

APPLICATION OF ACUTE PHASE PROTEINS AS BIOMARKERS IN MODERN VETERINARY PRACTICE

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Received : 17.05.2013

Accepted : 05.11.2013

ABSTRACT

Animal body reacts to all kinds of injuries and stress to keep the homeostasis. Such resistance can be specific or non specific. Non specific innate resistance of the body like cytological and cytokine reactions including fever, leukocytoses etc. are known as acute phase response. In this response, there will be increase or decrease of serum concentration of particular proteins. These proteins are known as acute phase proteins. Measurement of serum concentration of these acute phase proteins are found to be useful in assessment of health status and prediction of diseases of the man and animals. Acute phase proteins like Serum amyloid A, C- reactive proteins, Haptoglobin, alpha 1 acid glycoprotein etc. are found to be more sensitive and specific than the routine diagnostic methods like white blood cell count. Use of acute phase proteins as biomarkers for animal disease diagnosis and health status assessment has got high potential in modern veterinary practice. Usefulness of the acute phase protein analysis in veterinary practice especially in dog, cat, cattle and pig practices is discussed in this review.

Key words: acute phase proteins, biomarker, animal disease diagnosis, Serum amyloid A, C-reactive protein

INTRODUCTION

Current researches in disease diagnosis are aimed to identify ailments well before the clinical manifestation. In case of animals this early identification is important not only for the well being of the animals but also for the

humans who consume the animal and its products. Scientists are consistently in search of such predictive biomarkers in man and animals. Research on serum acute phase proteins (APP) provides a lime light in the area of non specific biomarkers.

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Animal body shows two types of immune reaction to any type of injury. One is specific immune reaction mediated by antibodies and the other is innate nonspecific immune reaction like fever, cytological reactions etc. This innate nonspecific immune reaction of the body is otherwise known as acute phase response. The main aim of acute phase response is to maintain homeostasis and tissue healing. In the acute phase response serum/ plasma level of some kind of proteins are found to decrease while the levels of some other proteins increase many folds. These proteins are known as acute phase proteins (APP). These proteins include protease inhibitors (eg. alpha 1 antitrypsin, alpha 1 antichymotrypsin), coagulation proteins (e.g. fibrinogen, prothrombin), complement proteins (e.g., C2, C3, C4, C5, etc.), transport proteins (e.g., Haptoglobin (Hp), Ceruloplasmin (Cp), hemopexin) and some other kind of proteins, like C reactive protein (CRP), serum amyloid A (SAA), serum amyloid P (SAP), acid glycoprotein (AGP) etc.

Those APP whose serum levels decrease in acute phase response are called as negative acute phase proteins (eg. albumin, transferrin etc.) and whose serum levels increase are called as positive acute phase proteins (Kaneko, 1997). Among the positive acute phase proteins, the serum level of some APP increase 10 to 100 or even 1000 folds within a few hours after injury. They are called as major APPs. Proteins whose levels increase 2-10 times and their value decline to normal after longer period are known as moderate APPs and those with slight increase in serum level (approximately 2 times or lesser) are

known as minor APPs. Moderate and minor APPs are more pronounced in chronic inflammation (Ceron *et al.*, 2005).

Acute phase proteins are primarily synthesized by the liver. Their production is triggered by different stimuli including trauma, infection, stress, inflammation and neoplasia. The mechanism for stimulation of the hepatic production of the acute phase protein is mainly by pro-inflammatory cytokines. Induction of the acute phase proteins by IL-6, following binding to the IL-6 receptor, is via the phosphorylation of the transcription factor, NF-IL6 which is then translocated to the nucleus, where it mediates the transcription of acute phase genes. IL1 and TNF alpha, after linking to their respective receptors cause phosphorylation and degradation of I κ B, the inhibitor of transcription factor NF κ B leading to release of NF κ B and subsequent activation of acute phase genes in the nucleus.

The functions of positive acute-phase proteins (APP) are regarded as important in optimization and trapping of microorganisms and their products, in activating the complement system, in binding cellular remnants like nuclear fractions, in neutralizing enzymes, scavenging free hemoglobin and radicals, and in modulating the host's immune response.

