modifications to the reagent components.
- Follow the logic of the steps from observation of haptoglobin in canine fecal extract to human intestinal disease and relevance to haptoglobin in bovine milk.
- Describe the links between bovine haptoglobin, human pre-haptoglobin 2 and zonulin.

Abstract

Major modifications have been made to the haptoglobin (Hp) assay, that can be used for all relevant species where Hp is an acute phase protein and which was first described two decades ago in Eckersall et al (1999; *Comp Haem Intl* 9: 117-124). These modifications are the use of a more stable chromogen, use of cysteine as reducing agent and use of a serum-based blank reagent. The validation yielded intra and inter assay coefficients of variance of <3% and <10% respectively and a limit of quantification of 0.05 g/L for measuring Hp in bovine serum. The opportunity has also been taken to integrate recent research findings on Hp in animals with related revelations from across the scientific and clinical literature. Initial findings from a proteomic analysis of canine fecal extracts from dogs with chronic inflammatory enteropathy revealed that Hp was present in the feces of this group but was absent from extracts of feces from healthy dogs. In human gastrointestinal diseases, it is known that a particular Hp isoform (pre-Hp2) is also present in the intestine, but crucially its biological activity had already been investigated and named as zonulin (Zon). This protein has been shown to actively stimulate the opening of tight junctions of the intestine epithelia, an action it shares with the zona occulens toxin of Vibrio cholera. Zonulin has been implicated as being associated with a number of intestinal diseases in humans such as celiac disease and inflammatory bowel disease. As human Hp2 is known to be closely related to bovine Hp in structure and sequence (70% homology) and the latter is known to be present in milk from cows with mastitis and in colostrum during the first hours of milking, an investigation was undertaken of milk from cows with mastitis to search for any indication that a zonulin-like bovine Hp could be in present in these secretions. Western blot analysis of bovine milk from cows with mastitis identified the α and β subunits of Hp but also detected an extra band of higher molecular weight in the milk samples. This extra band was not present in serum samples from cows with an acute phase response.

Introduction

Over the last few decades, analysis of the acute phase proteins (APP) has come ever closer to the forefront of valuable diagnostic tests in veterinary clinical pathology laboratories. One of the building blocks for this progress has been the use of a method than is able to measure haptoglobin (Hp) in the serum of numerous species of animals. First described at the end of the last century[5], the method depends on the preservation of the peroxidase activity of hemoglobin at low pH, by its binding to haptoglobin. Recently improvements to the assay have been reported [2] and the improving modifications and its validation are reported here. There have also been developments, not only in diagnostic applications but also in understanding of the structure, function and evolution of Hp. Widely regarded as its major function being its binding affinity to hemoglobin, there have been substantially differing activities ascribed to certain isoforms of the protein, which could have implications for understanding of apparently unrelated disease processes. The steps in developing a rationale to integrate these revelations into the known pathophysiology of Hp in veterinary species are described herein.

Haptoglobin Assay

Although widely available the Hp assay based on hemoglobin binding has had some drawbacks since it was first invented[5]. Notably, the requirement to use three separate reagents has reduced its use when many biochemical analysers are limited to carrying only two reagents. It is possible to mix two of the three reagents, but this could affect their stability and cause lower precision and higher than acceptable coefficients of variance for routine analysis. In addition, the chromogen reaction measuring the peroxidase activity of hemoglobin in the assay when 4-aminoantipyrine reacts with 1-anilinonaphthalene-8-sulfonic acid leading to a blue/red product, is inherently unstable, losing colour intensity in a few moments. A recent modified method[2] makes use of a novel peroxidase substrate (SAT3, Dojindo Inc, Japan) which has a more stable chromogen development product. Other modifications included the use of cysteine to replace dithiothreitol as a reducing agent and the inclusion of bovine or ovine serum from healthy animals as the assay blank. This is important for analysis of Hp in ruminants where monitoring low levels of Hp is vital as healthy ruminants have minimal Hp in the circulation. The modified assay has shown acceptable intra and inter assay CVs of <3% and <10% respectively and a limit of quantification of 0.05 g/L for measuring Hp in bovine serum.

