COMPARATIVE STUDY AND STORAGE STABILITY OF HEPATOBILIARY ENZYMES IN RUMINANTS AND DOGS OF HUMID-TROPICS

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DECLARATION

I hereby declare that the thesis entitled **"COMPARATIVE STUDY AND STORAGE STABILITY OF HEPATOBILIARY ENZYMES IN RUMINANTS AND DOGS OF HUMID-TROPICS"** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis, entitled **"COMPARATIVE STUDY AND STORAGE STABILITY OF HEPATOBILIARY ENZYMES IN RUMINANTS AND DOGS OF HUMID-TROPICS"** is a record of research work done independently by **Dr. Divya P. D.,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, associateship or fellowship to her.

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We, the undersigned members of Advisory Committee of **Dr. Divya P. D.**, a candidate for the degree of **Master of Veterinary Science in Veterinary Biochemistry,** agree that the thesis entitled **"COMPARATIVE STUDY AND STORAGE STABILITY OF HEPATOBILIARY ENZYMES IN RUMINANTS AND DOGS OF HUMID-TROPICS"** may be submitted by **Dr. Divya P. D.**, in partial fulfilment of the requirement for the degree.

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Divya P. D.

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LIST OF ABBREVIATIONS

AAP	Alanine aminopeptidase
ACP	Acid phosphatase
AChE	Acetyl cholinesterase
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ARG	Arginase
AS	Arginino succinate synthase
AST	Asparatate aminotrasferase
BUN	Blood urea nitrogen
ChE	Pseudocholinesterase
CK	Creatine kinase
DGKC	German Society of Clinical Chemistry
FMD	Foot and mouth disease
GGT	Gamma glutamyl transferase
GDH	Glutamate dehydrogenase
GSTA	Glutathione S- transferase
ICD	Isocitrate dehydrogenase
IFCC	International Federation of Clinical Chemistry
IU	International Unit
LAP	Leucine aminopeptidase
LDH	Lactate dehydrogenase
MDH	Malate dehydrogenase
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide Dehydrogenase
NTP	5' Nucleotidase
OCT	Ornithine carbamoyl transferase
PBB	Poly brominated biphenyl
PPR	Peste des petits ruminants
SDH	Sobitol dehydrogenase
TAG	Triacyl glycerol

Introduction

1. INTRODUCTION

Enzymes are considered as markers of cellular damage and their measurement is an important tool for the diagnosis of diseases in veterinary and human clinical practice. Some of these clinically important enzymes are widely distributed in the body, but some occur only in the cells of a few organs, sometimes in single one. Most of the enzymes with diagnostic application are predominantly intracellular and are retained within the cells by the plasma membrane. Any process that impairs energy production, either through deprivation of oxidizable substances or by restriction of oxygen necessary for energy production, promotes deterioration of the cell membrane allowing the enzymes to leak out (Burtis and Ashwood, 2001). Direct attacks on cell membrane by agents like viruses or organic chemicals also cause enzyme release, which is particularly significant in the case of liver. Increased serum activity of these enzymes act as a sensitive marker of cellular damage. So the study of clinically important enzymes has immense practical importance in diagnosis and monitoring the progress of tissue damage.

Liver, the principal organ of the body, is involved in almost all the biochemical pathways helping growth, supply nutrients, provide energy, detoxification of xenobiotics and excretion of metabolic end products. Liver cells go through thousands of biochemical reactions every second in order to perform these activities. The clinical manifestations of hepatic diseases are directly attributable to the alteration in the functions of liver. Important hepatic diseases in domestic animals involve acute and chronic forms of hepatitis, cirrhosis, bile duct obstruction and neoplasia. Since liver possess enormous regenerative capacity, the signs of hepatic failure do not develop until 70 % of the functional capacity of liver is lost. Numerous tests have been developed to assess liver function either by measuring the concentrations of

substances produced by the hepatocyte, measuring serum content of substances that are changed by hepatocyte damage, assessing the ability of liver to perform metabolic tasks such as detoxification or by measuring serum enzyme activity.

Many enzymes have been used to appraise hepatic injury. Increase in serum concentration of these enzymes provides important clues about the involvement of hepatocytes. The major clinically important hepatic enzymes used in diagnostic enzymology includes Alanine aminotransferases(ALT), Aspartate aminotransferases (AST), Alkaline phosphatase (ALP), Gamma glutamyltransferases (GGT), Sorbitol dehydrogenase (SDH), Lactate dehydrogenase (LDH), Ornithine carbamoyl transferase (OCT) and 5' Nucleotidase (NTP). These enzymes are grouped under two headings as enzymes indicating hepatocellular damage and those involved in bile duct obstruction / cholestasis (Kaplan and Pesce, 1989). Since hepatocytes are in direct contact with plasma of the sinusoid capillaries, the enzyme released from the cells as a result of hepatic injury immediately enters the plasma compartment. The cytosolic enzymes spill into the blood first, followed by mitochondrial enzymes depending upon the severity of the hepatic injury. In cholestasis condition when the bile flux is slowed or blocked, the pressure in the bile duct cause paracellular reflux of the bile content into the sinusoid capillaries. So the enzymes such as GGT and ALP which are present in high concentration in the membrane of biliary pool of the hepatocytes, reach plasma.

The enzymes routinely used in human beings for disease diagnosis may not give true indications of hepatic injury in veterinary practice. There is also lack of standard reference values for some species. Each animal species have their own specific hepatobiliary enzyme levels which vary from one species to another (Kaneko *et al*., 1997). The available data on hepatobiliary enzyme levels from literature shows widely divergent values among different species and these data are mainly procured

from the animals reared in temperate climate. Even though considerable information is available on normal serum hepatobiliary enzyme levels of domestic animals of exotic breeds, kept under different environment and management conditions, use of these serum enzyme levels for monitoring health status of indigenous breeds may mislead the diagnosis. So for more accurate clinical interpretation of hepatic diseases, it is a prerequisite to establish the reference values of these enzymes.

When blood samples are being collected in large numbers or when many different analysis are required or in the event of analyzer break down and for transportation of specimens, it is inevitable to store the samples and do the analysis on a later date. So it is important to get information about the ideal storage condition of the sera samples without altering the concentration of the analytes particularly for enzyme activities. Many studies on the storage stability of enzymes showed discrepancy in their results and most of the works in this field were performed on human sera samples. The information available on the storage stability of hepatobiliary enzymes from different animal species is also contradictory. At present there is no referred standard condition to preserve the sera samples of animals meant for hepatobiliary enzyme assay. Hence, the present study is taken up with the following objectives:

1. To establish appropriate physical baseline values for hepatobiliary enzymes in adult ruminants and dogs of hot humid climatic condition
2. To find out the most ideal condition for preserving sera samples for hepatobiliary enzyme analysis

Review of Literature

2. REVIEW OF LITERATURE

2.1 CLINICAL IMPORTANCE OF HEPATOBILIARY ENZYMES

Serum enzymes have been used as biochemical markers of hepatobiliary diseases. Abnormal increase in these enzymes in serum provides a sensitive means to evaluate liver diseases either due to hepatocellular damage or bile duct obstruction.

2.1.1 Enzymes indicating hepatocellular damage

Altered cell membrane permeability of hepatocytes due to various disease conditions results in leakage of cytosolic enzymes into the extra cellular fluid and subsequently to blood and if the process continues and necrosis occurs, mitochondrial enzymes are also released. This phased release act as an indicator of time and severity of damage. The activities of serum enzymes elevated during hepatocellular damage are Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Glutamate dehydrogenase (GDH), Arginase (ARG), Sorbitol dehydrogenase (SDH), Ornithine carbamoyl transferase (OCT), Isocitrate dehydrogenase (ICD), Lactate dehydrogenase (LDH) and Glutathione S- transferase (GSTA) (Burtis and Ashwood, 2001).

2.1.1.1 Alanine aminotransferase

Alanine aminotransferase is considered to be liver specific in primates and small animals. This enzyme is present in high concentration in the cytoplasm of hepatocytes and so serum concentration increases with a mild hepatocellular damage.

Graded hepatic damage was induced in mature lactating Holstein Friesian dairy cows to measure the sensitivity of several liver function tests (Bartholomew *et al.*, 1987). They used carbon tetrachloride as the hepatotoxicant and observed elevated serum activities of AST, GGT and SDH by 24 h postdosing, indicating severe hepatic damage caused by carbon tetrachloride whereas, activities of ALP, ALT and LDH were not significantly different. They also reported lower ALT activity in the liver of cattle and the enzyme showed marked increase in activity only in cases of severe hepatic necrosis.

Laker (1996) reported clinical importance of ALT in diagnosing hepatic diseases in human beings. They found highest amounts of ALT within the liver and were more liver specific as compared to AST. Elevated serum ALT levels were found in many of the hepatic diseases.

Hepatobiliary enzyme activity in various species of domestic animals was presented by Kaneko *et al.* (1997). In primates, dogs, cats, rabbits and rats, they reported ALT as a liver specific enzyme, but not in pig, horse, goat, sheep and cattle in order to have a diagnostic significance. They reported an elevated serum ALT activity in dogs and cats suggestive of hepatocellular injury including chronic hepatocellular disease, cirrhosis, parasitic hepatopathy, primary or metastatic neoplasia.

Ettinger and Feldman (2000) reported ALT as liver specific cytosolic enzyme in dogs and cats and the largest increase was observed with acute hepatocellular necrosis and inflammation, which was directly proportional to the number of injured hepatocytes. Increased serum ALT activity was also found in hepatic neoplasia.

Latimer *et al.* (2003) investigated the alterations in serum chemistry parameters after hepatic injury. During hepatocellular injury and necrosis they observed an elevated ALT activity in dogs and cats but a lower activity in horses, pigs, ruminants and birds.

Mohan (2005) reported normal serum level of ALT in animals, within the range of 0 to 35 IU/L and also proposed serum ALT as the specific enzyme for diagnosing hepatic injury in domestic animals.

2.1.1.2 Aspartate aminotransferase

Aspartate aminotransferase occurs in the cytoplasm and mitochondria of a wide variety of tissues, with high concentrations especially in liver, skeletal and cardiac muscles. It is useful in evaluating muscle and liver damage in small and large animals (Kaplan and Pesce, 1989).

Laker (1996) presented and compared the clinical importance of hepatobiliary enzyme activities in serum for detecting hepatic and skeletal muscle damage in human beings. They reported that AST is widely distributed in heart, liver, skeletal muscles and kidney. They suggested that AST estimation has a major diagnostic application in investigation of myocardial infarction, viral hepatitis and muscle diseases.

Kaneko *et al.* (1997) reported the activity of various liver enzymes in domestic animals and observed that AST activity is mainly located in liver, kidney, pancreas and erythrocytes. They found high AST activity in the liver of all domestic species and elevated serum activity in acute and chronic liver injury.

Kennedy *et al.* (1997) studied the histopathological and ultra structural alterations of white muscle diseases in sheep experimentally depleted of cobalt. They reported reduced growth rate, anorexia, lacrimation, alopecia and elevated plasma bilirubin, AST and GGT activities in cobalt depleted lambs. The AST activities were 92 IU/L and 45 IU/L in cobalt deficient and cobalt supplemented lambs, respectively.

Ettinger and Feldman (2000) reported that AST is more sensitive enzyme in the detection of hepatobiliary diseases in dogs and cats. Increased serum AST levels were also observed in muscle damage and in cases of corticosteroid and anticonvulsant therapy.

Radostits *et al.* (2000) assessed the clinical significance of serum hepatic enzymes concentration for detection and evaluation of hepatic diseases. They suggested AST, GGT, Alkaline phosphatase (ALP), SDH and GDH as the most sensitive indicators of chronic hepatic diseases in adult cattle.

Burtis and Ashwood (2001) reported that serum AST activities were elevated in viral hepatitis and other forms of liver diseases in human beings associated with hepatic necrosis, myocardial infarction, progressive muscular dystrophy and dermatomyositis before the appearance of clinical signs. In addition to the cytoplasmic isoenzyme of AST an elevated level of mitochondrial AST activity was observed in hepatic necrosis.

Harr (2002) interpreted the results of standard biochemical analytes including Blood Urea Nitrogen (BUN), ALT, AST, GGT, ALP, bilirubin, ammonia, cholesterol, glucose and proteins in different avian species. They reported AST as a highly sensitive indicator in detecting liver damage, caused by

ethylene glycol toxicity in pigeons, while as a non specific indicator of hepatocellular diseases in other avian species.

Smith (2002) reported high concentration of AST in a variety of tissues including skeletal and cardiac muscles, erythrocytes, kidney and liver of large animals and elevated serum AST levels were found in liver diseases such as, acute and chronic liver failure, cholangiohepatitis, cholelithiasis, parasitic infestation and in muscle diseases such as, rhabdomyolysis and polysaccharide storage myopathy. The elevated level persisted for ten days after an episode of myonecrosis and liver damage.

Mohan (2005) reported an elevated serum AST level in liver cell injury, acute necrosis of myocardium and skeletal muscle. He also observed very high AST level in extensive acute hepatic necrosis such as, in severe viral hepatitis and acute cholestasis. In alcoholic liver diseases and cirrhosis mild to moderate rise in ALT level was observed.

2.1.1.3 Glutamate dehydrogenase

Glutamate dehydrogenase is highly concentrated in liver tissues and is located in the cell mitochondria. So its measurement is important in identifying severe hepatic damage.

The studies conducted by Roseman and Lieber (1994) in human beings with alcoholic liver diseases reported the use of plasma GDH as a laboratory marker of hepatic necrosis, a mitochondrial enzyme predominantly located in the peri venular area of the hepatic lobule.

The clinical usefulness of GDH enzyme for assessing hepatic necrosis in sheep, goat and cattle were reported by Kaneko *et al.* (1997). They showed high concentration of GDH in ovine and bovine liver and increased serum GDH activity during hepatic necrosis and bile duct obstruction.

Interpretation of the results of standard biochemical analyses, including BUN, ALT, AST, GGT, GDH, CK, ALP, bilirubin, cholesterol and serum proteins of various avian species were established by Harr (2002). Out of the various analytes studied, he found that GDH was located specifically within the hepatocyte mitochondria and was the most specific indicator of hepatocellular damage in birds.

2.1.1.4 Arginase

Arginase is one of the important enzyme of urea cycle and is considered to be liver specific in ureotelic animals. Increased serum activity of ARG indicates hepatic necrosis.

Ikemoto *et al.* (1993) examined the clinical application of ARG in diagnosing hepatic damage and reported a greater concentration of ARG within the liver of human beings. They observed an increase in concentration of ARG in patients with various hepatic disorders such as hepatoma, viral or alcoholic hepatitis and hepatocellular damage.

Kaneko *et al.* (1997) reported ARG as a mitochondrial enzyme, found in high concentration in the liver of man, other primates, horse, dog, sheep, cattle, rat and pig. Elevated serum ARG activity was found in naturally occurring liver diseases of horses, cattle, sheep and dogs.

Ikemoto *et al.* (2001) conducted studies on hepatic enzymes in serum, such as ARG, OCT and Arginino succinate synthase (AS) in four week old male Wistar rats after inducing chemical liver injury by intra peritoneal injection of carbon tetrachloride. They observed a drastic increase in the serum ARG within 30 minutes after the injection which was 45 fold higher than the concentration before treatment, whereas concentration of all other hepatic markers remained low.

2.1.1.5 Sorbitol dehydrogenase

Sorbitol dehydrogenase is distributed in a wide variety of tissues but largest amounts are found within the cytosol of the hepatocytes. Marked increase in serum levels are found in liver necrosis and moderate increase seen in pancreatitis, cirrhosis, obstructive jaundice and diabetes mellitus.

The serum activity of SDH, a cytoplasmic enzyme, in assessment of hepatocellular injury in most domestic species including dogs, horses and ruminants were reported by Kaneko *et al.* (1997). They suggested SDH as a liver specific enzyme in large domestic species and was the best enzyme to assess liver damage in horse.

The studies conducted by Latimer *et al.* (2003) reported high SDH activity in the liver of all animals and found SDH as the enzyme of choice to detect hepatocellular injury in horses, sheep, goat and cattle.

2.1.1.6 Ornithine carbamoyl transferase

Ornithine carbamoyl transferase is a liver specific enzyme, mainly located in the mitochondria of hepatocytes. The enzyme functions in the urea cycle and elevated levels are found in liver necrosis.

High hepatic activities of OCT, a mitochondrial enzyme have been demonstrated in cattle, sheep, pigs and dogs. In ruminants this enzyme responds in a similar fashion to GDH and SDH (Kaneko *et al.,* 1997). They also reported OCT as the useful marker of hepatocellular injury in swine.

2.1.1.7 Other enzymes indicating hepatocellular damage

Nathan *et al.* (1973) examined the clinical usefullness of measuring LDH activity in the serum for the diagnosis of hepatic diseases and found increased LDH activity in patients with hepatitis, primary and metastatic neoplasms of liver.

Serum Isocitrate dehydrogenase (ICD) was estimated in dairy cattle as a clinical measurement indicative of hepatic injury (Schanbacher *et al.,* 1987a). Assays of ICD in normal cattle showed average activity of 0.814 IU/ml. Moderate elevations in the level of ICD was observed in cows lethally dosed with 25 g poly brominated biphenyl (PBB) and a ten fold elevation of ICD was noted in calves with thioacetamide induced hepatotoxicity.

Clinical chemistry changes during thioacetamide induced hepatotoxicity in calves were studied by Schanbacher *et al.* (1987b). It showed marked changes in serum ICD levels which increased sharply by 18 h and about two fold by 24 h.

The usefulness of GSTA as an indicator of acute and chronic liver damage in human beings was investigated by Dajani *et al.* (2001). They reported that measurements of GSTA in serum samples could provide new information on possible hepatotoxic effects of high doses of chemotherapy.

Smith (2002) reported that liver diseases such as, acute and chronic liver failure, cholangiohepatitis and cholelithiasis are the common cause of elevated

serum LDH levels in large animals. He also observed massive release of LDH enzyme and high serum enzyme activities in cases of extensive muscle damage and rhabdomyolysis.

2.1.2 Enzymes associated with bile duct obstruction / cholestasis

Elevated serum enzyme activity that suggest cholestasis, intra or extra hepatic bile duct obstruction are, ALP, GGT, Leucine aminopeptidase (LAP) and 5' Nucleotidase (NTP).