The first APP identified was C reactive protein (CRP) in pneumococcal infection of monkeys and human in early 1930s (Tillett and Francis, 1930). Now the CRP has become a major biomarker in human for trauma, tumor, myocardial infarction and wellness assessment.

Applications of APP assay in animals

Up to 1990s the uses of APP in animals were limited to research work. By the mid of 1990s use of APPs have gained importance in veterinary medicine. APPs are used in veterinary medicine with the following objectives

Objectives of APP assay in animals

1. Checking the health status of the individual animal or herd in a farm
2. Quantification of inflammatory activity - it indicates how intense or serious the inflammation is.
3. Monitoring inflammatory activity - during the course of treatment, efficacy of treatment can be monitored at the earliest by detecting a drop in APP levels.
4. Detection of post operative recovery / complications- normally the APP levels decline 48 to 72 hours after the surgical procedure. A contradiction to same may indicate complication and need for changing antibiotics / procedure.
5. Helps in ante-mortem inspections of large herds in slaughter houses. Animals with varied APP values need in-depth checking.

Assay of APP provides better sensitivity and specificity in finding whether, the inflammation is acute or chronic. In comparison, neutrophil counts had much lower sensitivity and specificity (30% to 70%) (Horadagoda *et al.*, 1999). In a retrospective evaluation of inflammatory conditions in dogs, APP showed significant changes even in the absence of changes in the total or differential WBC count (Solter *et al.*, 1991). In a review of more than 900 cases of inflammation in dogs with various diseases, CRP concentrations were significantly correlated with disease, whereas only slight or no correlation was found with total WBC and band neutrophil counts (Nakamura *et al.*, 2008).

Acute phase proteins of importance in animals

Albumin is considered to be one of the major negative APP in all classes of animals. Paraoxonase (PON) is another negative APP. Transferrin is considered to be a positive APP in birds but negative APP in most of mammals. Alpha 1 acid glycoprotein is a positive APP in animals except in pigs where it has been recently identified as a negative APP (Heegaard *et al.*, 2013). The major APP of different animals are listed in the Table 1.

Table - 1 Acute phase proteins of significance in different species

Species	Major APP	Moderate APP
Human	CRP, SAA	AGP, fibrinogen, Hp
Dog	CRP, SAA	AGP, Cp, Hp
Cow	SAA, Hp	AGP, CRP, fibrinogen
Cat	AGP, SAA	Hp
Goat	Hp, SAA	Fibrinogen
Pig	Hp, SAA, MAP	AGP
Chicken	None	AGP, Cp, SAA

SAA: Serum Amyloid A

Hp: Haptoglobin

CRP: C- reactive Protein

AGP: alpha 1 acid glycoprotein

Species Specific Application of APP

Studies on acute phase response of many species of animals are made worldwide. Species of importance as companion animals like dog and cat and farm animals like cattle and swine are reviewed in this article.

Dog : In canines CRP is the major APP used as marker for systemic inflammation / infection. Normally the level of CRP is less than 1.5 mg/dL or even lower than 0.5 mg/dl. The normal range may be 0.08 to 2.26 mg/dl (Otabe *et al.*, 1998). The level rises within 4 to 6 hrs after onset of inflammation / infection. Serum CRP level above 3.5 mg/dl, indicates presence of systemic inflammation. Level above 5 mg/dl is a strong evidence of systemic inflammation.

Elevated concentration of CRP at the time of diagnosis of lymphoma carries a poorer prognosis in humans. Similar study of relation between elevation of CRP level and lymphoma in canines by Joana (2013) revealed that, the

level of CRP is elevated in lymphoma of canines both at times of diagnosis and at the time of relapse of the disease but did not show any prognostic significance. But a trend of reduced survival time was observed for those dogs with CRP concentration of more than 3 mg/dl. Some diagnostic manufacturing companies have recently introduced lymphoma diagnostic kits using the CRP and Haptoglobin (eg. Tridelta Development Ltd)

Cases like pyometra, panniculitis, acute pancreatitis, polyarthritis, leptospirosis, babesiosis, parvo infection, glomerulonephritis, immune mediated disease and malignant neoplasia show significantly high CRP (Nakamura *et al.*, 2008). Rise in CRP may not be observed in local tumors like leiomyosarcoma, upper respiratory tract infection, diabetes, neurological problems involving intracranial disorders. Since the CRP concentration did not increase in patients with intervertebral disk protrusion, it might be useful in distinguishing arthritis from spinal / brain

diseases in patients with lameness. Thus, although CRP is a nonspecific inflammatory marker, it could facilitate diagnosis by indicating the presence and the extent (e.g., localized vs. generalized, neurological vs. other) of inflammation. In canine babesiosis, the serum CRP is significantly higher while serum Haptoglobin is significantly lower when compared to healthy dogs (Ulutas *et al.*, 2005).