Observations on Haptoglobin Across Multiple Species

The major function of Hp has been thought to be the removal of hemoglobin following haemolysis in order to eliminate damage to cells due to the innate peroxidase activity of hemoglobin and in addition to conserve the iron content contained in the haem group. However a number of other actions have been ascribed to Hp such as having an antimicrobial action on Gram negative bacteria and also having angiogenesis and chaperone activities[3]. More recently a particular isoform of human Hp has been recognised as having a functional role in the pathophysiology of the gastrointestinal tract. Identification of evidence that a similar action

could occur in the canine intestine has led to speculation on the relevance of such observations to further species.

Haptoglobin in Canine Chronic Enteropathy

A quantitative proteomics study (O'Reilly et al, submitted[1]) of canine fecal extracts, comparing the fecal proteome between healthy dogs and those with food responsive enteropathy (FRE) and those with chronic inflammatory enteropathy (CIE) revealed that the fecal extracts in the latter group contained high abundances of canine Hp, compared to the healthy or FRE group. The proteomic results were validated by use of western blots using antibody specific for canine Hp which revealed that samples of the CRE group had a major Hp band at 40kDa the molecular weight of β-Hp, while samples from healthy dogs gave no reaction in the western blot. There were also bands of lower Mw, likely to be breakdown product of the β-Hp chain. The amount of Hp in fecal extracts was determined by a SPARCL immunoassay for canine Hp (Life Diagnostics Inc, West Chester, USA) and showed a significant increase in the content (µg Hp/mg total protein) in samples from CIE compared to extracts from FRE or healthy dogs. An initial consideration was that this finding could be explained by leakage of the Hp from the circulation into the gastro-intestinal tract, however exploration of the literature leads to other explanations.

Human Intestinal Disease

Even a cursory examination of the scientific literature using 'haptoglobin' and 'intestine' as keywords for search engines such as Google or databases such as Web of Knowledge, came up with information on the relationship between Hp and an intestinal protein called zonulin (Zon)[8,11]. Zonulin was discovered as a host protein of identical sequence and structure to the zona occulens toxin (Zot) from *Vibrio cholera* which is known to weaken the tight junction between cells in the intestine via binding to receptors on the external membrane of epithelial cell. Zot is thus instrumental in the development of diarrhoea and fluid loss by this infection. Although being a host derived protein Zon, which has been identified by immunohistochemistry to be present in intestinal epithelial cells, has been also characterised as having a similar action on intestinal tissues as Zot with the ability to loosen the tight junction required for establishing a barrier between the intestinal lumen and the circulation. Zonulin has been associated with a number of human diseases that involve inflammatory reaction in the intestine and implicated in a range of diseases such as celiac disease, inflammatory bowel disease and ankylosing spondylitis [8].

Human Pre-Haptoglobin 2 and Zonulin

The primary structure of the amino acid sequence of human Zon has been determined and is identical to that of pre-haptoglobin 2 of humans[8]. Serum Hp is synthesised and secreted from the liver and is produced as a single amino acid chain; a short signal peptide is removed and a further cleavage at an arginine residue separates the remainder into an α-Hp and β-Hp held

[1] O'Reilly E.L. et al Faecal proteomics in the identification of biomarkers to differentiate canine chronic enteropathies, 2021 submitted.

together by disulphide bridges[1]. Most mammalian species have a single gene product for Hp but human Hp is derived from two genes, Hp1 and Hp 2 with the difference that in Hp2 there is a partial duplication of the sequence leading a larger α-Hp subunit than is present in Hp1. In humans the distribution of Hp genotypes in healthy people is Hp 1-1, 17.1%; Hp 1-2, 50.1%; Hp 2-2 32.8%[11]. Pre-human Hp 2-2, which is 100% aligned with Zon, is the total primary sequence including the signal peptide and without the cleavage into the α-Hp and β-Hp subunits[8]. The similarity between pre-Hp2 and Zon and its evolutionary benefit in humans has not been fully explained.

Bovine Haptoglobin and Human Haptoglobin 2

It has been known, certainly within the community of experts in animal acute phase proteins, that human Hp2 closely resembles bovine Hp in primary structure, with 70% homology of amino acids and with a similar partial gene duplication leading to an extension on the bovine α-Hp subunit[12,9]. It is well known that Hp is a major acute phase protein in cattle and other ruminants with the serum concentration rising from the undetectable to reach levels of up to 1 g/l within a 24-48 hours of infection or other stimulation to the innate immune response[3]. On electrophoresis and western blotting bovine Hp separates into its components of the α-Hp and β-Hp with Mw of 23kDa and 43kDa respectively[4]. Whether a bovine pre-Hp exists and has a zonulin-like activity, for instance in the intestinal epithelia is an unanswered question.