2.1.2.1 Alkaline phosphatase

Highest activity of ALP is seen in the microvilli of secretory and absorptive cells like osteoblasts, hepatobiliary system, GI mucosa, renal tubules and placenta. Its biological role is detoxification of endotoxins. Elevated ALP concentration is generally due to cholestasis in most domestic animals where the enzyme level may increase by about 10 to 20 times than the normal level.

The isoenzymes of ALP present in the sera of one hundred and sixteen patients with hepatobiliary diseases were studied by Rhone *et al.* (1973). Wide variations were observed in their levels depending upon the disorders such as, nutritional cirrhosis, infectious hepatitis, obstructive jaundice, cholecystitis, cholelithiasis, cholangitis and hepatocellular carcinoma.

Schwartz (1973) evaluated the serum enzyme levels in neoplasia in human beings and found elevated serum ALP activity in patients with various liver diseases. He found higher ALP activity in cancer involving bile duct than with viral hepatitis and cirrhosis.

Laker (1996) reported high concentration of ALP in the liver, bone, intestine, placenta and kidney and suggested physiologically increased ALP levels during the periods of active bone growth and pregnancy whereas pathological increases in hepatobiliary diseases and bone diseases.

The serum ALP activities in different domestic animals was presented by Kaneko *et al.* (1997) and found increased serum ALP level in both acute and chronic liver diseases with marked elevation indicative of cholestasis, cholangitis, biliary cirrhosis, extra and intra hepatic bile duct obstruction particularly in dogs and cats.

Radostits *et al.* (2000) reported serum ALP levels as the most suitable test of hepatic excretory function in horses and elevated serum ALP levels were found during biliary obstruction.

Smith (2002) suggested ALP as a marker of intra and extra hepatic obstruction of biliary system in most species and as the important hepatobiliary enzyme in evaluating liver diseases in large animals, particularly in horses with pyrrolizidine alkaloid intoxication, chronic active hepatitis, cholangiohepatitis and cholelithiasis.

Elevated serum ALP activities were found in diseases of bone, liver and in pregnancy (Mohan, 2005) and proposed high serum ALP activity as a marker of hepatobiliary diseases in the absence of bone diseases and pregnancy in human beings. Elevated levels of 3 to10 times than normal were found in biliary tract obstruction whereas the increase was slight to moderate in parenchymal liver diseases such as hepatitis, cirrhosis and hepatic neoplasia.

Pal and Dasgupta (2006) studied haemato-biochemical profiles in *Fasciola gigantica* infected buffaloes. Haemetological analysis showed reduced erythrocytic count, haemoglobin percent and PCV level and eosinophilia and biochemical analysis revealed hypoalbuminemia, hypoglobulinemia, increased levels of total serum bilirubin, AST, ALT and ALP as compared to healthy animals.

Hilali *et al.* (2006) conducted studies on the hematological and biochemical changes in water buffalo calves (*Bubalus bubalis*) infected with *Trypanosoma evansi*. The blood and sera of all calves were examined for liver and kidney function tests and it revealed significant elevation in the activity of LDH, globulin and total bilirubin levels while significant decrease in activity of ALP.

Watanabe *et al.* (2008) investigated serum ALP activity and its role as an indicator to detect hepatobiliary disorders in male and female Beagle dogs. The established serum ALP activity was 460 IU/L at five months of age and 160 IU/L at twelve months of age irrespective of sex and breeding colony. They reported increased serum activity of ALP as a sensitive indicator of hepatobiliary diseases with the exception of growing animals or animals with bone diseases.

2.1.2.2 Gamma glutamyl transferase

Gamma glutamyl transferase has been found to be a valuable tool in the diagnosis of hepatobiliary diseases. It is a cytoplasmic enzyme found especially in kidney, liver and pancreas, but most of the serum GGT activity is derived from liver. The enzyme is involved in glutathione metabolism and elevations are quite specific for intra and extrahepatic cholestasis.

Serum GGT, LAP, ALP, ALT and AST activities were assayed in human beings with liver, pancreatic and bone diseases (Lum and Gambino, 1972). Increased GGT activity was observed in cases of viral hepatitis, cholecystitis, chronic hepatitis, fatty liver, cholangitis, cholelithiasis, metastatic carcinoma of liver, congestive heart failure and chronic alcoholism. They suggested GGT as a sensitive marker of intra or extra hepatic biliary obstruction than LAP, ALP, ALT and AST.

Rico *et al.* (1977) studied the hepatic specificity of GGT in ten healthy adult cows and reported that GGT activity is mainly located in kidney, bile duct, brain capillaries, leucocytes and blood. They suggested GGT assay as a valuable test for assessing hepatocellular damage in cows. Increased serum GGT activities could be observed with a maximum increase of 40 to 76 times than normal in acute fasciolosis, ketosis, angiomatosis and cholestasis.

Braun *et al.* (1983) presented and compared GGT activity in many organs such as, kidney, pancreas, liver, spleen, small intestine, skeletal muscle and lungs of domestic animals. Relatively high GGT activity was observed in cows, horses, sheep and goats while very low activity in dogs, cats and birds. They suggested serum GGT as a valuable marker of hepatobiliary diseases such as, cholestasis, fasciolosis, liver necrosis and other liver disturbances in domestic animals whereas urinary GGT contribute to the evaluation of kidney damage.

The activity of GGT, LAP and ALP in the serum of patients with different types of liver diseases in human beings was measured by Wenham *et al.* (1985). They reported increased activity of GGT in patients with extra hepatic bile duct obstruction whereas other enzymes predominated in patients with other liver diseases.

Numerous tests including serum enzyme assays, dye excretion tests, prothrombin time, serum bile acids and bilirubin were evaluated to detect hepatic injury induced by hepatotoxicant, carbon tetrachloride in twelve mature Holstein bred cattle (Bartholomew *et al.*, 1987). They suggested that analyzing the levels of ALP, AST, GGT, LDH, globulin, albumin and bilirubin might help in diagnosing hepatic diseases in human beings and domestic animals. They also reported that serum GGT can be used as an important marker to detect biliary obstruction and hepatic damage.

Kaneko *et al.* (1997) reported that GGT is a membrane bound enzyme with significant activity in liver, kidney, pancreas and intestine. They reported the clinical usefulness of GGT as serum marker for diagnosing hepatic diseases in animals. They also observed a high GGT activity in liver of cows, horses, sheep and goats but a lower activity in dogs and cats.

Sing *et al.* (2007) estimated the serum level of GGT in human beings with cholecystitis, chronic bile duct obstruction, fatty liver and viral hepatitis. A statistically significant increase in serum concentration of GGT was observed between patients with cholecystitis and bile duct obstruction.

Lim *et al.* (2007) investigated possible interaction between serum GGT and body mass index and their effects on the risk of type 2 diabetes mellitus. They observed increased serum GGT activity in patients with hepatobiliary diseases and alcoholic liver diseases. They also suggested serum GGT activity could predict future diabetes, hypertension, stroke and myocardial infarction in addition to bile duct obstruction.

2.1.2.3 Leucine aminopeptidase and 5' Nucleotidase

Leucine aminopeptidase and 5' Nucleotidase are specific for obstructive liver diseases and elevated levels are observed in hepatobiliary diseases and obstructive jaundice.

Schwartz (1973) examined serum activity of ALP, LAP, NTP and GGT in various neoplastic conditions affecting liver. They observed elevated serum LAP activity in a variety of hepatobiliary diseases including obstructive jaundice caused by biliary stones, carcinoma of liver and toxic or viral hepatitis.

Kawai *et al.* (1998) studied serum aminopeptidases such as, LAP and Alanine aminopeptidase (AAP) and found that LAP was located in various tissues including small intestine, renal proximal tubules and the canalicular domain of hepatic cell membranes. They also reported a high activity of serum aminopeptidase in intra or extra hepatic biliary obstruction.

2.2 REFERENCE VALUES OF HEPATOBILIARY ENZYMES

Reference values are of great importance for the correct interpretation of biochemical data. Standard serum biochemical parameters provide information that serves as the basis for the diagnosis, treatment and prognosis of diseases. Available data on normal reference values of hepatobiliary enzymes showed wide variation between different species and within a species.

2.2.1 Transaminase enzymes (ALT and AST)

The serum biochemical and hematological measurements of heat tolerant and cold tolerant cattle breeds were compared and found variation in many

parameters between the breeds (Olbrich *et al.*, 1971). They reported an average AST activity of 96 and 136 IU/L in heat tolerant and cold tolerant heifers, respectively.

Canine hematology and biochemistry reference values proposed by Lumsden *et al*. (1979) in different breeds of dogs suggested an ALT activity ranging from 3 to 20 IU/L and AST activity in a range of 7 to 8 IU/L irrespective of breed and sex.

Lumsden *et al.* (1980a) reported the normal reference values of many serum analytes of clinical importance for 60 thorough bred mares and 12 thorough bred foals. They reported an ALT activity of 0 to 5 and 3 to 4 IU/L in thorough bred mares and foals, respectively. The AST reference activity for thorough bred mares and foals were within a range of 99 to 231 and 124 to 171 IU/L, respectively. Many biochemistry variables were found to be significantly higher, including AST activity during pregnancy period.

The studies conducted by Lumsden *et al.* (1980b) on hematology and biochemistry variables from different age groups of female Holstein cattle, reported a reference range of 2 to11, 3 to18, 6 to 9 and 5 to 18 IU/L for ALT activity and 12 to 48, 18 to 50, 26 to 48 and 24 to 45 IU/L for AST activity in 1 to 14 days, 2 weeks to six months, six months to two years and above two years aged Holstein cattle, respectively.

Caisey and King (1980) presented the mean serum values of proteins, minerals, enzymes, cholesterol and triglycerides for some common laboratory animals. The ALT activity reported for mouse, rat, guinea pig, rabbit, cat, dog and monkey were 19, 36, 47, 79, 27, 60 and 94 IU/L, respectively whereas the AST activity reported were 37, 83, 45, 47, 11, 32 and 31 IU/L, respectively.

Friendship *et al.* (1984) established biochemical reference values for Ontario swine of various age groups including weaner pigs, feeder pigs, gilts and sows and the results showed an ALT activity of 8 to 46, 15 to 46, 17 to 56 and 19 to 76 IU/L, respectively and AST activity of 21 to 94, 16 to 67, 12 to 65 and 36 to 272 IU/L, respectively. They also reported that most of the biochemical and hematological variables were strongly influenced by chronic diseases and nutritional deficiencies.

Reference values for twelve blood serum components were determined in beef cattle of different ages and stages of lactation (Doornenbal *et al.,* 1988). They reported an increase in some serum constituents like urea, protein, bilirubin and serum activity of AST from birth to the age of ten years and the reported AST activity ranged from 94.4 to 157.9 IU/L.

Hematological and serum biochemical measurements were carried out in one year old hairless and haired hybrids derived from Mexican hairless dogs (Kimura *et al.*,1992). There was no significant difference between the two groups in all the analytes measured. Results also indicated a mean ALT and AST activity of 3.7 to 10.1 and 8.2 to 10.1 IU/L, respectively.

In an attempt to develop biochemistry reference values for different age groups of cows and pigs, Dubreuil and Lapierre, (1997) observed variations in ALT activity during the last two months of lactation. The results reported a serum ALT activity of 17.7 to 25.5, 21 to 26, 5.6 to 11.6 and 15.8 to18.8 IU/L in growing pigs, nursing sows, growing calves and lactating cows, respectively.

Biochemical reference values of serum ALT for large and small animals including horse, cow, sheep, goat, llama, pig, dog, cat and monkey were reported by Kaneko *et al.* (1997) and it ranged from 3 to 23, 11 to 40, 26 to 34, 6 to19, 6

to14, 31 to 58, 21 to102, 6 to 83 and 0 to 82 IU/L, respectively and the reported AST activities were with in the range of 226 to 336, 78 to132, 60 to 280, 167 to513, 216 to 378, 32 to 84, 23 to 66, 26 to 43 and 13 to 32 IU/L, respectively.

Ottol *et al.* (1998) studied the normal reference values of serum proteins, cholesterol, urea, creatinine, minerals like sodium, potassium and serum ALT, AST, Creatine kinase (CK) and LDH activities for Angoni cattle in Mozambique. They suggested a serum ALT and AST activity of 36.9 and 77.67 IU/L, respectively for Angoni cattle. They also determined the effect of age, sex and physiological state on these blood constituents.

Borjesson *et al.* (2000) published reference intervals for a wide range of hematological and biochemical analytes for twenty normal free ranging desert bighorn sheep. They reported a wider reference range for AST activity (78 to 312 IU/L) as compared to free ranging desert bighorn sheep.

Radostits *et al.* (2000) published serum biochemical concentration of many constituents in ox, sheep, swine and horse and the reported AST activity were within the range of 60 to150, 260 to 350, 25 to 57 and 200 to 400 IU/L, respectively.

Harr (2002) presented and compared many clinical biochemistry parameters in different avian species and found variation in many analytes including AST activity among different avian species and the observed AST activity in avian species ranged from 12 to 396 IU/L.

The mean values of hematological and biochemical indices of clinically healthy male and female Alsatian and local dogs were established by Ariyibi *et al.* (2002) and found no significant difference in serum ALT and AST levels

between these two breeds of dogs. The reported values for ALT activity for Alsatian and local dogs were 11.8 to 17.4 and 12.6 to 17.2 IU/L, respectively whereas AST activities were 14.0 to 21.2 and 16.2 to 20.0 IU/L, respectively.

Osman and Al-Busadah (2003) analysed the major constituents in the sera of she camels, cows and ewes in Saudi Arabia and reported significantly higher ALT activity in cows as compared to she camels and ewes. The observed ALT activities in the serum of she camels, cows and ewes were 17.2, 34.0 and 21.0 IU/L, respectively whereas a significantly higher AST activity was observed in she camels (164.6 IU/L) as compared with cows (72.4 IU/L).

Grasso *et al.* (2004) examined the influence of intensive and traditional systems of management on blood metabolites in water buffaloes. They found no significant changes in the blood metabolite levels between different housing systems. The reported ALT and AST levels were 146.84 to 164.68, 55.35 to 58.49, 370.11 to 443.12 and 26.95 to 27.43 IU/L, respectively.

Daramola *et al.* (2005) reported an ALT and AST activity of 2 to 22 and 12 to 38 IU/L, respectively in their study on West African Dwarf goats.

Serum biochemical reference ranges for many constituents, bicarbonate, enzymes, serum bilirubin, minerals and cholesterol were published by Kahn (2005). The serum ALT activities for dog, cat, cow, horse, pig, sheep, goat, rabbit and ostrich were 8.2 to 57, 8.3 to 53, 6.9 to 35, 2.7 to 21, 22 to 47, 15 to 44, 15 to 52, 48 to 80 and 20 IU/L respectively and AST activity within the range of 8.9 to 49, 9.2 to 40, 45 to110, 116 to 287, 15 to 55, 49 to123, 66 to 230, 14 to113 and 131 to 486 IU/L respectively.

Concentrations of total proteins, AST, ALT and GGT activities in the blood plasma of Holstein mares aged from five to ten years during pregnancy and lactation were monitored by Milinkovic *et al.* (2005). The ALT activities during first, second and third stages of pregnancy and lactation were 3.69, 4.08, 3.97 and 5.63 IU/L, respectively. The corresponding AST activities were 126.88, 124.15, 97.00 and 117.27 IU/L, respectively.

The activities of ALT, AST and GGT and their relations in blood plasma of highly productive Holstein bred dairy cows during lactation and dry period were determined by Stojevic *et al.* (2005). The results showed a significant increase in ALT activity from 46th day of lactation until dry period and a statistically higher activity in the second and third periods of lactation than in the dry period. A highest activity of AST was recorded during early lactation and as lactation progressed the activity of these enzymes decreased. The reported ALT and AST activities were 5.24 to 29.68 and 32.9 to 57.79 IU/L, respectively.

Terzano *et al.* (2005) established metabolic and hormonal parameters like cholesterol, glucose, bilirubin, urea, minerals and hepatobiliary enzymes such as, AST and GGT for adult healthy buffaloes. They reported AST and GGT activities of 101.2 and 21.2 IU/L, respectively for adult healthy buffaloes.

Zvorc *et al.* (2006) described the changes in the serum hemato- biochemical concentrations during pregnancy and lactation period of 240 sows of different ages. The result showed an ALT activity of 33.9 to 40.5 and 43.1 to 53.3 IU/L, respectively during lactation and gestation, whereas AST activities were 15.7 and 12.2 IU/L, respectively.

Braun *et al.* (2008) reviewed the clinical interpretation of serum enzyme activity and presented their concencentration in large and small animals. They

reported a wide range of inter and intra individual variations in serum enzyme levels including AST activity.

In the study of the effect of breed, age, sex and season on serum enzyme levels in healthy goats from three indigenous goat breeds of Ethiopia by Tibbo *et al.* (2008), reported mean serum ALT and AST levels were within the range of 14.0 to 20.2 and 43.2 to 49.3 IU/L, respectively in all the three goat breeds. They also suggested a significant influence of sex on ALT levels for Arsi- Bale goats with higher values in males than females, whereas season had no significant influence on ALT and AST levels.

2.2.2 Alkaline phosphatase

Olbrich *et al.* (1971) monitored serum biochemical and hematological measurements of heat tolerant (Zebu) and cold tolerant (Scotch Highland) heifers. Differences were found in many of the biochemical parameters between the two breeds including ALP activity. They reported higher mean serum levels of ALP in Zebu cattle (653 mu/ml) as compared to cold tolerant Scotch Highland breeds (266 mu/ml).

Lumsden *et al.* (1980a) in their study for the preparation of normal reference hematology and biochemistry parameters for thorough bred mares and foals found wide variation in ALP enzyme levels between mares and foals. The suggested ALP values for thorough bred mares and foals ranged from 26 to 92 IU/L and 116 to 198 IU/L, respectively.

Lumsden *et al.* (1980b) found many biochemistry variables to be significantly different between different age groups of Holstein cattle. They reported ALP activity for the age groups, 1 to 14 days, 2 weeks to 6 months, 6

months to 2 years and above 2 years as 29 to 187, 16 to 129, 22 to 82 and 3 to 46 IU/L, respectively which revealed a marked increase in ALP activity in young ones in comparison with adult animals.

The studies conducted by Caisey and King (1980) reported mean serum ALP activity of 439, 713, 876, 406, 291, 173 and 1134 IU/L for mice, rat, guinea pig, rabbit, cat, dog and monkey, respectively.