Albumin, a negative APP is a significant biomarker for bacterial infection. The serum level of fibrinogen, a positive APP is more reliable than albumin as its level persistently raised up to 21 days of post inoculation with staphylococci (Zapryanova *et al.*, 2013).

PON 1 is a negative acute phase protein in canines. But contrarily to CRP and α 2-globulins, PON1 activity was not significantly different between dogs with and without inflammation. Hence PON1 does not provide any diagnostic value as a negative acute phase protein in dogs (Gabriele *et al.*, 2013).

Examination of CRP concentration as a routine diagnostic test is needed to be evolved and incorporated in canine practices, as it is more sensitive and specific than the WBC counts.

Cat : SAA, AGP and Hp are the main acute phase proteins of cat. Concentration of acute phase proteins in healthy cat is not yet well standardized. APP values vary widely in different studies. Kann *et al.* (2012) reported normal concentration of APP in cats as follows: SAA- $1.8 \pm 2.3 \mu\text{g/ml}$; AGP- $532.8 \pm 204.1 \mu\text{g/ml}$; Hp- $2.5 \pm 2.1 \text{ mg/ml}$ and Albumin $28.2 \pm 4.7 \text{ g/l}$. But the concentrations reported by Giordano *et al.* (2004) were 10.21 ± 8.32

$\mu\text{g/ml}$, $1200 \pm 620 \mu\text{g/ml}$, $1.3 \pm 0.64 \text{ mg/ml}$ for SAA, AGP and Hp respectively. The difference may be due to the different methodology adopted by different scientists. In general concentration of APP in healthy cats increases with age and there is variation with gender.

Values of SAA, AGP and Hp are found to be increased in a variety of pathological conditions like infections, injuries, renal failure, hospitalization, surgery and neoplasms (Paltrinieri, 2008). SAA level is found to be increased in pancreatitis (Tamamoto *et al.*, 2009) and neoplasia like malignant mesothelioma. Serum level of AGP increases in feline immunodeficiency diseases, feline chlamydiosis, non symptomatic feline corona virus infections, carcinoma, sarcoma, round cell tumour etc. SAA value increases in conditions like renal failure, injury, and hospitalization while Hp level increases in inflammations, feline infectious peritonitis and splenectomy.

Though there is report of decrease in concentration of albumin in many feline inflammatory reactions (Ottenjann *et al.*, 2006), confirmatory data are not yet available for considering albumin as negative acute phase protein in cats.

Cattle : SAA, Hp and fibrinogen are the major APPs of cattle while albumin and PON are negative APPs. Unlike canines, clinical application of acute phase proteins in large animals has not been sufficiently standardized in routine practice. Many studies have been made on the serum concentration of APPs of cattle and its significance in different age groups, during parturition, lactation period,

acute and chronic infections, non-infectious and metabolic diseases.

Calves show higher concentration of APPs than the adult cattle. This is due to various physiological needs and challenges faced by the calves in its growing stage. The average concentration of Hp and SAA in one month old healthy calf is 6.8 mg/dl and 5.9 mg/dl respectively which will lower to about 2.1 mg/dl and 1.9 mg/dl respectively in the age of six months (Tothova *et al.*, 2011 B). Even though CRP is not a major APP in cattle, high concentration of CRP is noticed in healthy calves believed to be transferred through colostrum (Schroedl *et al.*, 2003).

