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# Evaluation of Haptoglobin as a Prognostic Marker in Dogs Affected with Hepatobiliary Disorders

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**ABSTRACT**

In the present investigation, the mean levels of the haptoglobin concentration was evaluated among 140 dogs affected with hepatobiliary disorders and compared with healthy control group dogs. Haptoglobin values were significantly declined among dogs affected with diffuse hepatic parenchymal disorders with ascites. While, a significant elevation of haptoglobin concentration was observed in focal parenchymal disorders, biliary tract disorders and diffuse hepatic parenchymal disorders without ascites as compared to the healthy control group.

**HIGHLIGHTS**

- Haptoglobin evaluation was carried out to assess the prognosis of the dogs affected with hepatobiliary disorders.
- Haptoglobin values in dogs diagnosed with hepatobiliary disorders were recorded.

**Keywords:** Dogs, Haptoglobin, Hepatobiliary disorders, prognosis

Liver plays a central role in a diverse array of processes including carbohydrate, lipid and protein metabolism; storage of vitamins, trace minerals, fat, glycogen and immune regulation. Liver is uniquely susceptible to damage as a consequence of its role as a filter for portal blood, metabolism of endogenous metabolites and xenobiotics. Symptoms, clinical signs and diagnostic results reflect impairments in these functions (Meyer and Rothuizen, 2013). Liver has great functional reserve capacity; detection of hepatic function or impairment by conventional means is possible only when ≥ 55% hepatic dysfunction is present (Hall and German, 2005).

Hepatobiliary dysfunctions usually seen among metabolic disorders, drug induced hepatotoxicity, congenital or neoplastic diseases, degenerative processes, number of acute and chronic clinical conditions, infectious diseases, auto-immune diseases, vascular injury (Kumar *et al*., 2013). The WSAVA (World Small Animal Veterinary Association) liver standardization group recently categorized hepatic disorders in dogs into four main types viz., parenchymal, biliary tract, vascular and neoplastic liver disorders (Geschen, 2009). Acute phase proteins are synthesized primarily by the liver. Their production in animals is triggered by different stimuli including infection, trauma, inflammation, neoplasia and stress. Various acute phase proteins are used as biomarkers for disease diagnosis and health status assessment (Pradeep, 2014). Haptoglobin is an acute phase protein which increases markedly in various types of canine liver

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diseases but in later stage of the disease the haptoglobin fraction decreases. Decreased albumin in association with significantly decreased haptoglobin values indicates poor prognosis. On the other hand, decreased albumin with increased haptoglobin values indicates good prognosis (Sevelius and Jonsson, 1995). Assessment of haptoglobin (acute phase proteins) in dogs had clinical use in the diagnosis, prognosis and monitoring of therapy in a diverse range of canine conditions. Haptoglobin can be considered to be one of the canine acute phase protein with moderate in nature among the dogs that increases approximately 2 to 3 fold in inflammation, infection or trauma (Crawford *et al.,* 2013).

## MATERIALS AND METHODS

Haptoglobin was estimated in the serum using a dog haptoglobin solid phase enzyme linked immunosorbent assay (ELISA). The assay for solid phase (microtiter wells) advocates affinity of the purified anti dog haptoglobin antibodies, immobilization and conjugated anti dog haptoglobin antibodies with horse radish peroxidase (HRP) for detection. The test sample was diluted and incubated in the micro titre wells for a period of forty five minutes. HRP conjugate was added to the wells and incubated for 30 minutes after washing the microtiter wells. Later, this causes sand witch among haptoglobin molecules detection antibodies and immobilization. Unbound HRP labelled antibodies are removed in the wells after proper washing of the wells and reagent TMB was added and at room temperature incubated for twenty minutes. It resulted in blue colour development. Addition of stop solution was done to stop Colour development, colour changes to yellow and optical density was read using spectrophotometer at 450 nm. The measured haptoglobin concentration is proportional to the test sample optical density.

### Materials

Anti dog haptoglobin antibody coated microtitre plate with 96 wells (12 detachable strips of 8), Enzyme conjugate reagent: 11 ml, Reference standard (lyophilized) containing 2 µg/ml dog haptoglobin when reconstituted as detailed on the vial label, 20 x Wash buffer, 50 ml, 10x Diluent, 25 ml, TMB Reagent (one step), 11 ml, (1N Hcl) Stop solution 11 ml, Precision pipettes and tips, Distilled water/Deionised water, Polypropylene/ glass tubes**,** Vortex mixer, Absorbent paper, Micro plate shaker mixing speed of 150 rpm, ELISA Plate reader with a range of 0-4 at 450 nm optical density.

