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# Serosurvey and Enzymatic Evaluation of Canine Hepatitis B in Herding Dogs

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**Abstract**

For our knowledge, this is the first study targeted serological detection of canine hepatitis among herding (stock) dogs in Iraq using the technique of indirect enzyme-linked immunosorbent assay (ELISA). Association between infection and hematology, liver enzymes, antioxidants and lipid peroxides, and demographic risk factors (age and sex) was evaluated, also. In total, 27 (14.67%) of the 184 dogs selected randomly from many rural, sub-urban, and urban areas during July (2020) to January (2021), were confirmed as seropositives for the presence of anti canine adenovirus-2 antibodies. Among seropositive dogs, the findings of demographic risk factors revealed a significant increase in values of rural and sub-urban compared to urban region, and in > 1- ≤ 3 years more than > 6-12 months, ≤ 6 months, and > 3 years age groups; however, no significant differences were detected between seropositive females and males. In comparison to seronegatives, the concentrations of ALT, AST, GGT, and TB of seropositives were showed a significant elevation whereas TP was decreased significantly. For antioxidants and lipid peroxides, significant decrease in levels of CAT, GPx, and SOD with significant increase in MDA were reported in seropositive dogs. In conclusion, there is an obvious prevalence of canine hepatitis among herding dogs in study areas in Iraq; however, furthermore studies are required to support our findings. Additionally, ELISA can be used as a rapid diagnostic tool in detection the prevalence of infection.

***Keywords:*** Adenovirus-2, Stock dogs, Liver enzyme, Antioxidant, lipid peroxides, Iraq.

## Introduction

Canine hepatitis or Rubarth's disease is an infectious disease caused by a non-enveloped, icosahedral double-stranded DNA virus, canine adenovirus type-1 (CAV-1), which classified under Mastadenovirus genus of Adenoviridae family. Worldwide, CAV-1 can infect a wide range of domestic and wild animals of *Canidae* (dogs, fox, wolf and coyote), *Ursidae* (bear), and *Mustelidae* (marten and weasel) (**Kahilo et al., 2012; Sykes, 2014; De Jonge et al., 2020**). The transmission of infection occurs by shedding of CAD-1 during acute phase of disease through the blood, nasal discharge, saliva, urine, or feces (**Decaro et al., 2008; Bulut et al., 2013**). For pathogenesis, the virus replicates in oronasal cavity resulting in pharyngitis and tonsillitis, which followed by variemia and translocation of virus in many organs especially liver, kidney and eye (**Boomkens et al., 2004; Greene, 2012**). Clinically, the disease characterized by in apparently healthy to rapidly fatal disease due to the massive destruction of hepatocytes, hepatocellular

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necrosis, hemorrhage, edema of the gall bladder, and multifocal vasculitis (**Caudell et al., 2005; Greene, 2012**). Chronic neurological and ocular signs may develop in complicated cases after recovery from acute or sub-clinical stages resulting in encephalitis, ocular discharges, blepharospasms and photophobia (**Caudell et al., 2005; De Jonge et al., 2020**).

Different assays have been described to diagnosis of canine hepatitis. Histology through impression smears of liver biopsies or tissues to detect inclusion bodies can be used at necropsy. Data of hematology, urinalysis, coagulation profile, and serum biochemical tests may facilitate the diagnosis. For confirmation, virus isolation from any body fluid or tissues in acutely diseased dogs, in addition to serology [serum-neutralization (SN), hemagglutination-inhibition (HI), and enzyme-linked immunosorbent assay (ELISA)], and molecular techniques [conventional and Real-Time polymerase chain reaction] were applied (**Kiss et al., 1996; Caudell et al., 2005; Bexfield, 2017**). In Iraq, dogs are not usually adopted as pets for different traditional and religious reasons. On the other hand, stray and herding dogs are common even inside urban areas; therefore, cross-species transmission of infection is existed between different hosts (**Bonvicino et al., 2014**). It appears that there is only one recent serological study targeting canine distemper in stray dogs in Iraq (**Al-Jumaa et al., 2020**). Thus, this seems to be the first Iraqi study aimed to detect canine hepatitis B in herding dogs using the technique of indirect ELISA with estimation association between infection and functions of liver enzymes, antioxidants and lipid peroxides, in addition to the demographic risk factors (age and sex).