Bovine Haptoglobin in Mastitis Milk and Colostrum

In dairy cows it has been demonstrated that Hp is present in milk during mastitis[6,7] and it has also been shown to be present in colostrum[10], the first milk produced by the cow and from which a calf derives maternal transfer of antibody. Western blotting with antibody to bovine Hp, of milk from cows in the early post parturient period showed a high concentration on the first day post calving and a rapid decline in Hp thereafter[10]. There was evidence in the western blot of the milk post calving in this study, of an additional band at a higher Mw that that of β-Hp. Recently, milk from dairy cows with mastitis was subjected to electrophoresis and western blotting with antiserum to bovine Hp, with the result that an extra stained band at 48kDa was clearly and consistently found in the samples of mastitis milk analysed. There was no detectable Hp in samples of milk from healthy cows. The nature and function of this high Mw band of Hp in milk from cows with mastitis awaits elucidation.

Discussion

These observations of Hp biology lead to interesting speculation as to the function of this protein in bovine physiology and pathophysiology. Questions that arise include: is there a link between Zon, pre-Hp2 in humans and bovine Hp? Could the action of Zon in opening tight junctions be relevant to the presence of Hp in bovine milk and colostrum? If Hp in colostrum could loosen the tight junctions in the intestine of calves in the first 24-48 h of suckling, then it could aid the transfer of IgG in the passive transfer of maternal antibody from maternal milk to

the calf. As the concentration of Hp in milk reduces the tight junctions would be re-established and normal digestion would proceed after 2-3 days.

In a mammary gland of a cow with mastitis a major pathophysiological action is the rapid transfer of white blood cells such as phagocytes from the circulation into the mammary gland contributing to a somatic cell count in milk of over 2,000,000 cells/ml. It has been shown that a rise in milk Hp is an early response to experimental infection of the udder[7]; could Hp cause a loosening of the tight junctions in the blood mammary border aiding the influx of these cells?

Biological functions often have endogenous control and feedback mechanisms. A feedback mechanism for the action of zonulin-like molecules that have caused loosening of tight junctions in barriers between blood circulation and external spaces, such as the lumen of intestine or mammary gland, could be that hemoglobin released from red blood cells binds to the Hp/Zon, causing its release from intestinal receptors and lead to the re-establishing of the tight junctions. The binding of hemoglobin to Hp has high affinity, being one of the strongest interactions of blood proteins[1].

To further investigate the likelihood of bovine Hp having zonulin-like activity a range of investigation is required. The presence of bovine Hp in intestinal cells should be assessed by immunocytochemistry, the nature of the high Mw form of Hp should be determined and the effects of Hp and extracts of colostrum and milk from cows with mastitis on the integrity of tight junctions should be determined in vitro and in vivo.

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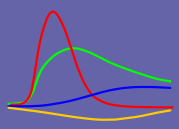

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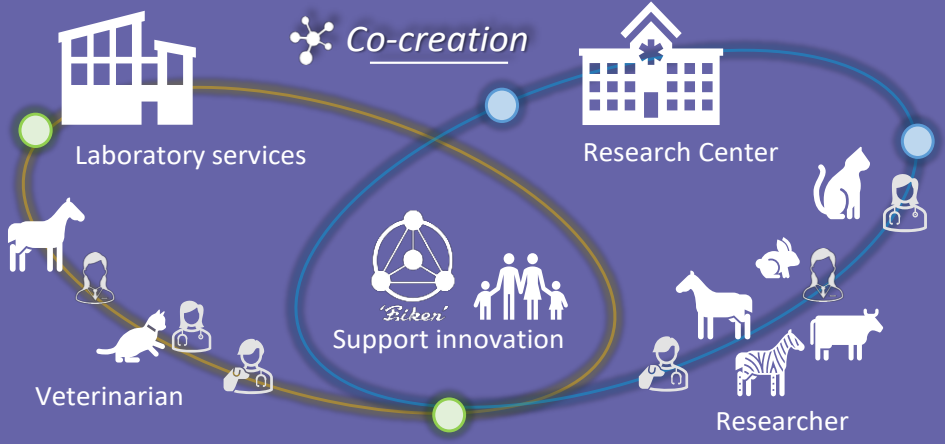