Friendship *et al.* (1984) in their study for the establishment of blood reference values of enzymes in the serum of Ontario swine found wide variation in many serum analytes among different age groups with more marked variation in ALP activity. The observed ranges for ALP activity in weaner pigs, feeder pigs, gilts and sows were 142 to 891, 180 to 813, 115 to 434 and 36 to 272 IU/L, respectively.

Doornenbal *et al.* (1988) assessed blood serum components in beef cattle of different ages and stages of lactation. They reported higher serum levels of ALP in young animals when compared with mature animals. The result showed a higher ALP activity of 430.5 IU/L at birth whilst a lower value of 212.6 IU/L in one year old animals.

Kimura *et al.* (1992) compared serum biochemical and hematological values in one year old haired and hairless hybrids of Mexican hairless dogs. They reported an ALP activity ranging 24.0 to 42.6 IU/L for the hairless and haired hybrids.

Dubreuil and Lapierre (1997) conducted studies on biochemistry reference values for Quebec lactating dairy cows, nursing sows, growing pigs and calves. They found wide variation in the levels of serum ALP between the four groups.

They also reported that aging in growing pigs caused a decrease in ALP concentration as compared to other groups. The reported values for ALP activity in growing pigs, nursing sows, growing pigs and lactating cows were 255 to 351, 67 to 88, 416 to 509 and 102 to 131 IU/L, respectively.

Ottol *et al.* (1998) published biochemical blood profile of Angoni cattle in Mozambique and also determined the effect of age, sex and physiological state on biochemical parameters. Results reported significant influence of physiological status of the animals on serum blood constituents with great differences in serum ALP levels which were highest in non lactating and non pregnant cows and also in young animals as compared to adults. They proposed a mean serum ALP concentration of 261.9 IU/L in Angoni cattle.

Borjesson *et al.* (2000) studied biochemical and hematological reference intervals for two hundred free ranging desert bighorn sheep. They reported great deal of variability in the reference values for many analytes among different animals and a greater ALP activity in individuals with less than one year old as compared to adult animals. The reference interval suggested for ALP in adult and young bighorn sheep were 73 to 575 and 184 to 627 IU/L, respectively.

Ariyibi *et al.* (2002) studied the effect of breed on serum biochemistry and hematological parameters for clinically healthy Alsatian dogs and local dog breeds of age 2 to 4 years and reported no significant difference between the two breeds of dogs for all the parameters including the serum ALP activity.

The activities of enzymes of clinical significance and the concentration of certain biochemical analytes were determined in the sera of she camels, cows and ewes in Saudi Arabia (Osman and Al-Busadah 2003). They reported significantly higher ALP activity in ewes as compared with she camels and cows. The

suggested ALP activities for she camels, cows and ewes were 60.0, 49.8, 112.4 IU/L, respectively.

In the study of the hematological and biochemical parameters of twenty West African Dwarf goats by Daramola *et al.* (2005), showed a reference range of 1.4-25.7 IU/L of ALP activity. They reported a higher serum ALP level in adult animals (11.7 IU/L) as compared to young animals (9.9IU/L).

Serum biochemical reference ranges for many analytes for small and large animals and birds were established by Kahn (2005). The reported ALP activity for dog, cat, cow, horse, pig, sheep, goat, rabbit, llama and ostrich were 10.6 to101, 12 to 65,18 to153, 70 to 227,41 to176, 27 to156, 61 to 283, 4 to16, 30 to 78 and 32 to 98 IU/L, respectively.

Tibbo *et al.* (2008) assessed the normal serum enzyme activities in healthy goats from three indigenous goat breeds of Ethiopia. The results reported a marked influence of age and season on serum ALP activities in goats. Higher ALP levels were found in young animals (119.36 IU/L) than adults (93.36 IU/L) and a lower ALP values during long rainy and dry seasons.

2.2.3 Gamma glutamyl transferase

The studies conducted by Caisey and King (1980) on serum biochemistry values of clinically important enzymes, proposed GGT activity for guinea pigs, rabbit and monkey as 10, 9, 62 U/L respectively. They also reported that GGT levels in the sera of rat, cat, mouse and dog were undetectable by their methods of estimation.

Kimura *et al.* (1992) presented and compared normal serum biochemical and hematological measurements in one year old haired and hairless hybrids of Mexican hairless dogs. The results showed a mean serum GGT activity of less than 10 IU/L in the haired and hairless hybrids.

In the published biochemistry reference values by Dubreuil and Lapierre (1997) suggested GGT activities within the range of 25.4 to 30, 24 to 29, 10.7 to 16.7 and 24.4 to 28.3 IU/L for lactating dairy cows, nursing sows, growing pigs and calves, respectively.

Among the different biochemistry reference values, Kaneko *et al.* (1997) reported GGT levels in horse, cow, sheep, goat, llama, pig, dog, cat, rabbit and monkey within the range of 4.3 to13.4, 6.1 to 17.4, 20 to 52, 20 to 56, 7 to 29, 10 to 60, 1.2 to 6.4, 1.3 to 5.1, 9 and 62 IU/L, respectively.

Ottol *et al.* (1998) published the normal blood composition and biochemistry reference values of Angoni cattle in Mozambique and also determined the effect of age, sex and physiological state on these values. The results showed significant differences between male and female Angoni cattle with GGT activity. The mean serum GGT activity for Angoni cattle was found to be 17.5 IU/L.

Biochemical and hematological reference intervals for free ranging desert bighorn sheep were determined by Borjesson *et al.* (2000). They reported a wide reference interval of 20 to 130 IU/L for GGT activity as compared to domestic ruminants.

Radostits *et al.* (2000) presented and compared many biochemical and hematological parameters of clinical significance in small and large animals. They

suggested a reference range of 0 to 31, 0 to 70, 0 to 25 and 0 to 25 IU/L for GGT activity in ox, sheep, swine and horse respectively.

Osman and Al-Busadah (2003) conducted studies on normal serum concentrations of twenty biochemical parameters of she camels, cows and ewes in Saudi Arabia. Reported results showed a significantly higher GGT activity in ewes as compared with the values of she camels and cows. No statistically significant differences were found in the mean serum GGT activities between she camels and cows. The proposed GGT concentrations for she camels, cows and ewes were 25.6, 29.9 and 77.0 IU/L, respectively.

In the study of Kahn (2005) for the development of the normal biochemistry reference values, reported GGT activity in dogs, cat, cow, horse, pig, sheep, goat, rabbit and llama were within the range of 1 to 9.7, 1.8 to 12, 4.9 to 26, 2.7 to 22, 31 to 52, 20 to 44, 20 to 50, 0 to 14 and 5 to 29 IU/L, respectively.

Milinkovic *et al.* (2005) investigated the activities of enzymes such as ALT, AST and GGT in the blood plasma of mares during pregnancy and lactation. The research results of GGT enzyme activities recorded no significant difference between pregnant and lactating animals. Reported reference range for GGT activity in these animals was 8.67 to 10.55 IU/L.

The serum enzyme activities in the blood plasma of 120 Holstein breed dairy cows during lactation and dry period were established by Stojevic *et al.* (2005). Results showed statistically significant difference in the GGT activities during different production periods. Reported ranges for GGT activity in the blood plasma of dairy cows was 8.11 to 27.79 IU/L.

2.3 STABILITY OF HEPATOBILIARY ENZYMES

Available data regarding storage stability of hepatobiliary enzymes in different species are scanty. Many investigations on the stability of enzymes activity *in vitro* showed widely divergent results.

Lazaroni *et al.* (1958) investigated the stability of LDH in human serum samples by preserving at temperatures of 25 ºC, 37 ºC and in freezer. The specimens kept in the frozen state showed no appreciable changes in LDH activity over a period of thirty days. In 80 % of cases the enzymes was stable for 8 days at room temperature (25 ºC) and seven days at 37 ºC.

Jull (1967) conducted studies on the stability of eight enzymes such as Acetyl cholinesterase (AChE), pseudocholineesterase (ChE), Acid phosphatase (ACP), AST, LDH, ALT, ALP and amylase. At – 20 ºC all the enzymes except LDH were stable for 8 days, while at 38 ºC all except amylase were unstable. At 4 ºC the stability of the enzymes differed in whole blood, plasma and serum.

Hartmann *et al.* (1981) evaluated storage stability of 22 commonly measured analytes in ethylene glycol stabilized serum during storage at 2 to 8 ºC and -15 to -20 ºC. They observed CPK, LDH, ALT, and AST to be stable at -15 to – 20 ºC for 55 weeks while other analytes except ALT were stable at 2-8 ºC for 24 days.

Ono *et al.* (1981) studied twenty five blood constituents in human serum during their storage along with clotted blood for different period at different temperatures. They found significant changes in the concentrations of total protein, glucose, calcium, inorganic phosphate and the activity of AST, ALT and LDH during their contact with clot for 24 to 48 h at different temperatures.

Cuccherini *et al.* (1983) assessed the effect of different temperature and duration of storage on the stability of the activities of AST, ALT of human serum at room temperature, refrigerator and freezer for 30 days. The activity of both AST and ALT was declined markedly beginning within 24 h of venipuncture. During their storage the ALT activity showed a rise during the first 3 to 4 days followed by a decline to below base line values. So they concluded that the samples should be analyzed on the day of venipuncture to ensure the accuracy of aminotransferase activity.

Davy *et al.* (1984) conducted studies on stability of plasma constituents of various Marmosets (*Callithrix jacchus*) during their storage at room temperature, 4 ºC and -20 ºC. Room temperature was optimal for ChE and AChE, 4 ºC for proteins and majority of enzymes including ALT and -20 ºC for ALP. For AST and GGT either 4 ºC or -20 ºC were most suitable.

Donnelly *et al.* (1995) studied the stability of twenty five analytes in human serum stored at room temperature, 4 ºC and -20 ºC over 48 h, 14 days and 4 months respectively. The activity of GGT was stable at all three temperatures for the specified times. While AST, ALT, ALP and LDH demonstrated some loss over time. They concluded that proper storage temperatures and times must be considered for these analytes if measurement is not possible immediately after specimen collection.

Heins *et al.* (1995) evaluated storage stability of serum analytes like Na, K, Ca, Cl, Mg, Creatinine , urea , uric acid, bilirubin, cholesterol, Triacyl glycerol (TAG), CK, AST, ALT, GGT, ALP, LDH and amylase kept at 9 ºC, and at room temperature for 7 days . They reported all analytes to be sufficiently stable for 4 days when stored at 9 ºC, where as decreased activity was seen for CK and AST stored at room temperature.

Saeed *et al.* (1995) investigated the effect of storage at room temperature and 4 ºC on various biochemical constituents of camel serum and found that AST, ALP, and GGT did not showed any change over 9 days when stored at refrigerator, where as decreased activities of CK, LDH and ALT were noticed after 1, 6, and 7 days respectively. The stability at room temperature for AST was 3 days, GGT and ALT 6 days and ALP 6 days.

Thoresen *et al.* (1995) assessed the changes in 24 blood constituents in frozen serum and heparinised plasma from 10 German shepherd army dogs. Out of the 24 analytes, the enzymes ALP, ALT, AST, CK, GGT, amylase, GDH and LDH showed significant changes during the storage period related to time and temperature.

Tornquist *et al.* (2000) studied the effect of temperature, storage time and sample type on activity of SDH in llama serum and plasma kept at room temperature (20 ºC), 4 ºC and -20 ºC for up to one week. They observed no significant changes in plasma and serum SDH activity at 4 ºC and -20 ºC for up to one week whereas plasma and serum SDH were stable only up to 24 and 8 h, respectively at room temperature.

Benjamin (2001) compared the stability of some of the animal serum enzymes with human serum enzymes during storage at different temperatures and reported great variation in stability of enzymes among different species.

Boyanton and Blick (2002) conducted studies on stability of 24 analytes in human plasma and serum maintained at room temperature. Out of 24 analytes plasma and serum AST, ALP, CK were stable over 56 hours where as plasma ALT was stable up to 40 h, then lost 20 % activity at 48 and 56 h.

Clark *et al.* (2003) conducted studies on stability of plasma analytes after delayed separation of whole blood and reported significant changes in activity of ALT, CK, Creatinine and GGT at room temperature and less than 4 % change during chilled storage up to 7 days whilst markedly increased AST concentration under both conditions.

Ehsani *et al.* (2008) studied the effects of storage time and temperature of clotted whole blood on 17 bovine serum analytes by keeping samples at room temperature and on ice. Results obtained for minerals, total proteins and GGT were not influenced by storage at room temperature and on ice for as long as 24 h whereas AST, CK and glucose showed significant variation upon storage.

Materials and methods

3. MATERIALS AND METHODS

The experiment was conducted at the Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences, Mannuthy to establish and compare the normal reference values of hepatobiliary enzymes in adult cattle, buffalo, goat and dog and also to identify the ideal storage conditions to preserve sera samples meant for enzyme assay.

3.1 EXPERIMENTAL ANIMALS

The study was carried out in adult healthy cattle, buffalo, goat and dog during the period of April to October 2008. A minimum of 10 animals were selected from each species for the study. Twelve female crossbred cattle of age of 3 to 7 years maintained at University Livestock Farm, ten female Murrah buffaloes between 2 to 3 years of age from University Buffalo Farm, eighteen female crossbred goats of age 3 to 5 years from University Goat and Sheep Farm, College of Veterinary and Animal Sciences, Mannuthy were selected randomly for the study. All the animals were apparently healthy, free of external parasites, dewormed regularly and vaccinated routinely against infectious diseases such as, foot and mouth disease (FMD) and haemmorhagic septicemia (HS).

A total of 13 apparently healthy dogs comprising 11 male and 2 females of 1.5 to 3 years of age belonging to different breeds such as, Golden Retriever, Great Dane, German Shepherd, Boxer, Labrador Retriever, Rottweiller, Chineese Pug, Cocker Spaniel and Daschund were selected. These dogs were maintained at nearby kennels and were fed on a balanced diet. All dogs were dewormed and immunized against rabies, distemper, parvo and hepatitis virus. They were also free from external parasites.

3.1.1 Collection of blood samples

Blood samples were collected from the animals between 8.30 and 9.30 a.m. prior to morning feeding. Blood was drawn from the animals at rest, with minimum disturbance. Approximately 15 ml of blood was taken by jugular venipuncture using sterile needles directly into clean dry sterile glass tubes without anticoagulants from all the animals except dogs. In the case of dogs blood was collected from the cephalic / saphenous vein aseptically in sterile disposable syringe, using scalp vein set and the blood was then transferred immediately into clean dry sterile glass tubes without anticoagulants.

The blood samples were handled gently to avoid hemolysis and kept in a slanting position at room temperature for about 1.5 h to allow proper clotting. The serum samples were harvested by centrifugation for 15 minutes at 3000 rpm and the clear sera separated were employed for the enzyme analysis.

3.2 ANALYSIS OF HEPATOBILIARY ENZYME ACTIVITY

All the sera samples were analysed for ALT, AST, ALP and GGT activities according to the standard methods described. From these values the mean, lowest and highest value, standard error and 95 % confidence interval for the mean values of each enzyme were determined statistically. These values also served as the base line fresh value (day 0 value) against each enzyme concentration in the stability study.

3.3 ASSESSMENT OF STORAGE STABILITY OF HEPATOBILIARY ENZYMES

The stability and storage characteristics of hepatobiliary enzymes such as, ALT, AST, ALP and GGT in sera samples of goat, cattle, buffalo and dog were

analysed. The sera collected from a minimum of six animals from each species were used for the study. After measuring the enzyme activities in fresh sera samples (day 0) the remaining sera was subsequently aliquoted into 18 ependorf tubes, capped and remained unopened until analysis. The aliquots of six sera samples each were kept upright at three conditions such as, room temperature with an ambient temperature ranged 22 to 27 ºC, refrigerator with a temperature of 4 ºC and freezer maintained at -20 ºC.

One of each sample stored at room temperature, 4 ºC and -20 ºC, were then analysed for enzyme activities on day 1, 2, 5, 8, 11 and 14. Prior to analysis, at each designated time, the aliquots of the frozen samples were left to stand at room temperature to thaw and the sample stored at 4 ºC were allowed to equilibrate to room temperature. The tubes were inverted several times to mix and centrifuged at 3000 rpm for 10 minutes. The clear sera then obtained were immediately analysed for enzyme assays.

The stability of an enzyme activity under each temperature condition and time was determined by calculating the percentage change in concentrations from the mean fresh value at each time-point for each animal. To test the significant differences in enzyme activity between storage temperatures and to assess the significant trends over time at each temperature, the data was analysed statistically.

3.4 ESTIMATION OF HEPATOBILIARY ENZYME ACTIVITY

3.4.1 Alanine aminotransferase

Alanine aminotransferase activity was determined photometrically based on reference method of International Federation of Clinical Chemistry (IFCC) in semi

automatic blood analyzer (microlab 200) using Ecoline ALAT kit (Merck Specialities Pvt. Ltd.)

Principle

$$\text{L-Alanine} + \text{2-oxoglutarate} \xleftrightarrow{\text{ALT}} \text{L-Glutamate} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + H^{+} \xleftrightarrow{\text{LDH}} \text{Lactate} + NAD^{+}$$

The rate of NADH consumption is measured photometrically at 340 nm and is directly proportional to the ALT concentration in the sample.

Reagents

Reagent 1:

TRIS buffer	pH 7.5	100 mM/L
L-Alanine		500 mM/L
LDH		≥ 1.2 KU /L

Reagent 2:

2-Oxoglutarate	15 mM/L
NADH	0.18 mM/L

Assay procedure

Wavelength	340 nm
Light path	1 cm
Temperature	25 ºC

Preparation of reaction solution

Mixed reagents 1 and 2 in the ratio 4:1 and proceeded as follows

Sample	100 µl
Reaction solution	1000 µl

Mixed well and after 1 minute read the decrease in absorbance every minute for 3 minutes.

Calculation

ALT activity (IU / L) = ΔA / min x factor

ΔA / min = Mean absorbance changes / minute

Factor = 1746

3.4.2 Aspartate aminotransferase

Aspartate aminotransferase activity was determined photometrically based on reference method of IFCC in semi automatic blood analyzer (microlab 200) using Ecoline ASAT kit (Merck Specialities Pvt. Ltd.)

Principle

$$\text{L-Aspartate} + \text{2-Oxoglutarate} \xrightarrow{\text{AST}} \text{L-Glutamate} + \text{Oxaloacetate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{L-Malate} + NAD^+$$

The rate of NADH consumption is measured photometrically at 340 nm and is directly proportional to the AST concentration in the sample.