## Materials and methods

### *Ethical approval*

The present study approved by the Scientific and Ethical Committee, College of Veterinary Medicine, Wait University / Iraq. The samples of study dogs were collected, processed, and tested according to the standard procedure and under the regulation of the Department of Internal and Preventive Veterinary Medicine and Department of Microbiology in College of Veterinary Medicine, Wait University.

### *Study animals*

Totally, 184 herding (stock) dogs of different ages and sexes were selected randomly from many rural, sub-urban, and urban areas during July (2020) to January (2021). Approximately, 6 ml of venous blood samples were collected from cephalic vein under aseptic conditions a free-anticoagulant glass gel tube. After centrifugation (5000 rpm for minutes), sera were kept frozen into labeled 1.5 ml eppendorf tubes. Concerning demographic data, age of study animals was estimated as described previously (**Tobias et al., 2000**) in addition to information the herd owners.

### *Serology*

*Canine hepatitis positivity*

Following the manufacturer instructions of Canine Hepatitis B virus surface antigen (HbsAg) indirect ELISA Kit (*Sunlong Biotech, China*), the serum samples and kit materials were prepared and processed. Briefly, 50µl of Positive (PC) and Negative (NC) Control was added in

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duplicate to the first wells of micrelisa stripplate; then, a total 10µl of sera in addition to 40µl of sample diluents were added to other wells except the last well that considered as blank. After incubation (37°C for 30 minutes), the micrelisa stripplate washed five times with diluted Washing Buffer. A total 50µl of HRP-Conjugate was added to each well except the blank, incubated (37°C for 30 minutes) and washed as previously mentioned. A total 50µl of each Chromogen Solution A and B were added to each well except the blank and incubated at 37°C for 15 minutes. Finally, 50µl of Stop Solution was added to all wells.

Absorbance of micrelisa stripplate was measured using the ELISA-reader (*BioTek, USA*) at an optical density (OD) of 450nm. After the OD value of the Blank Control Well set as a zero, we determined the test effectiveness, and calculated the Critical value (CUT OFF) as following: CUT OFF = Average value of NC + 0.15. The samples having an OD value ≥ CUT OFF were considered as positives.

*Antioxidants, lipid perioxides, and liver parameters*

Specific sandwich ELISA kits were applied to estimate the concentrations of MDA (Malondialdehyde) as a lipid perioxides, CAT (Catalase), GPx (Glutathion Peroxidase) and SOD (Superoxide Dismutase). Sera, Standards, micrelisa stripplate and buffers were prepared and processed; and finally, the ODs were measured following the manufacturer's instruction of each kit (*Assay Genie, Germany*). After the OD value of the Blank Control Well set as zero, the ODs of MDA, CAT, GPx, and SOD of each sample were blotted on the Standard Curve to determine their concentrations.

*Biochemical testing*

Abbott ARCHITECT Plus analyzer (Germany) was applied in current study to measurement the activities of liver function enzymes including ALT (alanine aminotransferase), AST (aspartate aminotransferase), GGT (gamma-glutamyl transferase), TP (total protein), and TB (total bilirubin). Negative dogs for HbsAg were considered as control group.

*Statistical analysis*

All obtained data were introduced and analyzed using the Microsoft Office Excel (2016), and GraphPad Prism (6.01) software. Chi-square ($x^2$) and *t*-test were used to detect the prevalence of positivity among study animals, and to examine degree of significance between the levels of enzymes, antioxidants and lipid peroxidases; while, the *Odd ratio* was served to evaluate significant differences between the groups of demographic risk factors. Statistically, variation between values considered significance at $P<0.05$.

**Results**

Of 184 serum samples examined by indirect ELISA, 27 (14.67%) were seropositives for canine hepatitis (Figure 1).