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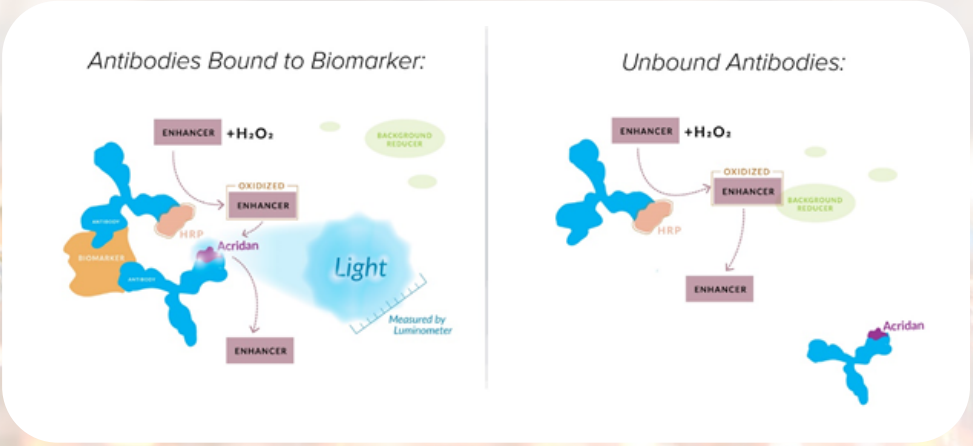

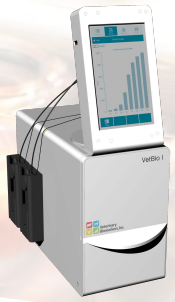

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VADDS

Software to Streamline Your Business

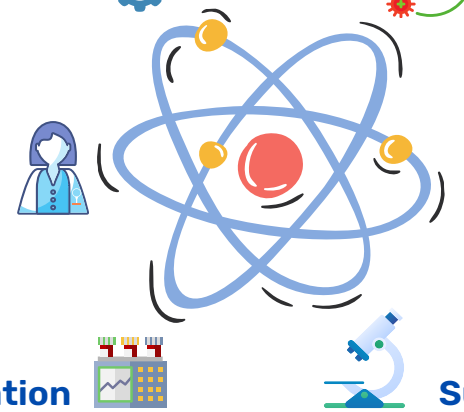
VADDS is a comprehensive, highly cost effective LIMS system designed for animal disease diagnostic laboratory management. VADDS is so flexible and highly customizable to your labs existing workflow it can be used in a wide variety of environments such as universities, state laboratories, small and large private practices and more! ATC is based in North America, with clients all over the world.

Best Value

Comprehensive package for larger labs and affordable core package for smaller labs.

Tremendously Customizable

Customizable Accession Screens & Results Reports. Used in Veterinary laboratories large and small



Consultation

Offices based in North America for immediate support. It's our desire to complement your workflow, not to limit it or dictate it. On-site consulting services available with a few weeks lead time.

Data Analytics

VADDS data analytics reports provide powerful business intelligence for your laboratory and organization. Queries can be exported to excel for further analysis and management

PIMS & Instrument Integration

Fully integratable with Advanced Technology Corp's Hospital Management System (**Vetstar**). VADDS currently interfaces with over 60 laboratory instruments and data sources. New interfaces are added as needed.

Subject Matter Experts

in Veterinary Software. Our team has over 50 years' combined experience and makes a constant commitment to understanding and meeting the evolving needs of the veterinary industry.

VADDS Messenger Service radically simplifies NAHLN messaging with the optional ability to use a JSON format for sending data instead of the HL7 message.

In response to the COVID-19 pandemic, Advanced Technology has rolled out a specially modified version of VADDS. **COVID VADDS** specifically addresses a variety of key business concerns including HIPAA and patient confidentiality, high volume testing and automation to name just a few.

Want to Learn More?

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201-934-7127 ex. 323

Ask for Elaine and mention this ad for ASVCP special pricing!

ADVANCED Technology Corp

Vetstar • VADDS

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