Reagents

Reagent 1:

TRIS buffer	pH 7.8	80 mM/L
L-Aspartate		240 mM/L

MDH	≥ 420 U/L
LDH	≥ 600 U /L
Reagent 2:	
2-Oxoglutarate	12 mM/L
NADH	0.18 mM/L

Assay procedure

Wavelength	340 nm
Light path	1 cm
Temperature	37 ºC

Preparation of reaction solution

Mixed reagents 1and 2 in the ratio 4:1 and proceeded as follows

Sample	100 µl
Reaction solution	1000 µl

Mixed well and after 1 minute read the decrease in absorbance every minute for 3 minutes.

Calculation

AST activity (IU / L) = ΔA / min x factor

ΔA / min = Mean absorbance changes / minute

Factor = 1746

3.4.3 Alkaline phosphatase

Alkaline phosphatase activity was measured photometrically based on reference method of German Society of Clinical Chemistry (DGKC) in semi automatic blood analyzer (Sinnowa 3000) using Ecoline ALP kit (Merck Specialities Pvt. Ltd.) using P- Nitro phenyl Phosphate as the substrate.

Principle

$$\text{P-Nitro phenyl Phosphate} + H_2O \xrightarrow{\text{ALP}} \text{Phosphate} + \text{P-Nitro phenol}$$

The increase in absorbance due to formation of 4- Nitrophenolate is measured photometrically at 405 nm and is directly proportional to the ALP activity in the sample.

Reagents

Reagent 1:

Diethanolamine	pH 9.8	1.0 mM/L
Magnesium Chloride		0.5 mM/L

Reagent 2:

P-Nitro phenyl Phosphate	10 mM/L

Assay procedure

Wavelength	405 nm
Light path	1 cm
Temperature	37 ºC

Preparation of reaction solution

Mixed reagents 1 and 2 in the ratio 4:1 and proceeded as follows

Sample	20 µl
Reaction solution	1000 µl

Mixed well and after 1 minute read the increase in absorbance every minute for 3 minutes.

Calculation

ALP activity (IU / L) = ΔA / min x factor

ΔA / min = Mean absorbance change / minute

Factor = 2754

3.4.4 Gamma glutamyltransferase

Gamma glutamyltransferase activity was determined photometrically according to the method of IFCC in semi automatic blood analyzer (Microlab 200) using Ecoline Gamma GT kit (Merck Specialities Pvt. Ltd.)

Principle

GGT catalyses the transfer of glutamic acid to acceptors like glycyl glycine.This process releases 5-amino 2-nitro benzoate, which absorbs light at 405 nm. The increase in absorbance at this wavelength is directly proportional to the activity of GGT in the sample.

L-Gamma glutamyl 3-carboxy 4-nitroanilide + Glycylglycine

↓ GGT

Gamma glutamyl glycylglycine + 5-amino 2-nitro benzoate

Reagents

Reagent 1:

TRIS buffer	pH 8.25	100 mM/L
Glycylglycine		100 mM/L

Reagent 2:

L- Gamma glutamyl- 3 carboxy- 4 nitroanilide 4 mM/L

Assay procedure

Wavelength	405 nm
Light path	1 cm
Temperature	37 ºC

Preparation of reaction solution

Mixed reagents 1 and 2 in the ratio 4:1 and proceeded as follows

Sample	100 µl
Reaction solution	1000 µl

Mixed well and after 1 minute read the decrease in absorbance every minute for 3 minutes.

Calculation

GGT activity (IU / L) = ΔA / min x factor

ΔA / min = Mean absorbance changes / minute

Factor = 1309

3.5 STATISTICAL ANALYSIS

The experimental results obtained were analyzed by using analysis of variance (ANOVA) technique followed by Duncan Multiple Range Test and paired t-test as described by Snedecor & Cochran (1994) using computerised software programme, SPSS .

Results

4. RESULTS

In the present study, the reference values for the hepatobiliary enzymes such as, ALT, AST, ALP and GGT of adult healthy ruminants and dogs maintained under hot humid climatic condition were analysed and the storage stability of the above mentioned enzymes were determined by keeping sera samples at room temperature, refrigerator and freezer for two weeks.

4.1 REFERENCE VALUES OF HEPATOBILIARY ENZYMES

Ten to eighteen healthy animals from each species were selected to establish the normal level of serum hepatobiliary enzyme activity. The mean serum hepatobiliary enzyme activities, number of observations (n), standard error (SE), 95 % confidence interval for the lower and upper bound values of enzyme activities (reference range) studied for these enzymes are listed in Table 1 to 2 and is graphically presented in Fig. 1.

4.1.1 Cattle

The mean serum activities of ALT, AST, ALP and GGT observed in twelve clinically healthy female crossbred cattle with an age of age 3 to 7 years are 19.46 ± 1.54, 68.67 ± 2.29, 113.7 ± 7.59 and 13.15 ± 0.78 IU/L, respectively. The reference range observed for ALT, AST, ALP and GGT was 16.11 to 22.81, 63.61 to 73.72, 96.53 to 130.87 and 11.45 to 14.86 IU/L, respectively in 95 % of the population studied (Table 1 and 2).

4.1.2 Buffalo

Table 1 and 2 illustrates the reference levels of the hepatobiliary enzymes such as, ALT, AST, ALP and GGT of ten adult healthy female Murrah buffaloes of age 2 to 3 years. The results showed that the mean serum activities of ALT, AST, ALP and GGT were 50.0 ± 3.53, 130.0 ± 7.29, 323.6 ± 32.09 and 10.11 ± 1.28 IU/L with 95% confidence interval of 42.02 to 57.98, 113.51 to 146.49, 251.0 to 396.19 and 7.15 to 13.07 IU/L, respectively .

4.1.3 Goat

A total of 18 adult healthy female crossbred goats of age 3 to 5 years were studied and the results are presented in Table 1 and 2. The mean serum activities for ALT, AST, ALP and GGT were 15.94 ± 0.84, 80.87 ± 3.71, 175.92 ± 20.09 and 35.27 ± 1.73 IU/L, respectively. The reference range observed was 14.17 to 17.72 IU/L for ALT, 72.89 to 88.84 IU/L for AST, 131.71 to 220.13 IU/L for ALP and 31.41 to 39.13 IU/L for GGT.

4.1.4 Dog

The animals composed of thirteen apparently healthy dogs of different breeds and sex with an age of 1.5 to 3 years were studied for the reference level of serum hepatobiliary enzymes. From the results it was found that the mean serum ALT, AST, ALP and GGT activities in dogs as 33.56 ± 3.38, 35.83 ± 2.49, 92.9 ± 7.53 and 4.0 ± 0.15 IU/L, respectively. Reference range obtained for ALT, AST, ALP and GGT were was 25.15 to 41.36, 30.36 to 41.31, 75.87 to 109.93 and 3.66 to 4.34 IU/L, respectively in 95 % of the population studied (Table1 and 2).

4.1.5 Comparison of hepatobiliary enzyme activities between different species

Mean serum ALT, AST, ALP and GGT activities obtained for cattle, buffalo, goat and dog were compared by Duncan Multiple Range test to assess the influence of species upon the hepatobiliary enzyme levels. Significant differences were noticed in most of the enzymes between different species (Table 1 to 2). The results are depicted in Fig. 2.

4.1.5.1 ALT

The results (Table 1) showed statistically significant difference ($P \leq 0.05$) in the activity of ALT between the four species studied. The highest ALT activity was observed in buffaloes with a mean of 50.00 ± 3.53 IU/L and the lowest value in goat (15.94 ± 0.84 IU/L). Serum ALT levels were significantly higher ($P \leq 0.05$) in the serum of buffalo and dog when compared to cattle and goat. The results revealed statistically significant difference ($P \leq 0.05$) in mean serum ALT levels between buffalo and dog, whereas no significant difference was found in mean serum ALT level of goat and cattle, but significantly different from that of buffalo and dog.

4.1.5.2 AST

Comparison of AST activities between different species are presented in Table 1. The activity of AST was also highest in the serum of buffaloes (130.00 ± 7.29 IU/L) but lowest value was observed in dogs (35.83 ± 2.49 IU/L). The results showed statistically significant difference ($P \leq 0.05$) between the mean serum AST levels of cattle, buffalo, goat and dog.

4.1.5.3 ALP

Serum ALP activities of cattle, buffalo, goat and dog are shown in Table 2. The highest ALP activity was observed in buffaloes with a mean of 323.6 ± 32.09 IU/L. Moreover, the results also showed wide variation in the enzyme activity in buffaloes with a baseline value of 251.00 IU/L and an upper value of 396.19 IU/L. The lowest ALP activity was observed in dogs (92.90 ± 7.53 IU/L). Significant differences were not found between the mean serum ALP values of cattle and dog, whereas the ALP activities of goat and buffalo were significantly different ($P \leq 0.05$), which were also significantly different from that of cattle and dog.

4.1.5.4 GGT

The results showed comparatively lower GGT activities (Table 2.) and the values obtained were in a narrow range in all the four species. The lowest GGT activity was observed in dog serum with a mean of 4.0 ± 0.15 IU/L whereas, goat showed the highest GGT activity (35.27 ± 1.73 IU/L). The mean serum GGT values of cattle and buffalo were under homogenous groups and were not statistically significant different. But significant differences were observed ($P \leq 0.05$) between the mean GGT values of goat and dog which were also significantly different from that of cattle and buffalo.

4.2 ASSESSMENT OF STORAGE STABILITY OF HEPATOBILIARY ENZYMES

The sera samples from cattle, buffalo, goat and dog were stored at room temperature, 4 ^{o}C and -20 ^{o}C and the results obtained for enzyme activities on days 0, 1, 2, 5, 8, 11 and 14 are presented in table 3 to 6. The stability of each enzyme under each temperature condition and days of storage was determined by calculating the

percentage change in activity from the mean fresh value (day 0 value) at each time point.

4.2.1 Cattle

Sera samples from six animals were analysed for enzyme stability study. The details of the storage stability of each enzyme are summarised in Table 3 to 6.

4.2.1.1 ALT

Table 3 and Fig. 3 illustrate the stability findings for cattle serum ALT activities stored at different conditions for 14 days. The serum stored at room temperature showed a rise from the 1st day onwards up to 5th day followed by a gradual decline and reached below baseline value on 14th day. Although a trend towards an increase in ALT activity was observed in samples kept at room temperature on 1st day itself, the change was insignificant. Significant variation ($P \leq 0.05$) in its activity was observed from day 2nd onwards ($P \leq 0.05$), with an increase in ALT activity by more than 48 %.

Alanine aminotransferase activity in sera samples stored at 4 ^{o}C also showed a tendency to go up. Even though, the variations observed were not statistically significant up to 14 days, the percentage change from initial activity was high after 24 h of storage.

A statistically significant variation in ALT activity was observed from 1st day onwards in sera samples kept -20 ^{o}C, which was prominent on 11th day, where 68.35 % increase in activity was noticed.

4.2.1.2 AST

Table 4 and Fig. 4 summarises the stability data for serum AST stored at room temperature, refrigerator and freezer for 14 days. The AST activities for sera samples stored at room temperature showed a steady decrease over the entire period of experiment. The activity declined prominently within 24 h of blood collection. More than 30 % of initial activity was lost within one day at room temperature and significant variations were seen from 2nd day onwards which was prominent on 11th day where about 55 % of the AST activity was lost.

AST activities were not altered significantly by preserving the sera samples at 4 ^{o}C and -20 ^{o}C for as long as two weeks and the change in activities was about 13 and 8 %, respectively.

4.2.1.3 ALP

The mean serum ALP activity (Table 5 and Fig. 5) on the day of venipuncture was 116.4 ± 12.67 IU/L, which showed a tendency to increase over time in samples kept at all the three storage conditions. At room temperature the initial ALP activity increased slightly on 2nd day (11.17 %) followed there after by a decline to below baseline values on day 11 and 14. But the changes were insignificant.

At 4 ^{o}C, a significant steady increase in ALP activity was noticed from 2nd day onwards up to 14th day, with a maximum increase of 34.71 %. In contrast, activities of ALP changed more markedly at -20 ^{o}C, significant increase was observed after 24 h of storage. The activity increased by more than 60 % after two days followed by a slight decline in activity from 5th to 11th day of storage as compared to second day. On 14th day there was statistically significant rise ($P \leq 0.05$)

in ALP activity by about 70 %. The storage characteristics of cattle serum ALP showed that the activity increased proportional to the decrease in storage temperature. At -20 ^{o}C, maximum increase in activity was observed but at room temperature, only mild variations were observed.

4.2.1.4 GGT

Gamma glutamyltransferase activities were not influenced by preserving serum at room temperature for one day, after which there was an upward trend in values which differed significantly ($P \leq 0.05$) from the mean baseline GGT activity (Table 6 and Fig. 6). Under 4 ^{o}C, however this analyte was more stable, but significant variations were observed on 14th day with 38.18 % increase in activity. At -20 ^{o}C, non significant changes were observed up to 8th day, thereafter, statistically significant changes were seen. The percentage changes in GGT appeared to be some what greater at -20 ^{o}C as compared to 4 ^{o}C.

4.2.2 Buffalo

The stability and storage characteristics of clinically significant enzymes, namely ALT, AST, ALP and GGT studied in eight sera samples from buffaloes are presented in Table 3 to 6.

4.2.2.1 ALT

The stability of ALT activity at room temperature was very less as compared to 4 ^{o}C and -20 ^{o}C (Table 3). The enzyme was highly unstable at room temperature and showed significant decrease ($P \leq 0.05$) in activity from the very next day of blood

collection. At the end of the experimental period only less than 30 % of initial activity was retained in the serum samples.

The storage of serum at 4 ^{o}C for two weeks did not result in any significant change in enzyme activity. Insignificant decrease in ALT activity was observed from the first day onwards at 4 ^{o}C. The storage of serum at -20 ^{o}C was also considered to be suitable for ALT assay in buffalo. The activity remained much unaffected up to the study period of two weeks. The results are graphically presented in Fig. 7.

4.2.2.2 AST

Sera samples stored at room temperature maintained the initial AST activity up to 8 days without any significant loss, but thereafter the values decreased to a point of statistical significance on 11th and 14th day of storage, more than 40 % decrease in activity was noticed during this period. The results are summarised in Table 4 and Fig. 8.

Only negligible changes were found in AST activity when the serum was stored at 4 ^{o}C up to 11th day and these changes were not statistically significant. After 11 days, a clinically acceptable significant decrease in AST values ($P \leq 0.05$) was seen.

Results obtained for AST stability at -20 ^{o}C revealed a negligible variation on enzyme activity up to 2 days. Beyond then, a statistically significant ($P \leq 0.05$) decline in activity was observed up to 14th day.

4.2.2.3 ALP

The ALP activity in the sera samples stored at room temperature did not show any statistically significant change up to first day, followed there after by a significant decline to below baseline values and only less than 23 % of initial activity retained at the end of the experimental period. The variations observed are presented in Table 5 and Fig. 9.

At 4 ^{o}C, ALP activities declined markedly beginning within 24 h of venipuncture, and the changes were statistically significant and the enzyme was totally unstable at this temperature. However, the percentage change in activity was comparatively lesser than that at room temperature.

The specimens kept in the frozen state showed great fluctuations in ALP activity over the entire period. Even after 24 h of storage, significant decline ($P \leq 0.05$) in ALP activity was observed.

4.2.2.4 GGT

Time of storage had significant effect on GGT activity in the sera samples kept at room temperature. The activities increased significantly over the time of storage with more pronounced degree of change on the 8th day with about 78 % increase in activity was observed. The results are presented in Table 6 and Fig. 10.

In the refrigerator and frozen state, the enzyme showed no appreciable change over a period of two weeks and the percentage change in mean activity was less than 23 % in both the conditions. Among these two conditions, the storage of serum at -20 ^{o}C was considered to be more suitable for GGT assay of buffalo serum.

4.2.3 Goat

The stability of sera samples collected from six healthy goats for hepatobiliary enzyme activity after storage is presented in Table 3 to 6.

4.2.3.1 ALT

ALT activity was significantly more stable in samples stored at room temperature, retaining about 86 % of initial activity at the end of the study period. No significant variations were observed up to the end of the study period (Table 3 and Fig. 11).

At 4 ^{o}C, the activity of ALT increased with 25.75 % excess of initial activity at the end of the experimental period, significantly higher change in ALT activity was noticed on days 11 and 14.

The values for the samples stored at -20 ^{o}C showed a decrease in activity during the initial period of investigation, followed by an increase in activity from 8th day up to 14th day (+17 to 18 %) but were not statistically significant.

4.2.3.2 AST

AST activities at room temperature remained almost the same for 2 days post collection with percentage change of only 0.46 %, which then gradually decreased up to 14th day of experiment, remaining only 50 % of initial activity. The significant change in activity was found from 5 th day onwards (Table 4 and Fig. 12).

At 4 ^{o}C, activities of AST differed by less than 5 % from the initial fresh value up to 8 days after blood collection. The activity increased by 10 % on day 11, which was not statistically significant. The change exceeded significantly ($P \leq 0.05$) by about 20 % on day 14. In serum samples stored at -20 ^{o}C, AST activities were sufficiently stable for two weeks without any significant variation.

4.2.3.3 ALP

Though, there was a significant decrease in activity of ALP at room temperature on day 1, it showed a progressive increase in activity with a marked change of 16.63 to 28.13 % increase from 5th to 14th day, respectively (Table 5 and Fig. 13). The enzyme was considered to be unstable at room temperature because of significant decrease in activity within 24 h of venipuncture and also due to great variations observed in the enzyme activity.

After two days of storage an increase in ALP activity was observed at 4 ^{o}C which was significant after 8 days. Only less than 10 % decrease was seen up to 2 days, thereafter, the activity increased by more than 25 % of the initial activity.

The effects of storage at -20 ^{o}C on ALP activities were similar to 4 ^{o}C during the first 2 days, about 15 % decrease in activity was observed on these days, but were not statistically significant. On 5th day the values showed 6.42 % increase in activity which was also not statistically significant. The greatest divergence from initial ALP values was seen on 8th and 11th day of storage with a significant decrease in concentration by about 26 %.

4.2.3.4 GGT

Table 6 and Fig. 14 summarises the stability data for goat serum GGT activities. Under the stated conditions GGT was found to be stable for as long as two weeks. The temperature of storage did not significantly affect the rate or degree of loss of enzyme activity. During the storage period, a negligible and insignificant increase in GGT activity was noticed at room temperature and 4°C, but at -20 °C about 4 to 14 % decrease in activity was observed from 2nd to 14th day of storage. But the variations were not significant.