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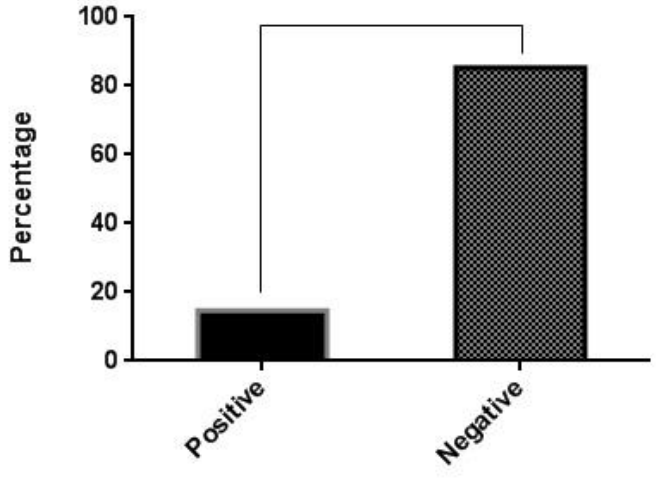


Figure (1): Total results of ELISA kit among 184 herding dogs

Regarding to demographic risk factors, the findings of region factor revealed that there was significant increase (P≤029) in seropositive dogs of rural (17.48%) and sub-urban (14.75%) when compared to urban (0%) regions. For age factor, significant elevation (P≤0.042) in seropositivity was detected of an age group of > 1- ≤ 3 years (25.53%) compared to other age groups, > 6-12 months (16.22%) as well as ≤ 6 months (9.99%) and > 3 years (9.09%). Concerning sex factor, no significant differences (P≤0.064) were detected between seropositive females (15.27%) and males (13.21%), (Table 1).

Table (1): Distribution of seropositivity among the demographic risk factors

| Factor (Group) | Total | Positive | Prevalence (%) | Risk | Odd Ratio | *P-value* |
|---|---|---|---|---|---|---|
| ***Region*** | | | | | | |
| Rural | 103 | 18 | 17.48 * | 1.54 | 1.696 | 0.029 |
| Sub-Urban | 61 | 9 | 14.75 * | 1.014 | 1.012 | |
| Urban | 20 | 0 | 0 | 0 | 0 | |
| ***Age*** | | | | | | |
| ≤ 6 months | 89 | 8 | 9.99 | 0.45 | 0.396 | 0.042 |
| > 6-12 months | 37 | 6 | 16.22 | 1.133 | 1.162 | |
| > 1 – ≤ 3 years | 47 | 12 | 25.53 * | 2.349 | 2.789 | |
| > 3 years | 11 | 1 | 9.09 | 0.607 | 0.565 | |

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| | | | | | | |
|---|---|---|---|---|---|---|
| ***Sex*** | | | | | | |
| Female | 131 | 20 | 15.27 | 1.159 | 1.184 | 0.064 |
| Male | 53 | 7 | 13.21 | 0.844 | 0.863 | |

**Significance * (P<0.05)**

The findings of liver parameters were revealed a significant variation (P<0.05) in their values (Mean ± Standard Error) (Figure 2). Significantly, the concentrations of ALT [(86.31 ± 5.92) U/L], AST [(72.31 ± 6.05) U/L], GGT [(12.58 ± 2.03) U/L], and TB [(9.25 ± 1.29) µmol /L] of seropositive dogs to canine hepatitis were higher that detected in seronegatives; [(59.7 ± 4.81) U/L], [(48.15 ± 4.81)] and [(6.26 ± 1.44) U/L], respectively (P<0.05). Whereas, the concentration of TP [(35.5 ± 5.45) g/L] of seropositives was lowered than reported in seronegatives [(49.14 ± 5.23) g/L].