### Wash solution preparation

Wash Solution preparation is available as a 20x stock. Before use contents of the bottle (50 ml) should be diluted with the 950 ml distilled water.

### Diluent preparation

The diluent was prepared as a 10x stock. Before usage, calculate the diluents final volume required for the assay and dilute one volume of the 10x stock with 9 volumes of distilled water.

### Standard preparation

The standard dog haptoglobin was available as a lyophilized stock. As indicated on the vial label, add the distilled water volume and mix gently until dissolved to obtain a 2 µg/ml dog haptoglobin stock. Label eight glass tubes or polypropylene tubes as 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.95 and 0 ng/ml. Then add 937.5 µl of diluents into the tube labelled 125 ng/ml and 300 µl of diluents into remaining tubes. Pipettes 2 µg/ml of the 62.5 µl haptoglobin standard into the tube labelled 125 ng/ml and mix. This provides the working 125 ng /ml haptoglobin standard. By diluting prepare 62.5 ng/ml standard and mix 300 µl of the standard (125 ng/ml) with 300 µl of diluents in the tube labelled 62.5 ng/ml. Similarly prepare the standards of 31.25, 15.26, 7.8, 3.9 and 1.95 ng/ml by serial fold dilution method.

### Sample preparation

Dispense 998 µl and 497.5 µl of 1x diluents into separate tubes. Pippette and mix 2 µl of the serum/plasma present in the tube with 998 µl of diluents. It creates a five hundred fold diluted sample. In the second tube, add 500 fold diluted sample 2.5 µl with the 497.5 µl of the diluents. Which forms a dilution of 1,00,000 fold of the sample. Continue the same procedure for each of the tested samples also.

### Haptoglobin assay procedure

Place the required number of coated wells into the holder. Dispense standards of 100 µl and samples diluted into the wells (All the samples should be tested twice). Place them on to an 100 -150 rpm orbital micro-plate shaker maintained at 18- 25ºC room temperature for 45 minutes. Later, the incubation mixture should be removed using by clicking the contents in the plate into an appropriate bio-waste container or with the plate washer. Empty and wash the micro titre wells five times using 1x wash solution. This was done using with squirt bottle or a plate washer (350 µl/well). Place the wells sharply onto a paper towels or an absorbent paper to discard the residual droplets. Then enzyme conjugate of 100 µl reagent was added into each well. Orbital micro plate shaker was incubated at room temperature (18-25ºC) with 100-150 rpm for 30 minutes. Wash as detailed as above and place the wells sharply onto paper towels or an absorbent paper or to discard the residual droplets. Add TMB reagent of 100 µl into each well. Mix gently on an orbital micro plate shaker at room temperature (18-25ºC) 20 minutes for 100-150 rpm. Add stop solution of 100 µl of to each well to stop the reaction. Mix gently and properly so that the blue color changes to yellow color. It is important to make sure that within 15 minutes, using micro titre plate reader we read the optical density at 450 nm.

## RESULTS AND DISCUSSION

In the present study, one hundred and forty dogs were diagnosed with hepatobiliary disorders by ultrasound imaging and histopathological studies. Out of which 24 (17.15%) dogs were diagnosed as focal parenchymal disorders, 52 (37.14%) as biliary tract diseases, 32 (22.86%) dogs diagnosed as diffuse hepatic parenchymal disorders with ascites and 32 (22.86%) as diffuse hepatic parenchymal disorders without ascites. Haptoglobin was measured in these dogs to analyze their utility in the diagnosis of hepatobiliary disorders apart from routine hemato-biochemical tests and imaging studies. Haptoglobin was measured using a dog haptoglobin Elisa kit method. Serum samples from healthy control and dogs with hepatobiliary disorders were used to run the test. The result of the test was noticed by the color development which was later quantified as depicted in Figs. 1 and 2. 2.