4.2.4 Dog

Table 3 to 6 illustrates the results of storage of six dog sera samples on the stability of ALT, AST, ALP and GGT activities under different conditions.

4.2.4.1 ALT

At room temperature, there was an upward trend in ALT values within 24 h of venipuncture and the values were substantially higher from 1st to 14th day when compared to mean fresh values. Significant increase ($P \leq 0.05$) in activity was found from the first day onwards (Table 3 and Fig. 15). During the experimental period, the observed increase in activity was from 48.16 % to 84.99 % .

Activity of ALT in serum stored at 4 °C remained unchanged for two weeks retained with more than 90 % of initial activity and there was no significant difference in mean ALT activities over the entire period of storage. Whilst, ALT activities changed significantly at -20 °C with an increase in activity from 2 nd day

onwards followed by a decrease in activity on 11th and 14th day and only about 63 % of the initial activity remained after two weeks.

4.2.4.2 AST

Results of the storage stability of AST at room temperature revealed a statistically significant increase ($P \leq 0.05$) in values from the very next day of blood collection up to 14th day. Table 4 and Fig. 16 represent the results of AST stability in dog serum. About 50 % increase in ALT activity was found after 24 h of storage, thereafter, the activity increased to 253.08 % on 2nd day. The enzyme was totally unstable at room temperature.

For AST, storage at 4 °C and -20 °C for two weeks did not result in any change in enzyme activity that would be considered statistically significant. It was found that AST activity changed by about 7 % and 10 % at 4 °C and -20 °C, respectively during their storage.

4.2.4.3 ALP

Table 5 and Fig. 17 depict the results of stability of ALP activity in dog serum. Storage of sera samples at room temperature significantly affected mean ALP activities through out the study period and the enzyme was found to be unstable. The enzyme showed significant decrease ($P \leq 0.05$) in activity after 24 h of venipuncture.

At 4 °C, decrease in activity was seen through out the study period. Statistically significant decrease in activity was observed from 2nd day onwards.

There was no significant change in ALP activity in sera samples overtime, when stored at -20 ^{o}C for up to 5 days, thereafter, significantly lower ALP activity was observed from 8th to 14th day.

4.2.4.4 GGT

There was virtually no significant change in GGT activity of sera samples stored at room temperature, refrigerator and freezer. The enzyme was sufficiently stable under all the three storage conditions up to two weeks (Table 6 and Fig. 18). During the study period only less than 12 % change in activity was observed in the samples.

4.3 COMPARISON OF STORAGE STABILITY OF HEPATOBILIARY ENZYME ACTIVITIES BETWEEN DIFFERENT SPECIES

Stability and storage characteristics of serum ALT, AST, ALP and GGT activities of cattle, buffalo, goat and dog were compared to find out the ideal storage condition of each enzyme for each species. The details of the comparison of storage stability of each enzyme for all the four species are presented in Table 3 to 6. Summary of storage stability in days for the activity of ALT, AST, ALP and GGT in sera samples of cattle, buffalo, goat and dog after storage at room temperature, 4 ºC and -20 ºC is listed in Table 7.

4.3.1 ALT

Comparative study of ALT activity over 14 days, revealed wide variation in its stability in different species at different temperatures (Table 3). There were significant variations ($P \leq 0.05$) in ALT activity in serum samples of buffalo and dog

after one day of preservation at room temperature and cattle serum lost stability after two days. Whereas, ALT activities in goat serum was found to be stable up to two weeks of preservation at room temperature.

At 4 ^{o}C, the enzyme was found to be stable through out the 14 days of study period for cattle, buffalo and dog, while in goat serum variations were observed with an increase of about 25 % of the initial activity from 11th day onwards. The serum samples stored at freezer showed significant variation in the enzyme activity after 24 h of preservation for cattle and 2 nd day onwards for dog. But buffalo and goat serum ALT activity was found to be stable in freezer through out the study period.

4.3.2 AST

Wide variations were also observed in the storage stability of serum AST activities between different species under each storage conditions (Table 4). At room temperature stability of AST showed differences between cattle, buffalo, goat and dog and the observed stability period were one day for cattle, 8 days for buffalo, 2 days for goat and only in fresh samples of dog.

The enzyme was comparatively stable at 4 ^{o}C, 14 days in the sera of cattle and dog and 11 days in that of buffalo and goat. At -20 ^{o}C, the activity of AST was not significantly altered in all the species under study except for buffalo serum where it was stable only for 2 days.

4.3.3 ALP

Among the hepatobiliary enzymes, ALP showed greatest variation in activity over the entire storage period in all the four species (Table 5). In cattle, the enzyme

did not show any statistically significant change up to 14 days at room temperature but the percentage change was highly variable, while ALP activity in goat and dog serum was stable only in the fresh samples. In buffalo, the serum ALP activity was found to be unstable after one day at room temperature and was also not stable even at 4 ^{o}C and -20 ^{o}C.

The enzyme was found to be unstable for cattle and dog serum after one day at 4 ^{o}C, whereas, the activity in goat serum showed stability up to 8 days. Like buffalo serum, the cattle serum samples also could not be stored at -20 ^{o}C as it showed instability within 24 h of preservation. In goat and dog the enzyme was stable up to 5 days at -20 ^{o}C.

4.3.4 GGT

The activity of GGT was found to be highly stable among the hepatobiliary enzymes studied in the present investigation (Table 6). In goat and dog, the enzyme was stable up to 14 days at room temperature and only negligible differences were found during the storage period in these species. In the same condition the enzyme activity was stable for one day for cattle and it was unstable in buffalo.

At 4 ^{o}C, the enzyme was stable up to 14 days for all the species studied except for cattle which showed variation after 11 days. At -20^{o}C, the enzyme showed significant differences in activity after 8 days in case of cattle. But a stability of 14 days was observed in buffalo, goat and dog. From the present study it has been found that, buffalo, goat and dog sera samples can be preserved at both refrigerator and freezer without affecting enzyme activity for as long as two weeks.

Table 1. Serum ALT and AST activities (IU/L) in cattle, buffalo, goat and dog

Species	ALT				AST			
	Minimum	Maximum	Mean ± SE	95 % confidence interval	Minimum	Maximum	Mean ± SE	95 % confidence interval
Cattle (n=12)	11	28	19.46 ± 1.54[a]	16.11 - 22.81	58	84	68.67± 2.29[b]	63.61 - 73.72
Buffalo (n=10)	30	64	50.00 ± 3.53[b]	42.02 - 57.98	105	172	130.0 ± 7.29[c]	113.51 -146.49
Goat (n=18)	11	24	15.94 ± 0.84[a]	14.17 - 17.72	61	114	80.87 ± 3.71[a]	72.89 - 88.84
Dog (n=13)	15	48	33.56 ± 3.38[c]	25.15 - 41.36	24	50	35.83 ± 2.49[d]	30.35 - 41.31

* Means with different superscripts differ significantly ($P \leq 0.05$) at each column

Table 2. Serum ALP and GGT activities (IU/L) in cattle, buffalo, goat and dog

Species	ALP				GGT			
	Minimum	Maximum	Mean ± SE	95 % confidence interval	Minimum	Maximum	Mean ± SE	95 % confidence interval
Cattle (n=12)	82	147	113.70± 7.59[b]	96.53 – 130.87	9	19	13.15± 0.78[b]	11.45 - 14.86
Buffalo (n=10)	175	479	323.60± 32.09[c]	251.00 - 396.19	4	15	10.11 ± 1.28[b]	7.15 - 13.07
Goat (n=18)	71	294	175.92± 20.09[a]	131.71 - 220.13	27	45	35.27 ± 1.73[a]	31.41 - 39.13
Dog (n=13)	45	128	92.90 ± 7.53[b]	75.87 – 109.93	3	5	4.00 ± 0.15[c]	3.66 - 4.34

* Means with different superscripts differ significantly ($P \leq 0.05$) at each column

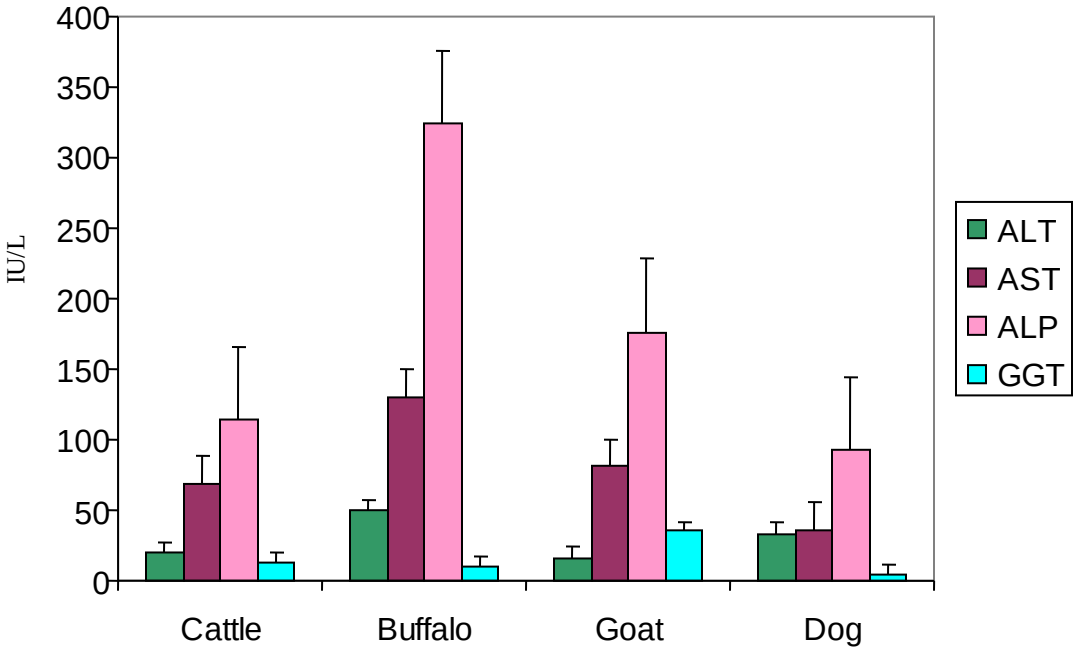

Fig.1. Normal serum ALT, AST, ALP and GGT activities of adult cattle, buffalo, goat and dog

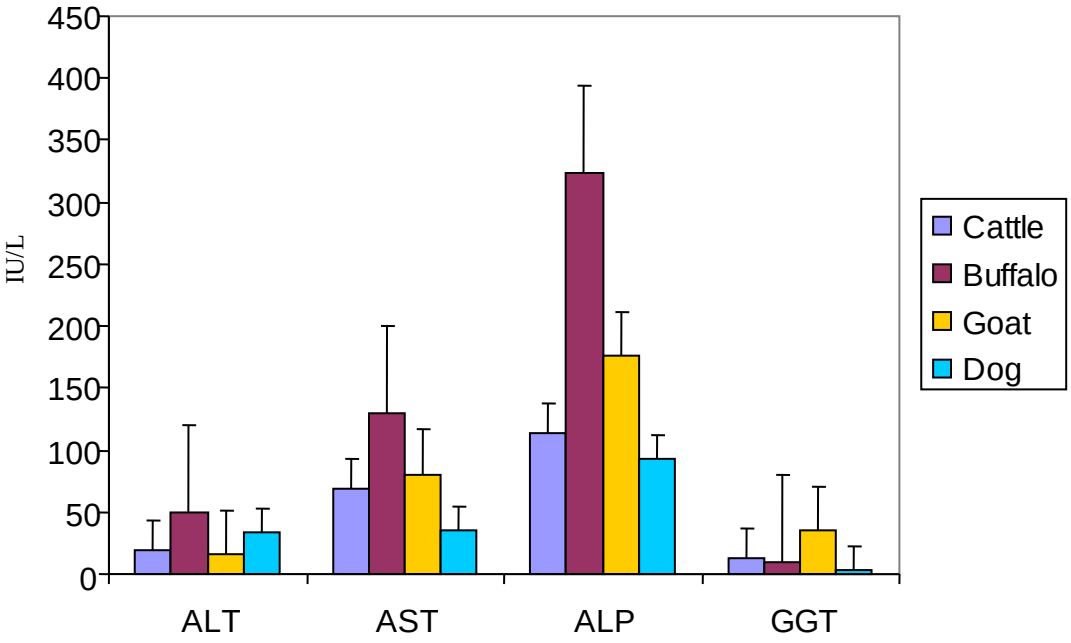

Fig.2. Species wise comparison of normal serum ALT, AST, ALP and GGT levels

Table 3. ALT activity (IU/L) in cattle (n=6), buffalo (n=8), goat (n=6) and dog (n=6) sera samples preserved at room temperature, 4 ºC and -20 ºC for 14 days

Days of storage	Room temperature				4 ºC				-20 ºC			
	Cattle	Buffalo	Goat	Dog	Cattle	Buffalo	Goat	Dog	Cattle	Buffalo	Goat	Dog
0 (Base line value)	15.80± 2.20 -	51.80± 3.43 -	16.70± 1.80 -	35.30± 4.93 -	15.80± 2.20 -	51.80± 3.43 -	16.70± 1.8 -	35.30± 4.93 -	15.80± 2.20 -	51.80± 3.43 -	16.70± 1.80 -	35.30± 4.93 -
1	19.20± 2.80 (+21.25)	44.50± 2.19* (-14.09)	16.50± 2.17 (-1.19)	52.30± 4.66* (+48.16)	21.80± 1.88 (+37.99)	47.30± 2.39 (-8.69)	17.80± 2.15 (+6.58)	35.80± 4.89 (+1.42)	23.40± 1.81* (+48.1)	51.30± 3.12 (-0.97)	15.80± 1.47 (-5.39)	35.30± 4.40 (0)
2	23.40± 1.29* (+48.1)	44.30± 2.46* (-14.47)	16.70± 2.29 (0)	65.30± 3.94* (+84.99)	21.40± 1.54 (+35.44)	47.40± 3.10 (-8.49)	17.80± 1.30 (+6.58)	38.30± 5.25 (+8.49)	21.20± 1.53* (+34.18)	50.90± 2.29 (1.74)	14.70± 2.32 (-11.98)	38.20± 4.95* (+8.22)
5	25.80± 1.39* (+63.3)	37.90± 2.16* (-26.83)	15.30± 2.04 (-8.38)	62.30± 4.83* (+76.49)	22.80± 1.07 (+44.30)	47.40± 2.61 (-8.49)	20.70± 1.43 (+23.95)	38.80± 6.51 (+9.92)	18.80± 1.43 (+18.99)	46.30± 1.74 (-10.62)	16.50± 2.14 (-1.19)	39.20± 4.32* (+11.05)
8	17.80± 0.58* (+12.6)	27.10± 1.52* (-47.68)	13.20± 0.83 (-20.95)	60.50± 5.09* (+71.39)	23.00± 3.22 (+45.57)	47.60± 1.91 (-8.11)	17.00± 1.61 (+1.79)	34.50± 6.79 (-2.27)	19.80± 3.38 (+25.31)	46.60± 2.03 (-10.04)	17.00± 1.03 (+1.79)	39.60± 4.87* (+12.18)
11	17.00± 1.58 (+7.59)	20.50± 2.09* (-60.42)	12.70± 1.45 (-23.95)	60.50± 5.09* (+71.39)	23.80± 2.22 (+50.63)	46.20± 3.22 (-10.80)	20.80± 1.54* (+24.55)	38.30± 5.69 (+8.49)	26.60± 3.89* (+68.35)	45.80± 3.80 (-11.58)	19.80± 1.30 (18.56)	26.50± 4.43* (-24.93)
14	14.00± 1.48 (+11.39)	14.80± 1.85* (-71.42)	14.30± 0.96 (-14.37)	59.80± 5.69* (+69.41)	17.80± 2.33 (+12.65)	45.10± 3.79 (-12.93)	21.00± 2.13* (+25.75)	36.00± 4.77 (+1.98)	20.00± 1.55 (+26.58)	51.1.00± 3.72 (-1.35)	19.70± 3.11 (+17.96)	22.20± 3.19* (-37.11)

Percentage change from initial activity in parenthesis, n= number of samples,* $P \leq 0.05$

Table 4. AST activity (IU/L) in cattle (n=6), buffalo (n=8), goat (n=6) and dog (n=7) sera samples preserved at room temperature, 4 ºC and -20 ºC for 14 days

Days of storage	Room temperature (Mean ± SE)				4 ºC (Mean ± SE)				-20 ºC (Mean ± SE)			
	Cattle	Buffalo	Goat	Dog	Cattle	Buffalo	Goat	Dog	Cattle	Buffalo	Goat	Dog
0 (Base line values)	63.00± 2.09 -	129.80± 8.70 -	74.67± 2.60 -	35.60± 2.14 -	63.00± 2.09 -	129.80± 8.70 -	74.67± 2.60 -	35.60± 2.14 -	63.00± 2.09 -	129.80± 8.70 -	74.67± 2.60 -	35.60± 2.14 -
1	43.20± 5.86 (-31.43)	132.80± 7.50 (+2.31)	75.17± 2.99 (+0.67)	53.10± 5.58* (+49.16)	65.80± 5.04 (+2.8)	127.00± 7.60 (-2.16)	76.20± 3.03 (+2.05)	37.90± 2.14 (+6.46)	68.40± 3.75 (+8.56)	128.30± 7.49 (-1.16)	80.00± 3.77 (+7.14)	38.10± 2.26 (+7.02)
2	34.20± 5.22* (-45.71)	130.80± 7.90 (+0.77)	74.33± 5.65 (-0.46)	125.70± 14.00* (+253.08)	64.60± 4.64 (+2.5)	125.80± 7.05 (-3.08)	76.00± 3.13 (+1.77)	38.30± 1.61 (-7.58)	59.20± 0.86 (-6.03)	121.90± 10.09 (-6.09)	79.80± 3.23 (+6.87)	39.00± 2.44 (+9.55)
5	32.40± 7.12* (-48.57)	125.60± 8.30 (-3.23)	53.33± 6.17* (-28.58)	103.40± 9.70* (+190.45)	65.60± 4.34 (+4.1)	125.30± 7.62 (-3.47)	75.80± 2.29 (+1.51)	35.40± 3.15 (-0.56)	59.60± 1.29 (-5.39)	118.40± 10.69* (-8.78)	74.50± 4.49 (-0.23)	38.40± 3.05 (+7.86)
8	31.00± 6.35* (-50.79)	114.30± 16.40 (-11.94)	48.67± 3.79* (-34.82)	67.10± 11.13* (+88.48)	60.20± 5.69 (-4.4)	127.60± 9.40 (-1.69)	70.80± 3.59 (-5.18)	35.60± 2.77 (0)	59.80± 2.62 (-5.08)	116.30± 7.90* (-10.40)	77.70± 3.36 (+4.06)	38.60± 1.79 (+8.43)
11	28.60± 7.90* (-54.60)	77.10± 12.90* (-40.60)	43.17± 8.78* (-42.19)	57.90± 10.13 (62.64)	60.80± 4.90 (-3.5)	123.60± 7.80 (-4.78)	82.30± 4.15 (+10.22)	33.50± 2.49 (-5.89)	58.40± 1.63 (-7.30)	112.80± 6.9* (-13.09)	73.50± 2.93 (-1.57)	35.90± 1.79 (+0.84)
14	42.40± 7.42* (-32.69)	68.00± 10.90* (-47.61)	37.50± 6.88* (-49.78)	55.40± 9.34 (+55.62)	54.60± 5.28 (-13.3)	121.40± 7.8* (-6.47)	89.70± 5.34* (+20.13)	35.40± 1.69 (-0.56)	60.60± 2.66 (-3.81)	109.10± 6.90* (-15.95)	71.60± 4.01 (-4.11)	38.30± 1.54 (+7.58)