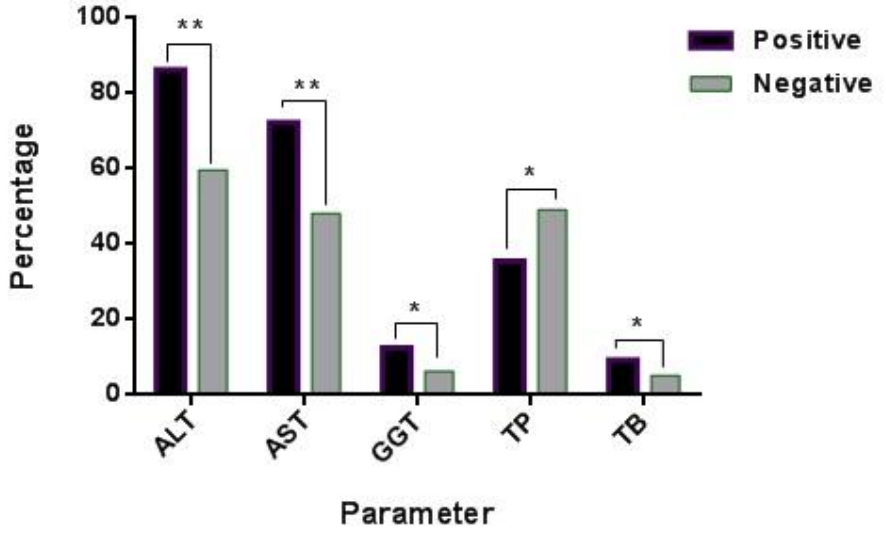


Figure (2): Concentration of liver parameters among seropositive and seronegative study dogs to canine hepatitis

Concerning to levels of antioxidants and lipid peroxides, the findings of seropositive dogs were showed a significant reduction (P<0.05) in values of CAT [(608.23 ± 24.32) U/gHb], GPx [(4.07 ± 0.34) U/L], and SOD [61.22 ± 3.18 (U/L)]; while, significant elevation (P<0.05) was seen in MDA [(1.89 ± 0.037) µmol /L] when compared to seronegatives (Table 2).

Table (2): Concentration of antioxidant enzymes and lipid peroxides among study animals

| **Parameter** | **Study animals** | | ***P-value*** |
|---|---|---|---|
| | **Positive** | **Negative** | |
| CAT (U/gHb) | 608.23 ± 24.32 * | 875.49 ± 29.71 | 0.039 |

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| | | | |
|---|---|---|---|
| | (433 - 885) | (587-1104) | |
| GPx (U/L) | 4.07 ± 0.34 * | 5.83 ± 0.19 | 0.046 |
| | (2.09 – 8.45) | (3.19 – 7.54) | |
| SOD (U/L) | 61.22 ± 3.18 * | 88.53 ± 5.15 | 0.026 |
| | (42.19 – 78.25) | (42.71 – 93.48) | |
| MDA (µmol /L) | 1.89 ± 0.037 * | 0.92 ± 0.023 | 0.011 |
| | (0.92 – 2.81) | (0.56 – 2.39) | |

**Significance * (P<0.05)**

**Discussion**

Hepatitis is one of the most frequent hepatic diseases in dogs which caused by several etiological agents that mostly unknown. Worldwide, there is a clear lack of search results in the field of canine hepatitis that is far less well defined than reported in human (**Evermann and Kennedy, 2011; Kahilo et al., 2012**). Although, viral hepatitis is widespread in canine population as a common cause of death, infectious canine hepatitis is an uncommonly recognized disease of dogs in spite of it cause a serious disease in dogs (**Poldervaart et al., 2009; Teshale and Hu, 2011**). Many diagnostic assays were described for diagnosing canine hepatitis; however, the sensitivity and specificity of each method are difficult to interpret particularly in stray and herding dogs due to absence the full case history (**Caudell et al., 2005**). In this study, case history data as well as clinical symptoms of each animal were unavailable. Based on serology, we detected that the prevalence of IgG antibodies against canine hepatitis was 14.67%, while other studies reported 9% in Iraq (**Al-Jumaa et al., 2020**), 33.34% in Brazil (**Vieira et al., 2019**), and 48.51% in China (**Wang et al., 2010**). In general, the prevalence of hepatitis B virus is controversial because several hypotheses have been populated regarding the emergence of the virus based on its viral evolution rate and hepadenovirus nucleotide sequences from hosts already described in the literature (**Paraskevis et al., 2015; Vieira et al., 2019**). Due to in part to the growth of the companion animal market, global travel and commerce, anthropogenic development of habitats, ecosystem disruption, climate change, and animal husbandry practices, human and animal relationships are likely to continue to intensify worldwide over the next several decades (**Hubálek, 2003; Messenger et al., 2014**). Dogs considered as one of the most closely related to human; hence, they the almost abundant and widespread mammals in the world which existed in agricultural, rural, and urban areas. The large distribution of dog population and decreasing geographical boundaries human and animals play a great role in increasing the possibility of pathogen transmission from human to animals (**Messenger et al., 2014; Guterres and de Lemos, 2018**). Existence of other viruses, antigenically similar to hepatitis B virus, may contribute erroneously in elevation the prevalence of infection in study dogs as reported previously in human and swine (**Blumberg, 1981; Li et al., 2010**).