SAA and Hp estimation is useful in differentiating acute and chronic inflammation in cattle. Seven different isoforms of SAA are noticed in chronic inflammation of cow and out of this only three - SAA 1, SAA 2 and SAA 3 are elevated in acute phase response. SAA1 and SAA 2 are produced by liver and SAA 3 is produced extra hepatically from adipose tissue, mammary duct etc. Serum Amyloid A has maximum clinical sensitivity while Hp has highest serum specificity (Horadagoda *et al.*, 1999). Various studies on serum concentration of APP in bronchopneumonia showed that SAA concentration rises rapidly while Hp concentration increases with severity of the disease (Heegaard *et al.*, 2000). Hp level in serum increases with the number of antimicrobial treatment given and estimation of Hp alone has got better predictive value (Berry *et al.*, 2004)

Anaplasma marginale infected cows showed significantly increased serum Hp (20.3 mg/dl) and serum amyloid-A concentrations

(13.4 mg/dl) (Coskun, 2012). In naturally infected brucellosis cows there is significant increase of SAA but no significant increase in Hp (Sharifiyazdia *et al.*, 2012).

Most important application of APP assay in cow is diagnosis of subclinical mastitis. SAA of serum and milk increases in mastitis. It is to be remembered that normal colostrum contains high concentration of milk associated SAA which provides protection to the gastrointestinal tract of the calf by stimulating mucin production and reducing the adherence of injurious agents. It was demonstrated that SAA levels increased much earlier in milk than in serum and preceded increased somatic cell count in milk (Pedersen *et al.*, 2003).

Milk SAA does not increase with inflammatory process outside the udder (Nielsen *et al.*, 2004). In experimental *E.coli* mastitis, the concentration of SAA increased after 12 hours and its highest concentration was observed 60 hours after the infection (Suojala *et al.*, 2008). In natural subclinical infection the milk level of SAA was found to raise to 0.65 - 22.16 mg/dl (control group-0.5 -1.9mg/dl) and that of Ceruloplasmin raised to 3.35 to 8.02 U/g of protein (control group-0.73 – 2.11 U/g of protein) and both were found to be good indicators of subclinical mastitis (Szczybal *et al.*, 2012).

Haptoglobin concentration in both serum and milk increase in clinical mastitis of cow (Petersen *et al.*, 2004). Hp in milk is found to be locally synthesized by the mammary gland and neutrophils. Using a threshold value of 0.02 mg/ml for milk Hp and 0.55 g/ml for milk SAA, both tests show a high specificity (100%) with

no false positive results, and a reasonable sensitivity for the diagnosis of mastitis. Wenz *et al.* (2010) observed that concentration of Hp in the group of cows affected by Gram-negative bacterial mastitis was approximately twice that of the group infected by Gram-positive bacteria (1,126 vs 575 mg/ml, respectively).

A significant rise of Hp and SAA concentrations in milk was observed in udder quarters with chronic subclinical mastitis (Gronlund *et al.*, 2005). Hp and SAA concentrations below the detection limit were considered as good indicators of healthy udder quarters.

SAA concentration was found to be enhanced in cows fed with high grain concentration (Emmanuel, 2008). Sub acute ruminal acidosis produced by reduced fibre diet has no role in rising serum APP (Mulligan and Doherty, 2008). Study by Cannizzol *et al.* (2012) indicated that ruminal pH / acidosis is not able to produce acute phase response. But development of fatty liver is indicated by a high APP level (Ametaj *et al.*, 2005)

Concentration of SAA and Hp is found to increase after parturition, attaining highest level within 3 days after calving (mean value more than 6.6 mg/dl and 13 mg/dl respectively). Chan *et al.* (2010) noticed high levels of SAA (more than 8.5 mg/dl) in cows with post partum metritis. Dubuc *et al.* (2010) opined that high blood Hp (above 80 mg/dl) is a marker for reproductive disorders in the first week of calving. Holstein cows diagnosed with uterine infection had reduced serum albumin concentration 21 days before calving, lower paraoxonase (PON) activity at 7 days after calving, and increased Hp in multiparous cows at 7 days in milk compared to healthy cows

(Schneider *et al.*, 2013). These studies show the significance of APPs in early diagnosis of uterine infection.