Percentage change from initial activity in parenthesis, n= number of samples,* $P \leq 0.05$

Table 5. ALP activity (IU/L) in cattle (n=6), buffalo (n=8), goat (n=6) and dog (n=6) sera samples preserved at room temperature, 4 ºC and -20 ºC for 14 days

Days of storage	Room temperature (Mean ± SE)				4 ºC (Mean ± SE)				-20 ºC (Mean ± SE)			
	Cattle	Buffalo	Goat	Dog	Cattle	Buffalo	Goat	Dog	Cattle	Buffalo	Goat	Dog
0 (Baseline values)	116.40± 12.67 -	310.20± 36.57 -	166.70± 13.92 -	102.30± 7.51 -	116.40± 12.67 -	310.20± 36.57 -	166.70± 13.92 -	102.30± 7.51 -	116.40± 12.67 -	310.20± 36.57 -	166.70± 13.92 -	102.30± 7.51 -
1	118.20± 11.27 (+1.55)	254.50± 44.10 (-17.96)	157.20± 13.36* (-5.69)	88.00± 8.54* (-13.98)	134.60± 14.74 (+15.64)	261.30± 45.17* (-15.76)	151.80± 11.54 (-8.94)	97.70± 6.53 (-4.49)	144.60± 20.3* (+24.23)	274.60± 44.7* (-11.48)	140.60± 8.96 (-15.66)	114.30± 9.61 (+11.73)
2	129.40± 11.08 (+11.17)	229.80± 38.31* (-25.91)	167.60± 14.02 (+0.54)	75.50± 8.66* (-26.19)	135.40± 10.43* (+16.32)	279.10± 42.38* (-10.03)	160.10± 22.34 (-3.96)	98.00± 7.47* (-4.20)	187.20± 16.02 (+60.82)	275.00± 44.62* (-11.35)	147.40± 18.22 (-11.58)	112.30± 14.96 (+9.77)
5	123.80± 10.85 (+6.36)	165.40± 38.59* (-46.68)	194.40± 17.04 (+16.63)	82.30± 8.53* (-19.55)	138.40± 16.04* (+18.90)	274.50± 43.75* (-11.51)	209.00± 21.90 (+25.37)	96.30± 7.56* (-5.86)	133.60± 15.00 (+14.78)	311.50± 34.19 (+0.42)	177.40± 33.49 (+6.42)	111.00± 15.23 (+8.50)
8	120.60± 10.93 (+3.61)	110.50± 24.59* (-64.38)	199.60± 21.96 (+19.75)	75.30± 7.08* (-26.19)	146.00± 9.88 (+25.42)	272.30± 43.32* (-12.21)	213.10± 21.70 (+27.83)	91.20± 6.89* (-10.85)	149.80± 21.51 (+28.69)	319.60± 32.30 (+3.03)	122.00± 14.52* (-26.81)	85.20± 11.50* (-16.72)
11	83.40± 22.17 (-28.35)	77.60± 14.99* (-74.98)	210.10± 24.56 (+26.03)	75.70± 9.94* (-26.00)	149.00± 14.79* (+28.01)	270.90± 41.95* (-12.67)	245.00± 32.63* (+49.97)	97.80± 4.74 (-4.39)	168.40± 28.97 (+44.67)	284.10± 41.22 (-8.41)	123.10± 29.05* (-26.16)	96.80± 7.57* (-5.38)
14	76.00± 25.38 (-34.70)	71.25± 11.84* (-77.03)	213.60± 26.61 (+28.13)	79.00± 9.78* (-22.78)	156.80± 17.17* (+34.71)	271.80± 43.34* (-12.38)	209.60± 26.38 (+25.73)	95.80± 4.25 (-6.35)	198.80± 29.16* (+70.79)	279.80± 42.93 (-9.80)	164.30± 18.42 (-1.44)	100.30± 10.39 (-1.96)

Percentage change from initial activity in parenthesis, n= number of samples,* $P \leq 0.05$

Table 6. GGT activity (IU/L) in cattle (n=6), buffalo (n=8), goat (n=6) and dog (n=6) sera samples preserved at room temperature, 4 ºC and -20 ºC for 14 days

Days of storage	Room temperature (Mean ± SE)				4 ºC (Mean ± SE)				-20 ºC (Mean ± SE)			
	Cattle	Buffalo	Goat	Dog	Cattle	Buffalo	Goat	Dog	Cattle	Buffalo	Goat	Dog
0 (Base line values)	11.00± 0.95 -	11.30± 1.02 -	36.40± 3.04 -	4.20± 0.17 -	11.00± 0.95 -	11.30± 1.02 -	36.40± 3.04 -	4.20± 0.17 -	11.00± 0.95 -	11.30± 1.02 -	36.40± 3.04 -	4.20± 0.17 -
1	12.80± 0.58 (+16..36)	13.60± 0.95* (+20..35)	38.60± 3.27 (+6.04)	4.50± 0.43 (+6.67)	13.40± 0.40 (+21.82)	12.80± 0.58 (+11.27)	35.60± 2.63 (-2.19)	4.70± 0.42 (+11.90)	12.20± 0.66 (+10..91)	12.70± 0.57 (+12.39)	38.20± 3.39 (+4.95)	4.70± 0.21 (+11.90)
2	14.00± 0.45* (+27..27)	15.80± 0.87* (+39.82)	36.40± 3.19 (0)	4.30± 0.33 (+2.38)	13.60± 0.60 (+23.64)	13.80± 0.43 (+22.12)	37.20± 2.39 (+2.19)	4.70± 0.42 (+11.90)	12.80± 0.58 (+16..36)	12.20± 0.039 (+7.96)	34.80± 3.04 (-4.39)	4.70± 0.21 (+11.90)
5	15.00± 0.71* (+36..36)	17.60± 0.80* (+55.75)	39.80± 3.09 (+9.34)	4.00± 0.26 (-4.76)	12.00± 0.93 (+9.09)	12.60± 0.56 (+11.50)	38.80± 3.52 (+6.59)	4.30± 0.33 (+2.38)	13.00± 0.56 (+18..8)	12.50± 0.41 (+10.62)	34.40± 3.69 (-5.49)	3.70± 0.21 (-11.05)
8	16.80± 1.59* (+52.72)	20.10± 2.48* (+77.78)	37.20± 2.46 (+2.19)	3.70± 0.20 (-11.05)	12.00± 0.43 (+9.09)	13.50± 0.38 (+19.47)	37.00± 3.18 (+1.64)	4.30± 0.55 (+2.38)	13.60± 0.40 (+23..64)	13.20± 0.40 (+16.81)	31.40± 2.93 (-13.74)	3.70± 0.33 (-11.05)
11	17.60± 3.17* (+60..00)	18.00± 0.52* (+59.29)	38.20± 2.52 (+4.95)	4.70± 0.42 (+11.90)	10.80± 0.80 (-1.82)	13.80± 0.58 (+22.12)	37.00± 2.12 (+1.64)	4.20± 0.40 (0)	14.20± 0.63* (+23..09)	13.80± 0.58 (+22.12)	31.80± 3.37 (-12.64)	3.50± 0.35 (-11.80)
14	20.60± 3.71* (+87..27)	17.70± 1.02* (+56.64)	38.80± 1.96 (+6.59)	4.30± 0.21 (+2.38)	15.20± 1.02* (+38.18)	13.00± 0.56 (-15.04)	36.60± 2.71 (+0.55)	4.00± 0.26 (-4.76)	16.80± 1.24* (+52..73)	11.00± 0.63 (-2.65)	31.20± 2.53 (14.29)	4.20± 0.17 (0)

Percentage change from initial activity in parenthesis, n= number of samples,* $P \leq 0.05$

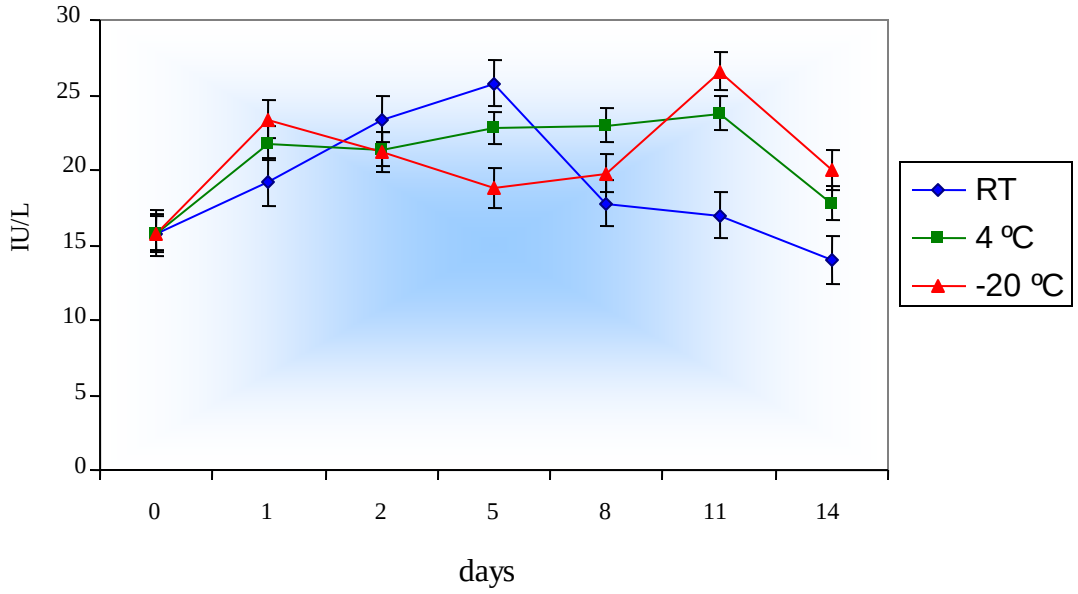

Fig.3. Stability of ALT activity in cattle serum after storage at different days and temperatures

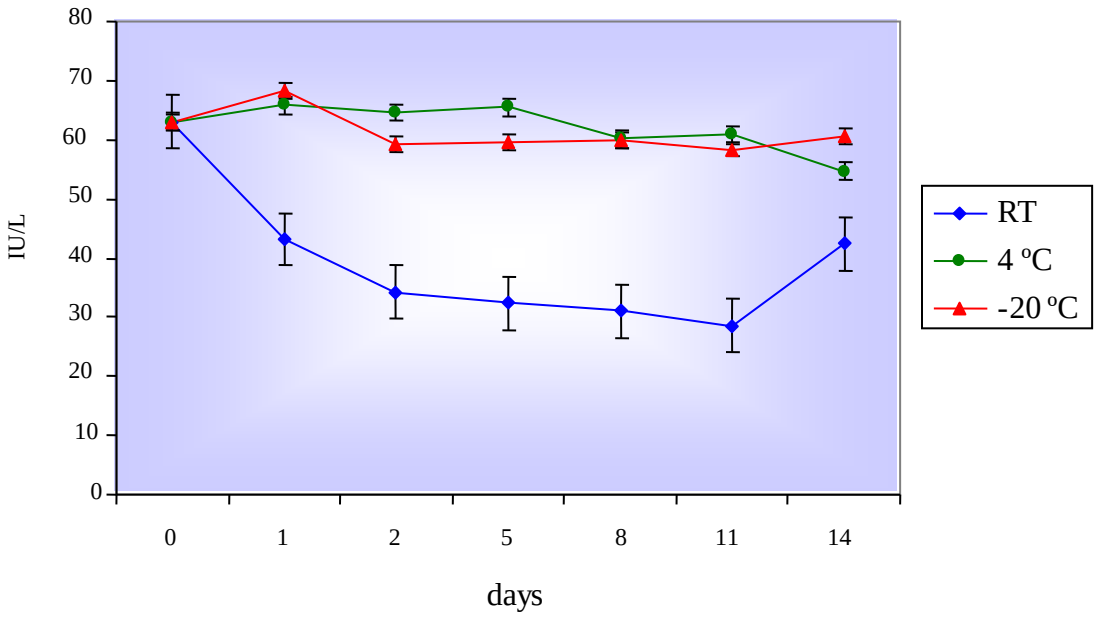

Fig. 4. Stability of AST activity in cattle serum after storage at different days and temperatures

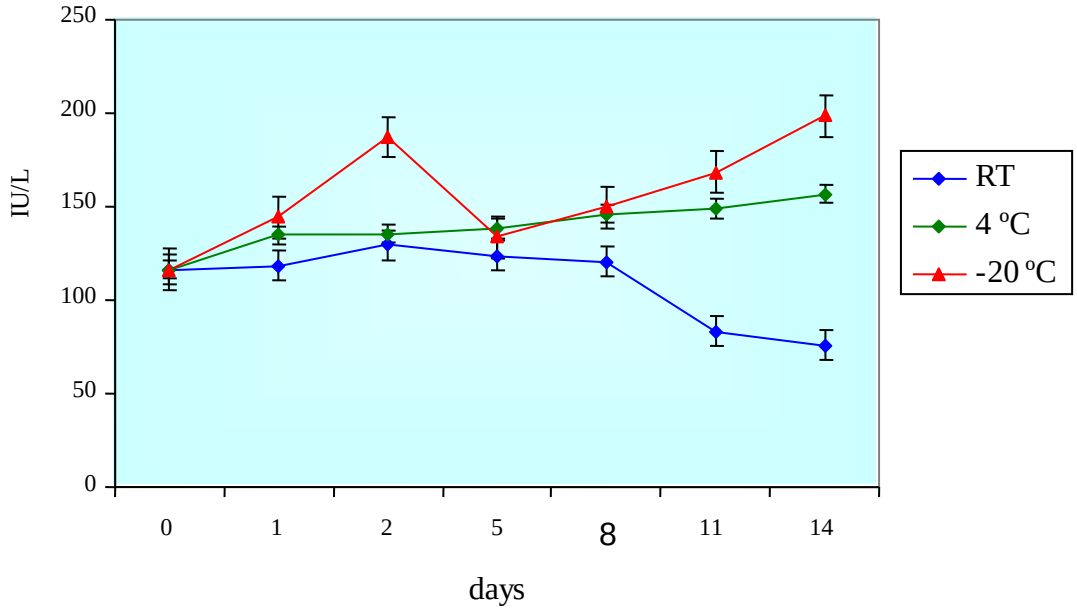

Fig. 5. Stability of ALP activity in cattle serum after storage at different days and temperatures

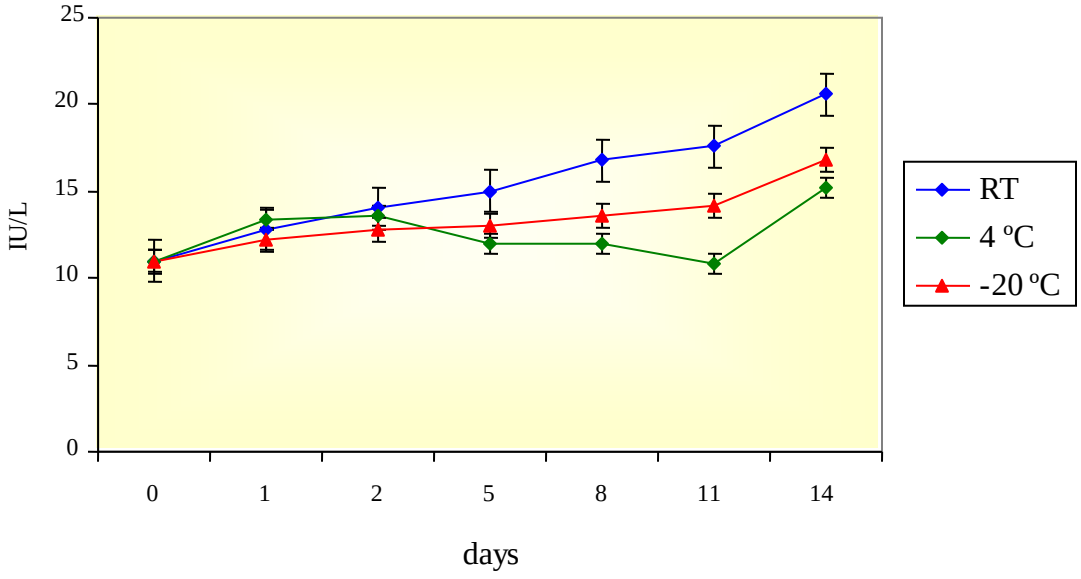

Fig. 6. Stability of GGT activity in cattle serum after storage at different days and temperatures