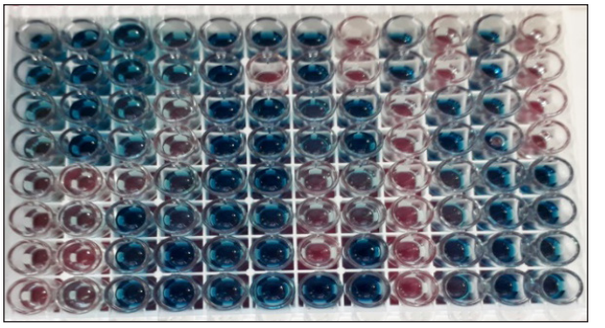

**Fig. 1:** Elisa plate of dog haptoglobin kit showing color development before addition of stop solution

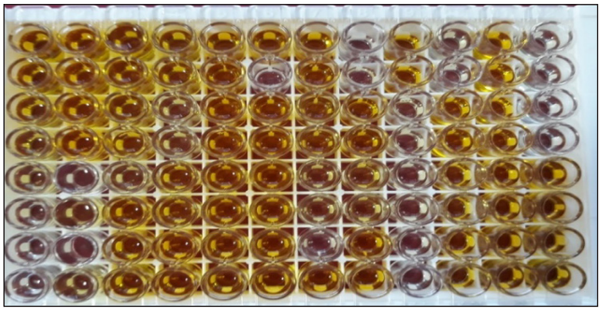

**Fig. 2:** Elisa plate of dog haptoglobin kit showing color development after addition of stop solution

The mean levels of haptoglobin concentration in dogs diagnosed with focal parenchymal disorders, biliary tract disorders, diffuse hepatic parenchymal disorders with ascites and diffuse hepatic parenchymal disorders without ascites were 1.23±0.13, 1.13±0.09, 0.70±0.08 and 1.27±0.09 ng/ml respectively (Table 1). A significant ($P<0.05$) elevation of haptoglobin values were seen in dogs affected focal parenchymal disorders, biliary tract disorders and with diffuse hepatic parenchymal disorders without ascites. While, haptoglobin concentration decreased significantly ($P<0.05$) among diffuse hepatic parenchymal disorders with ascites as compared to healthy control (0.93±0.15 ng/ml). The mean levels of haptoglobin concentration were significantly declined in dogs with diffuse parenchymal disorders with ascites. While, a significant elevation of haptoglobin was seen in diffuse parenchymal disorders without ascites, focal parenchymal disorders and biliary tract disorders as compared to the healthy control group. These findings were in accordance with the results of Sevelius and

Jonsson (1995) and Andersson and Sevelius (1991), who reported increased concentrations of serum haptoglobin in various forms of hepatitis and decreased concentrations in late stage cirrhosis.

**Table 1:** Mean values of haptoglobin as a Prognostic markers in healthy and various hepatobiliary disorders affected dogs

| Hepatobiliary disorders | Number (N) | Haptoglobin (ng/ml) |
|---|---|---|
| Healthy control | 10 | 0.93± 0.15 |
| Diffuse parenchymal disorders with ascites | 32 | 0.70± 0.08* |
| Diffuse parenchymal disorders without ascites | 32 | 1.27± 0.09* |
| Focal parenchymal disorders | 24 | 1.23± 0.13* |
| Biliary tract disorders | 52 | 1.13±0.09* |

* Significant at ($P < 0.05$).

Haptoglobin in dogs is considered as a acute phase protein with moderate in nature and which increases approximately 2 to 3 fold in inflammation, infection or trauma (Mc Grotty *et al*., 2003) and used in the diagnosis, prognosis and monitoring of therapy in diverse range of canine conditions (Mastrorilli *et al*., 2007; Crawford *et al.,* 2013). The Haptoglobin viz., acute phase proteins (APP) are the reactants which are usually synthesized during an acute phase response against several stimuli like stress, inflammation, trauma, tissue damage or infection. The concentrations of APP may increase or decrease after an appropriate stimulus being classified as positive, moderate or negative APP's depending on the enhancement of its concentration (Petersen *et al.,* 2004). Haptoglobin is considered as a glycoprotein composed of 2 α and 2 β chains connected by disulfide bridge. It binds to free haemoglobin which is released from red blood cells possessing high affinity and reduced oxidative damage to itself and albumin (Yang *et al*., 2003).

## CONCLUSION

Haptoglobin as a prognostic marker was useful in assessing the condition of the dogs affected with various hepatobiliary disorders.

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