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Statistical analysis of study data indicated that variation in distribution of seropositivity among groups of region and age factors was significant ($P<0.05$), but not between females and males ($P>0.05$). Compared with pet dogs, dogs from rural and sub-urban areas may expose frequently to the viscera and meat of freshly deceased animals. Herding dogs may expose to a greater diversity of wild animals especially at night, and this may increase contact with blood, saliva, and feces from these animals, which increases the likelihood the sharing of new pathogens (**Vieira et al., 2019**). In Iraqi rural and sub-urban areas, large number families have different animals such as chicken, horses, donkeys, cattle, sheep and / or goats, which may act as reservoir and play a role in increase the rate of infection in these regions. Regarding age of positives, **Al-Jumaa et al.** (**2020**) detected that there was a variation in prevalence of infection among positive dogs at different age groups but is insignificance. However, increasing rate of infection among young dogs (>1- ≤3 years) might be attributed to diminish maternal immunity at this age or increase chance of exposure with advancing age. Insignificant decreases of seropositivity at an old age group may be caused by increasing the rate of mortalities due to infection or other infectious diseases as well as eradication operations that performed usually to control stray and erroneously herding dogs. For sex factor, our results were similar to that reported recently as there were no significant differences in prevalence of canine hepatitis between females and males (**Lanave et al., 2019; Al-Jumaa et al., 2020**).

Significant elevation in concentration of ALT, AST, GGT, and TB and significant decreases in concentration of TP reported among seropositive study dogs could indicate hepatocellular injury as well as involvement of the bile canaliculi. **Dirksen et al.** (**2017**) demonstrate the sensitivity and specificity of these enzymes in detection of hepatitis in clinically, acute and chronic, and sub-acutely infected dogs. Elevated total serum bilirubin concentration has been identified as a negative prognostic indicator for severely affected dogs particularly those at chronic phase of hepatitis (**De Jonge et al., 2020**). Significant decreasing in TP could be attributed to the degenerative changes of the infected liver as well as increasing hepatocellular apoptosis and necrosis. Our findings were in agreement with the data of other studies (**Kahilo et al., 2012; Lawrence et al., 2018**).

Oxidative stress is increasingly implicated in the pathogenesis of many diseases, perhaps most notably in liver diseases. The liver is particularly vulnerable to oxidative stress due to its physiological role and anatomical placement, which make it susceptible to toxic, infectious and ischemic insults. In the current study, significant reduction in level of antioxidants (CAT, GPx, and SOD) and elevation in values of lipid peroxides (MDA) were detected. **Browning and Horton** (**2004**) mentioned that a common contributor to hepatocellular injury is oxidative stress, an imbalance between levels of antioxidant molecules and reactive oxygen species that are highly reactive with various organic molecules, and can direct hepatocytes toward necrosis or apoptosis. **Vince et al.** (**2014**) confirmed that the progression in formation of MDA is not an early important outcome in canine liver disease, although generalized diffuse pattern of MDA adduct formation was found to correlate strongly with grade of disease, suggesting that oxidative stress may progress to the formation of MDA adducts in those cases with profound inflammation

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and necrosis. In addition, its found that the use of antioxidants as an adjunct to standard therapy was advocated to reduce hepatic injury and fibrosis in dogs with chronic hepatitis (**McMichael, 2007**).

## Conclusion

Assessment of the clinical severity, pathogenesis, and prognosis of canine hepatitis poses significant challenges of clinicians relating in part to lack of standard diagnostic method. The present study demonstrated that the prevalence of canine hepatitis among herding dogs in Wasit province / Iraq, was higher than expected. The magnitude of increase liver enzymes and decrease of antioxidants might aid the clinician in determining the extent impact of infection on liver. Additional studies involving dogs in other regions as well as other animals are necessary. Utilization of molecular assays can support the diagnosis and provide furthermore information about the CAV-1.

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