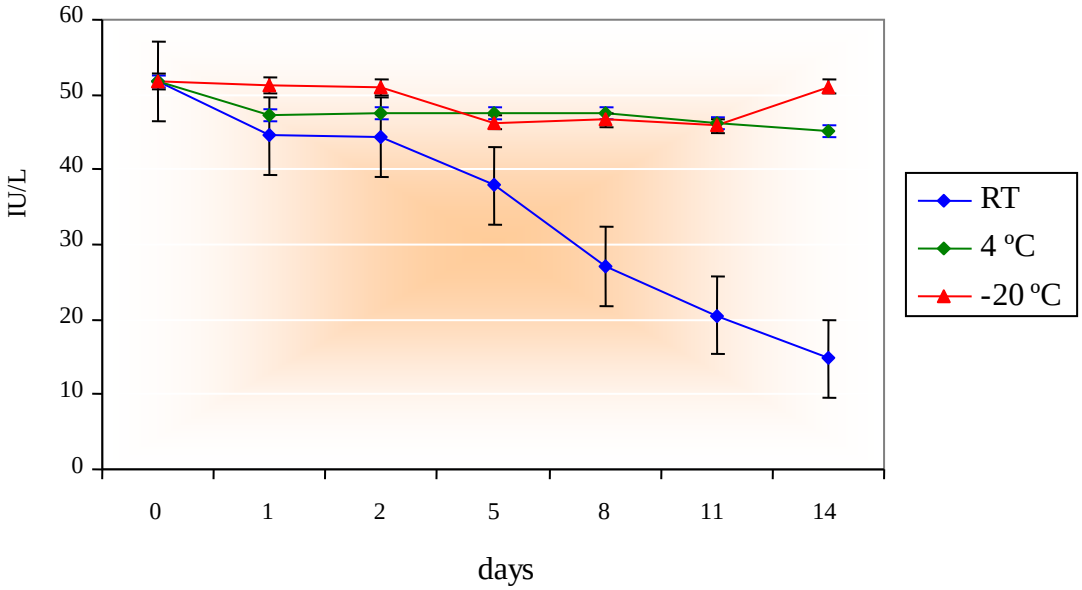

Fig.7. Stability of ALT activity in buffalo serum after storage at different days and temperatures

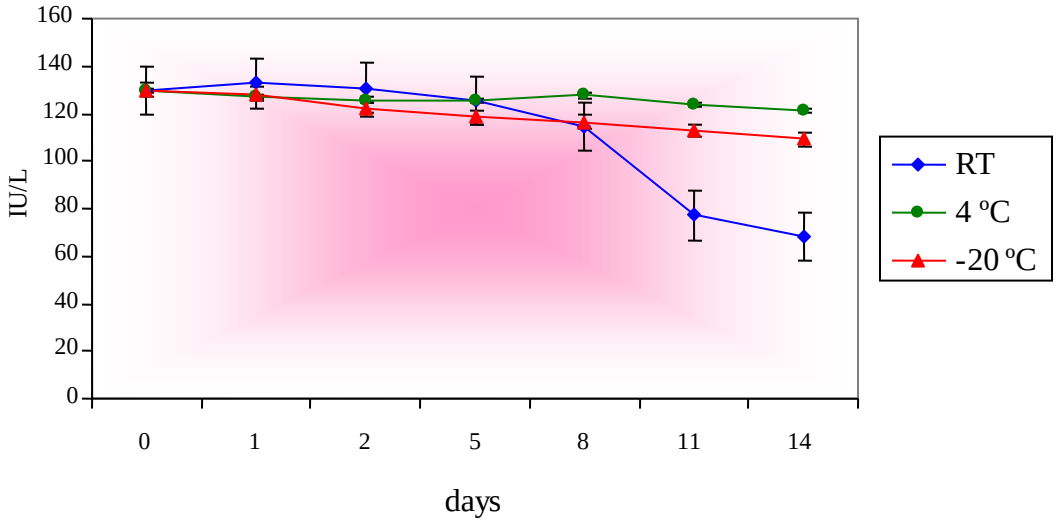

Fig. 8. Stability of AST activity in buffalo serum after storage at different days and temperatures

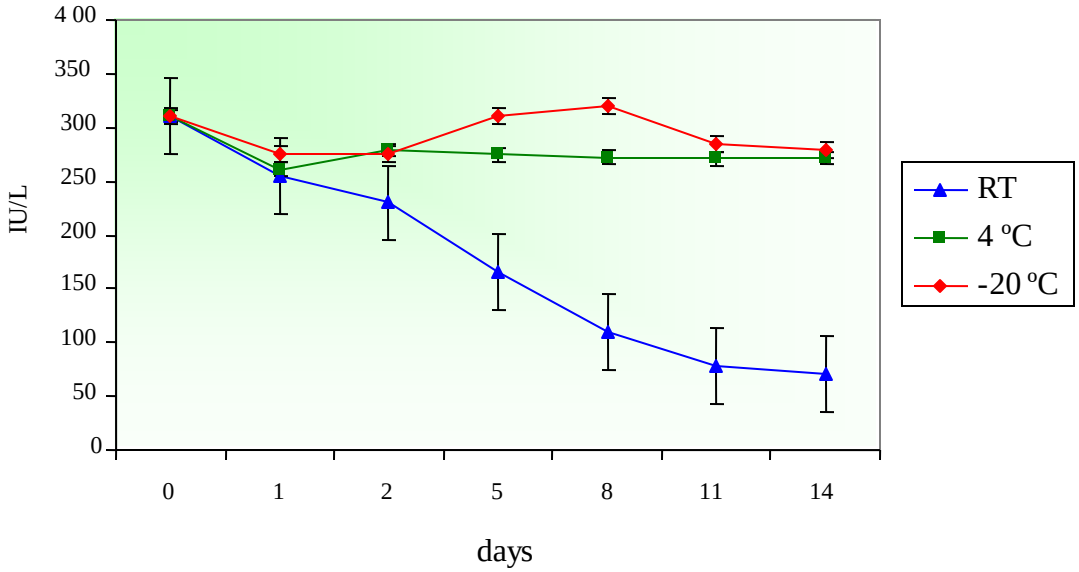

Fig. 9. Stability of ALP activity in buffalo serum after storage at different days and temperatures

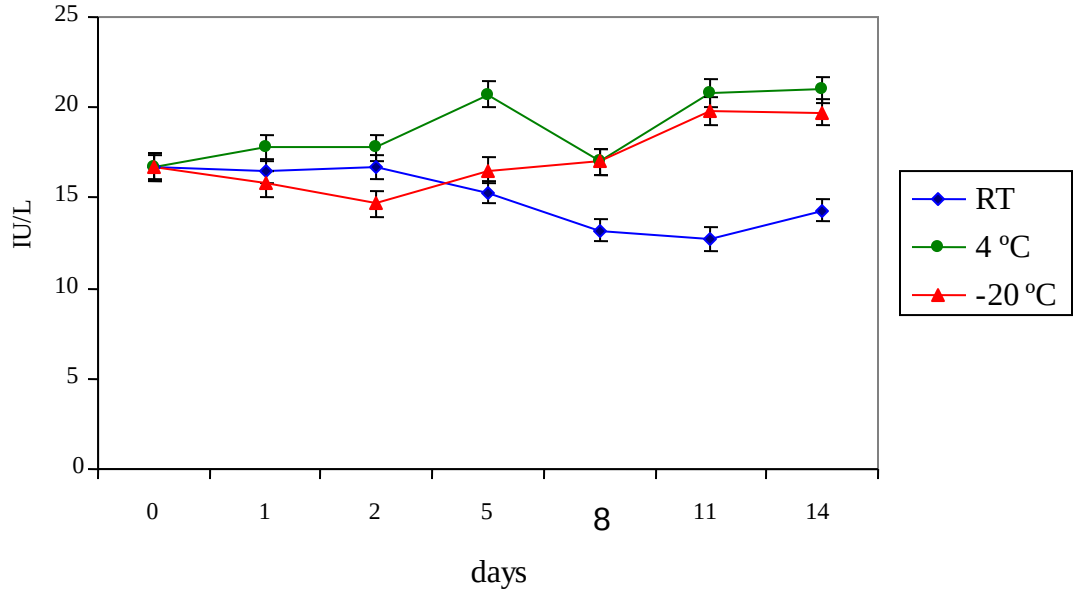

Fig. 10. Stability of GGT activity in buffalo serum after storage at different days and temperatures

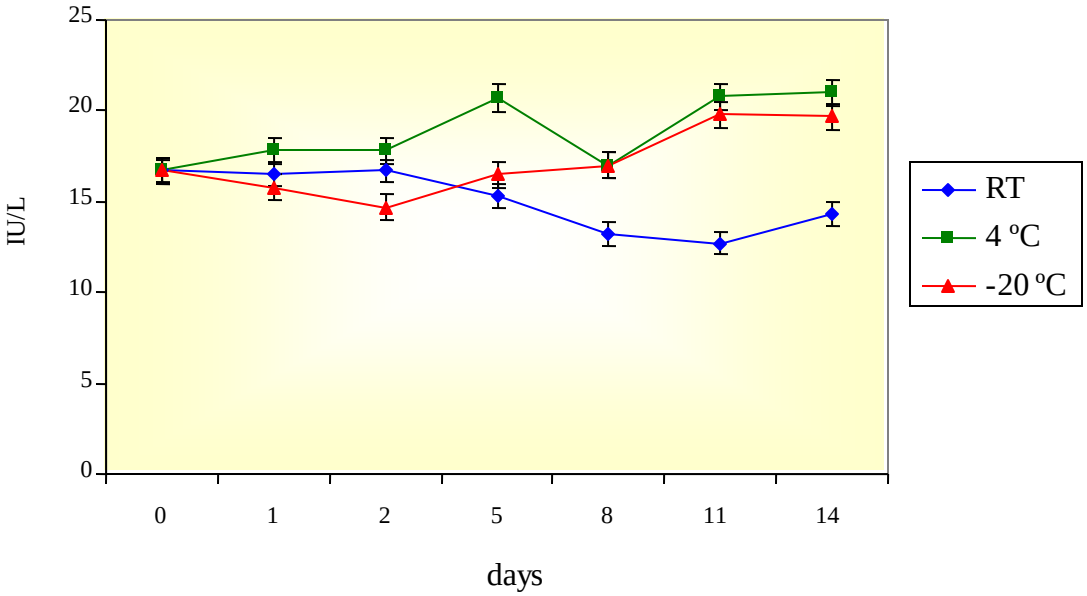

Fig. 11. Stability of ALT activity in goat serum after storage at different days and temperatures

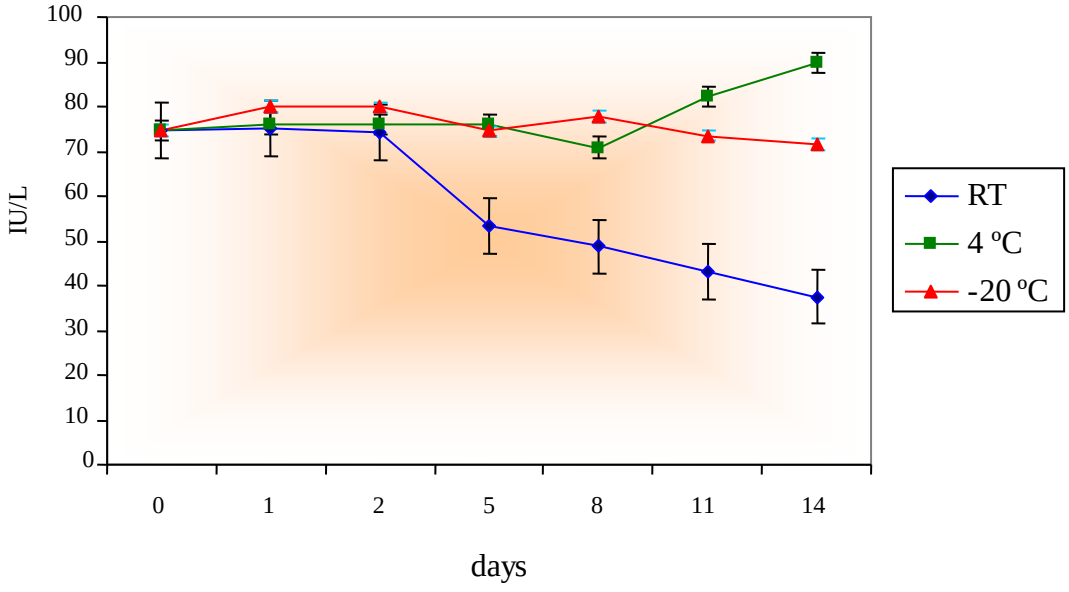

Fig. 12. Stability of AST activity in goat serum after storage at different days and temperatures

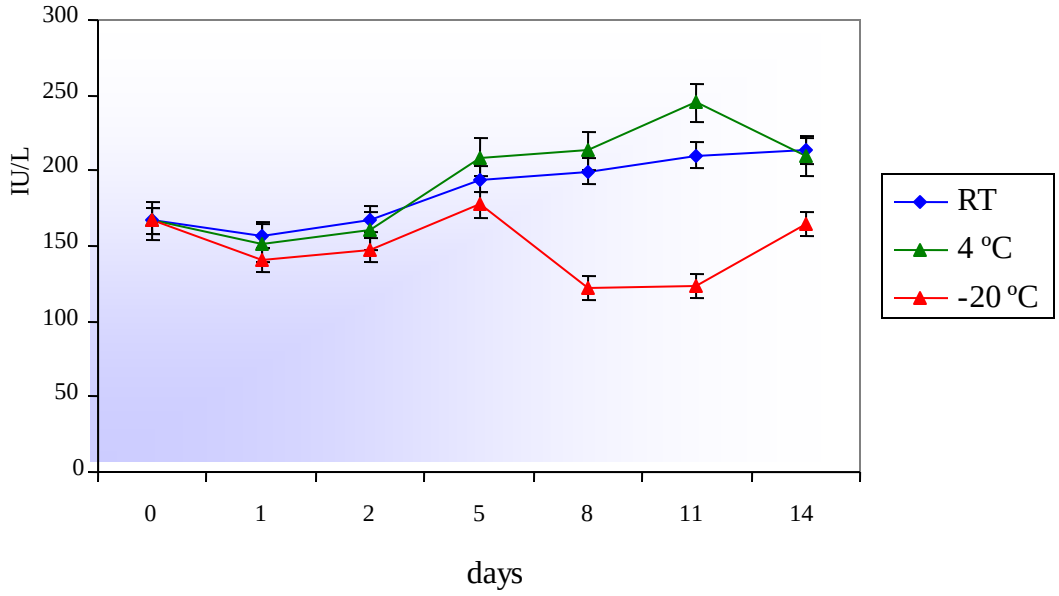

Fig. 13. Stability of ALP activity in goat serum after storage at different days and temperatures

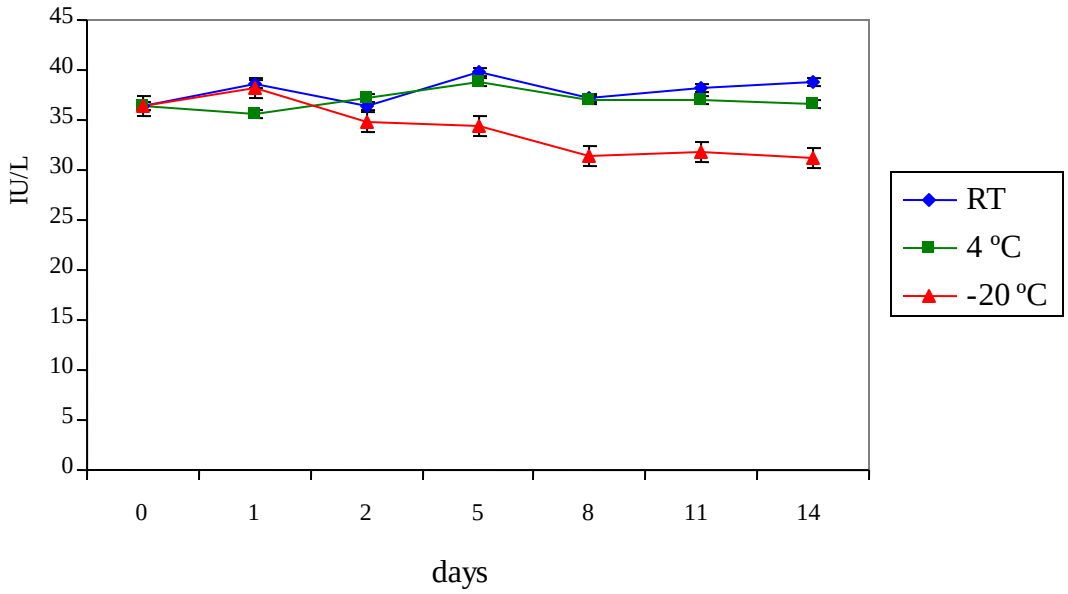

Fig. 14. Stability of GGT activity in goat serum after storage at different days and temperatures

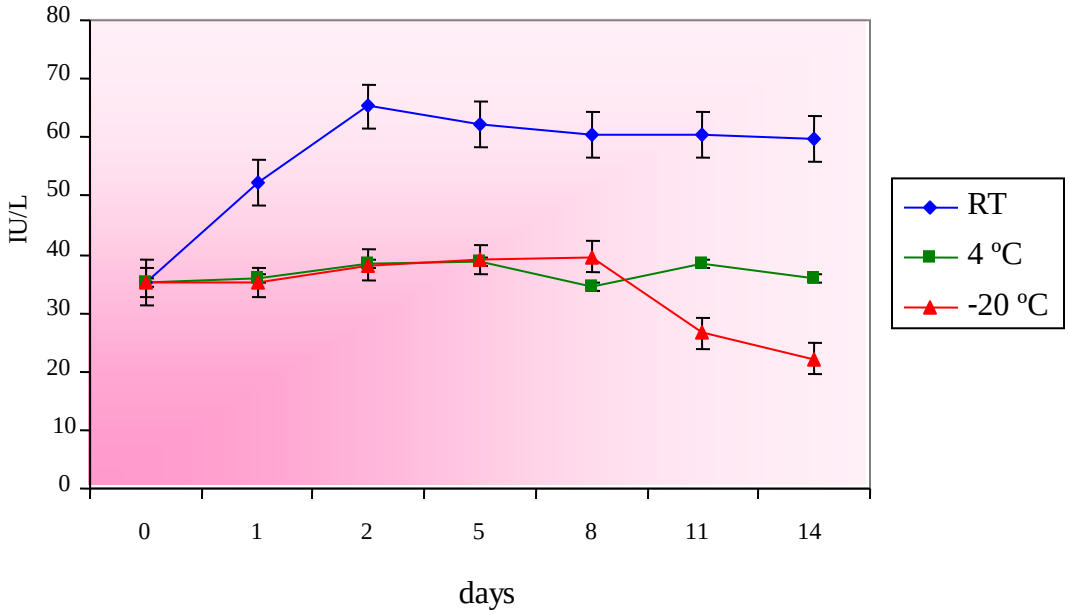

Fig. 15. Stability of ALT activity in dog serum after storage at different days and temperatures