High concentration of SAA is noticed in lame cows with sole ulcers (Kujala *et al.*, 2010). Serum Hp didn't show any difference between healthy and lame cows. But Tóthová *et al.* (2011a) found significantly higher concentration of SAA, Hp and fibrinogen in heifers with foot disease. It is to be noted that Serum Amyloid A is found to increase in stress condition like transportation, slippery floor, etc.

An age wise study of the activity of PON1 in cattle by Giordano *et al.* (2013) revealed that the activity increases from 2 to 21 days of age in healthy calves and from 21 to 120 days no significant increase in PON1 activity was reported. But activity is significantly high in adult cattle. The PON1 activity is significantly lowered in calves of less than 7 days of age with diarrhoea and in calves of 21 to 120 days of age with respiratory diseases, showing that PON1 is a diagnostically significant negative acute phase protein in cattle.

It is always better to include both major and moderate positive and negative APPs in an assay. An Acute phase index can be made using the formula given below to enhance specificity of APP to detect non healthy animal in the herd.

Acute phase index =

$$\frac{\text{Value of major APP} \times \text{Value of moderate APP}}{\text{Value of rapid negative APP} \times \text{Value of moderate negative APP}}$$

Pigs : In pigs five acute phase proteins namely C-reactive protein (CRP), serum amyloid A

(SAA), haptoglobin (Hp), pig-MAP and albumin are found to be significant. In porcine reproductive and respiratory syndrome virus (PRRSV) all the above APPs except the pig MAP level were found to be elevated. In Aujeszky's disease virus (ADV) infection, only Hp level was elevated. Porcine circovirus type 2 (PCV2) had elevation of all APPs. Haptoglobin has the highest sensitivity in pigs (Parra *et al.*, 2006) when compared to other APPs.

Increased level of CRP, SAA, Hp and Pig MAP were noted well before the rise of antibody titre in experimental inoculation with swine influenza(H1N1) and *Pasteurella multocida* in pigs (Pomorska-Mól *et al.*, 2013).

Pallares *et al.* (2008) found that serum concentration of Hp and CRP are high in fattening pigs with clinical disease than apparently healthy pigs with gross lesion (2.1 times) at slaughter and healthy pigs without gross lesion (2.6 times) and hence can be used as markers for presence of lesions at slaughter. Heegaard *et al.* (2013) revealed that Pig AGP behave as a negative acute phase protein during a range of experimental infections and aseptic inflammation with significant decrease in serum concentration.

Gutierrez *et al.* (2012) had made a longitudinal analysis of APP in pig saliva using time-resolved immunofluorometric assays (TR-IFMA) and found that their level is concomitant with the serum level and useful for monitoring of diseases in pigs.

METHODS AND MATERIALS

Total protein and Albumin are usually assayed using the automated chemical serum

analyzer. Protein electrophoresis has also been employed for assessing the APP for many years. ELISA is the widely used method for assaying the individual APP. This method is best employed for batch analysis of many samples. Species specific ELISA kits have to be used for reliable results. Serum collected for APP assay can be kept for 2 days under refrigeration at -4°C or for one month in deep freezer at -20°C. For prolonged storage it is better to keep the serum at -80°C. Radioimmunoassay, nephelometry, immunoturbidimetry, Western blot and mRNA analyses (Paltrinieri, 2008) have also been employed for the assay. For qualitative / semi quantitative methods latex test kits can be employed.

Immunosensor assay is found to be a quick and reliable technique for detection of Hp in mastitis milk (Tan *et al.*, 2012). Haptoglobin in haemolysed samples can be estimated using a correction equation, $\text{Hp corrected} = \text{Hp raw} - \text{Hp endogenous activity} - \text{Hp due to Hb}$; where $\text{Hp due to Hb} = 0.118 \times \text{Hb free} + 0.015$. (Slocombe *et al.*, 2012).

It is conclusion the acute Phase proteins are found to be elevated in animals before the manifestation of clinical signs in many diseases. As their elevation is a non specific reaction, the information cannot be used for diagnosing a specific condition but can be used as health monitoring tool. Researches so far conclude that APPs are more sensitive than the conventional methods like the WBC count, Somatic cell count in milk, etc. It is better to assay two or more APPs together to get a valid result. New technologies need to be developed for economically feasible estimation of APPs. More research is needed

to be done for making these biomarkers a perfect predictive tool for animal health status.

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