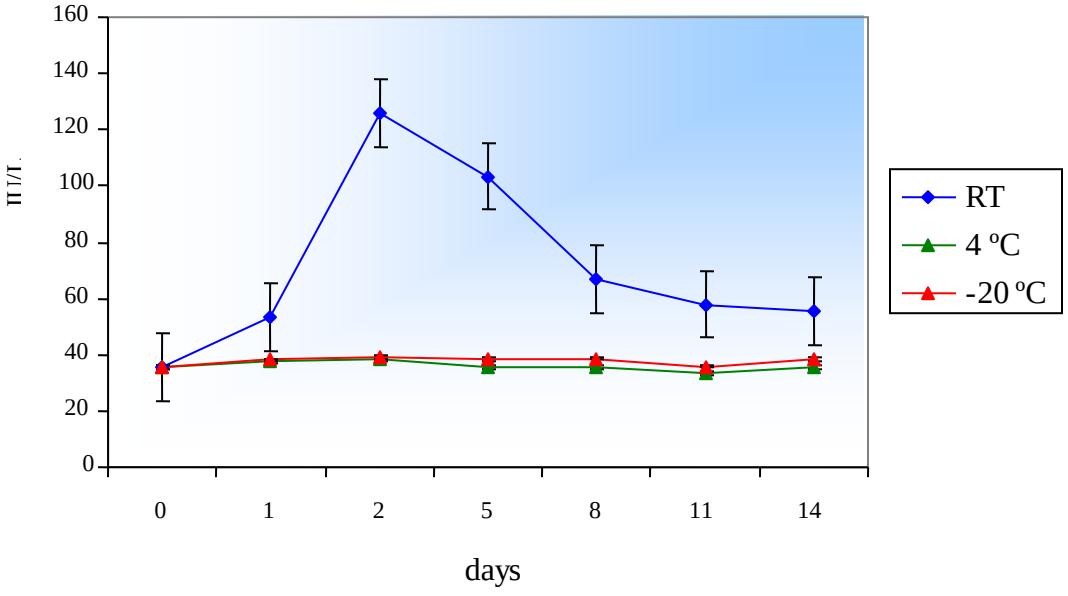

Fig. 16. Stability of AST activity in dog serum after storage at different days and temperatures

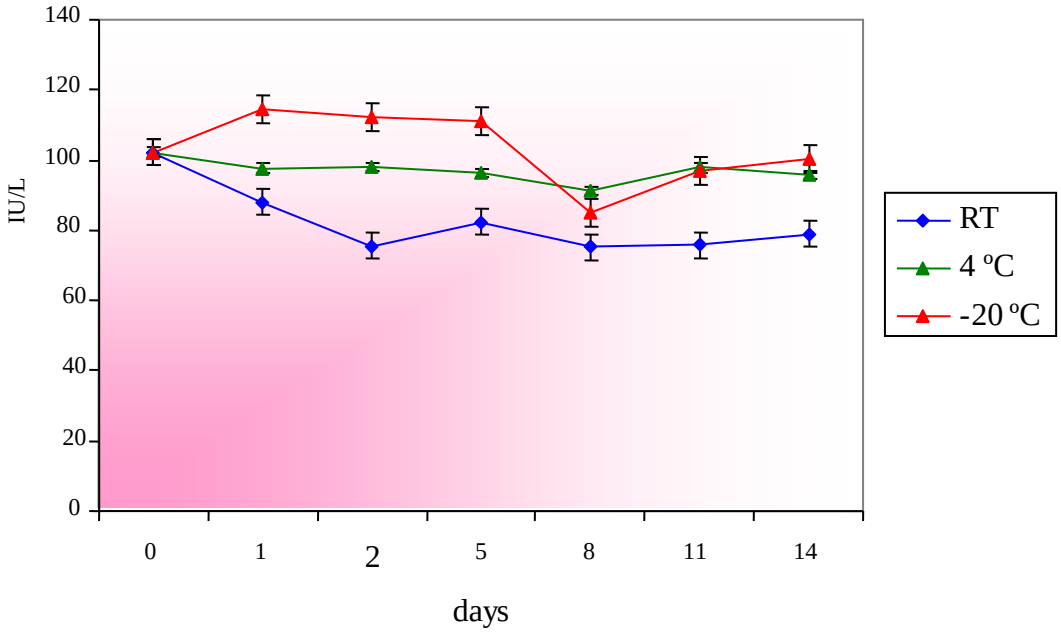

Fig. 17. Stability of ALP activity in dog serum after storage at different days and temperatures

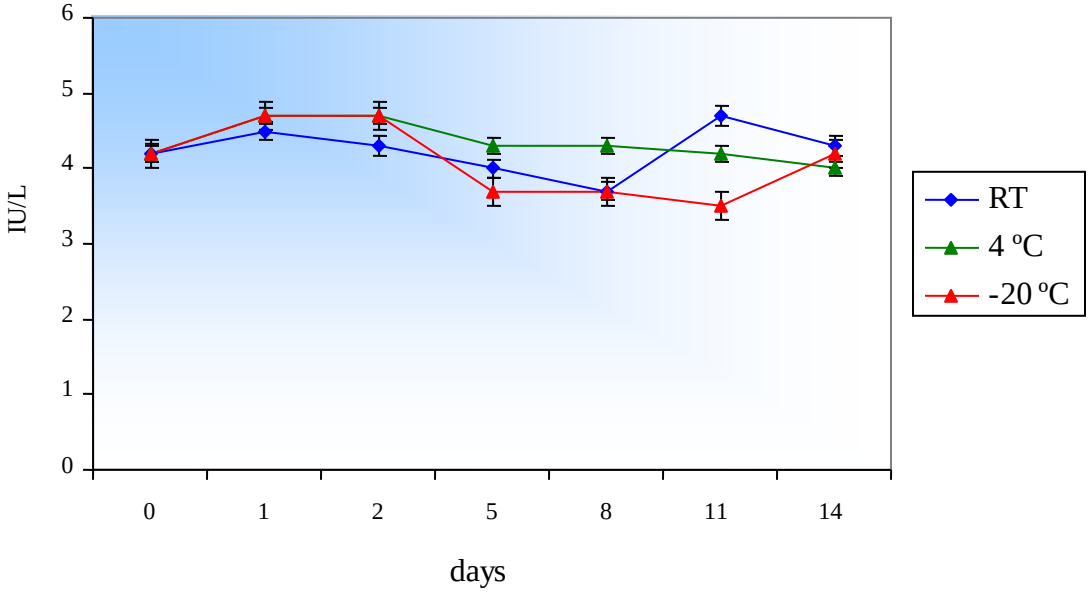

Fig. 18. Stability of GGT activity in dog serum after storage at different days and temperatures

Table 7. Summary of storage stability in days for the activity of ALT, AST, ALP and GGT in sera samples of cattle, buffalo, goat and dog at room temperature, 4 °C and -20 °C

Species	Storage conditions	Enzyme stability in days			
		ALT	AST	ALP**	GGT
Cattle	RT	1	1	14	1
	4 °C	14	14	1	11
	-20 °C	unstable	14	unstable	8
Buffalo	RT	unstable	8	1	unstable
	4 °C	14	11	unstable	14
	-20 °C	14	2	unstable	14
Goat	RT	14	2	unstable	14
	4 °C	8	11	8	14
	-20 °C	14	14	5	14
Dog	RT	unstable	unstable	unstable	14
	4 °C	14	14	1	14
	-20 °C	1	14	5	14

** Though, not significant, ALP showed wide variations in percentage change in activity and therefore, not recommended for storage as per the days mentioned

RT: Room temperature

Discussion

5. DISCUSSION

Enzymes are central to every biochemical process and most of the metabolic pathways in our body are regulated through the coordinated action of enzymes. Most of the enzymes with diagnostic applications, functions within the cells in which they are synthesised and are present in high concentration in specific tissues. Among the clinically important enzymes, hepatobiliary enzymes such as, ALT, AST, ALP and GGT act as a valuable tool in the diagnosis of liver disorders. These enzymes leak out into the serum when there is any damage to hepatic tissue. In small animals due to high liver ALT activity, it is considered to be liver specific and hence it has been used routinely for the diagnosis of hepatic damage. But in large domestic animals due to low ALT activity, it is not recommended for assessing hepatic diseases. Aspartate aminotransferase is present in many tissues and is useful in evaluating muscle and liver damage in small and large animals. Elevations of both ALT and AST can occur with states of altered hepatocellular permeability. Intra and extra hepatic biliary obstruction causes dramatic elevations of ALP concentration, by about 10 to 20 times the normal level. Gamma glutamyltransferase is also a sensitive marker of cholestasis and is highly liver specific in all animals.

The present study was undertaken, to assess the physical baseline values of above mentioned hepatobiliary enzymes in ruminants and dogs reared in humid tropics and also to establish the storage stability of these enzymes at room temperature, refrigerator (4 ^{o}C) and freezer (-20 ^{o}C) for a period of 14 days.

5.1 REFERENCE VALUES OF HEPATOBILIARY ENZYMES

Determination of the normal hepatobiliary enzyme levels is of primary interest in connection with the detection of liver problems. For evaluation of laboratory results, availability of reference value is a prerequisite. Interpretation of clinical data depends upon the reference values of each animal species, in

different regions and under the existing environmental conditions. In the present study ruminants such as, cattle, buffalo and goat maintained at University farms and dogs reared at nearby kennels were selected. Since, animals used in this study showed no clinical signs or pathological symptoms they were considered apparently healthy and the data obtained could serve as reference values for future use in veterinary medicine and animal production.

5.1.1 Cattle

The activity of serum ALT, AST, ALP and GGT concentration for adult healthy female cross bred cattle during dry period in the present study were 19.46 ± 1.54, 68.67 ± 2.29, 113.70 ± 7.59 and 13.15 ± 0.78 IU/L, respectively. The reference range obtained for these parameters were 16.11 to 22.81, 63.61 to 73.72, 96.53 to 130.87 and 11.45 to 14.86 IU/L, respectively.

5.1.1.1 ALT

Reference range of 16.11 to 22.81 IU/L serum ALT activity obtained in the study for female cross bred cattle is within the 3 to 23 IU/L reported by Kaneko *et al.* (1997) for adult cattle even though it is towards a higher side. The findings of the present study are in partial agreement with the 5 to 18 IU/L reported by Lumsden *et al.* (1980b) for adult female Holstein cattle and 15.8 to 18.8 IU/L reported by Dubreuil and Lapierre (1997) for lactating cows from Quebec. A higher range of ALT activity of 5.24 to 29.68 IU/L was reported in the blood plasma of highly productive Holstein dairy cows during lactation and dry period by Stogevic *et al.* (2005) and 6.90 to 35 IU/L reported by Khan (2005) in adult cattle. However, the values obtained in the present study were lower than the 36.30 IU/L reported by Ottol *et al.* (1998) for Angoni cattle in Mozambique and 34 IU/L reported for cows in Saudi Arabia by Osman and Al-Busadah (2003).

5.1.1.2 AST

The mean values of AST ranging from 63.61 to 73.72 IU/L for the female crossbred cattle in the present study is within the range of 45 to 110 IU/L for cows reported by Kahn (2005) but it is towards the lower margin of the reference range. The values obtained is slightly lower than the 96 IU/L for heat tolerant and 136 IU/L for cold tolerant Zebu heifers reported by Olbrich *et al*. (1971). It was also lower than the 84.6 IU/L, 87.2 IU/L and 86.2 IU/L obtained for non pregnant, lactating and pregnant Angoni cattle in Mozambique, respectively (Ottol *et al.*, 1998). However, it was in close agreement with the 72.4 IU/L reported by Osman and Al-Busadah (2003) for cows in Saudi Arabia. In contrast, Kaneko *et al.* (1997) reported a higher range of 78 to 132 IU/L in cattle. Earlier reports in female Holstein cattle of more than 2 years of age (Lumsden *et al*. 1980b) showed a lower value of 24 to 45 IU/L. The serum AST activity obtained in the present study is also higher than the 32.90 IU/L reported by Stojevic *et al*. (2005) for the Holstein dairy cows during dry period and 44.91 to 57.71 IU/L during lactation.

5.1.1.3 ALP

The overall mean values of ALP in the female crossbred cattle ranged from 96.53 to 130.87 IU/L (113.7 ± 7.59) and falls within the range of 18 to 153 IU/L for adult cattle (Kahn, 2005). It is higher than the 3 to 46 IU/L reported by Lumsden *et al*. (1980b) and 49.8 IU/L by Osman and Al-Busadah (2003) for cows in Saudi Arabia. The values obtained were in close accordance with 102 to 131 IU/L for lactating cows from Quebec (Dubreuil and Lapierre, 1997). The present results were not in agreement with the ALP values reported by some authors. Olbrich *et al*. (1971) reported a higher ALP activity of 653 IU/L for heat tolerant and 266 IU/L in cold tolerant cattle, Doornenball *et al.* (1988) reported an ALP activity of 430.50 IU/L and 212.60 IU/L for Short horn calves at birth and one year age, respectively. Ottol *et al.* (1998) also suggested a higher ALP activity of 261.90 for Angoni cattle in Mozambique.

5.1.1.4 GGT

The results of the present study showed a mean GGT activity of 13.15 ± 0.78 IU/L with a reference range of 11.45 to 14.86 IU/L for female cross bred cattle. The results were within the range of 6.10 to 17.40 IU/L reported by Kaneko *et al.* (1997). The values were within the 8.11 to 27.79 IU/L reference range for Holstein bred dairy cows (Stojevic *et al.* 2005) and 4.90 to 26.00 IU/L for adult cattle reported by Kahn (2005) even though it is towards the lower margin. Dubreuil and Lapierre (1997), Ottol *et al.* (1998) and Osman and Al-Busadah (2003) reported a higher GGT activity of 25.40 to 30 IU/L for lactating dairy cows in Quebec, 17.50 IU/L for Angoni cattle in Mozambique and 29.90 IU/L for cows in Saudi Arabia, respectively. Stojevic *et al.* (2005) observed statistically significant difference in mean GGT activities in dairy cows during lactation (16.28 ± 3.97 IU/L) and dry period (19.56 ± 4.09).

5.1.2 Buffalo

The research was carried out by using adult healthy Murrah buffaloes in order to assess the normal serum level of ALT, AST, ALP and GGT. According to the study results, concentrations of these enzymes observed were 50.00 ± 3.53, 130.00 ± 7.29, 323.60 ± 32.09 and 10.11 ± 1.28 IU/L, respectively. The reference range for ALT, AST, ALP and GGT was 42.02 to 57.98, 113. 51 to 146.49, 251.00 to 396.19 and 7.15 to 13.07 IU/L, respectively.

5.1.2.1 ALT

The mean values of ALT activity observed for female Murrah buffaloes in the present study was found to be 50.00 ± 3.53 IU/L with a reference range of 42.02 to 57.98 IU/L. The results support the findings of Terzano *et al.* (2005) and Grasso *et al.* (2004) who reported mean ALT values of 60 IU/L in buffalo heifers and with a range of 55.35 to 58.49 IU/L in adult female buffaloes kept at intensive

and traditional system of management. However, a higher ALT activity of 176 to 219 IU/L and 83 to 116 IU/L was observed for buffaloes at different pre-post partum time intervals and early lactation, respectively (Terzano *et al.*, 2005). A significantly lower ALT level was reported by Mudgal *et al.* (2008) who found a mean ALT level of 37.15 IU/L for 8 to 9 months old buffalo calves. Marked differences were also observed in mean serum ALT activity for adult buffaloes (Pal and Dasgupta, 2006) who reported 28.50 ± 1.32 IU/L which was significantly lower than the present findings.

5.1.2.2 AST

The 113.51 to 146.49 IU/L of AST reference range observed for adult healthy buffaloes in the present study is in close agreement the reports of Randhawa *et al.* (1997) and Grasso *et al.* (2004) and they reported a mean ALT value of 134.6 ± 4.36 IU/L for adult healthy buffaloes and 146.84 IU/L for buffalo cows maintained under intensive system of management, respectively. In contrast, a slightly increased AST value (164.68 IU/L) was observed for those under traditional system of management (Grasso *et al.*, 2004). The present findings were also comparable with the observations of Terzano *et al.* (2005) who reported 101.2 IU/L of mean AST activity for adult buffaloes even though it is towards the lower margins of the present reference range. Contrary to the results of the present study, a significantly lower AST values were reported by Pal and Dasgupta (2006) and Mudgal *et al.* (2008) who reported 54.00 ± 1.22 IU/L for adult healthy buffaloes and 62.47 IU/L for male buffalo calves, respectively.

5.1.2.3 ALP

The reference range of 251.00 to 396.19 IU/L ALP activity obtained in the present study is in close agreement with the studies of Grasso *et al.* (2004) who reported 370.11 IU/L of ALP activity in buffaloes maintained under intensive system of management, whereas a higher ALP values was observed for those

maintained under traditional system (443.12 IU/L). A similar study was conducted by Terzano *et al.* (2005) on adult healthy buffaloes and the present findings were within reference range of 200 to 650 IU/L established by them. But Randhawa *et al.* (1997) presented comparatively lower ALP values (113.9 ± 4.25 IU/L) for buffaloes. ALP activity of 76.34 IU/L suggested by Bharti *et al.* (2008) for male Murrah buffalo calves of 6 to 8 months of age was significantly lower than the present findings.

5.1.2.4 GGT

The mean GGT concentration of 10.11 ± 1.28 IU/L obtained for adult healthy buffaloes in the present study is within the range of 4.9 to 25.7 IU/L reported by Hilali *et al.* (2008). The findings of the present study are also comparable to the reports of Randhawa *et al.* (1997) who presented a GGT activity of 16.8 ± 0.82 IU/L for adult healthy buffaloes. However, the results of the present study were significantly lower than the reports of Terzano *et al.* (2005) and Grasso *et al.* (2004) and the reported GGT levels were 21.2 IU/L and 26.95 to 27.43 IU/L, respectively.

5.1.3 Goat

The values obtained for serum ALT, AST, ALP and GGT activities for adult healthy female crossbred goats were 15.94 ± 0.84, 80.87 ± 3.71, 175.92 ± 20.09 and 35.27 ± 1.73 IU/L with a confidence interval of 14.17 to 17.72, 72.89 to 88.84, 131.71 to 220.13 and 31.41 to 39.13 IU/L, respectively.

5.1.3.1 ALT

The mean ALT values of 14.17 to 17.72 IU/L obtained in this study fell within the range of 6 to 19 IU/L, established by Kaneko *et al.* (1997), 15 to 52 IU/L reported by Kahn (2005) for adult goats and 2 to 22 IU/L reported by

Daramola *et al.* (2005) for West African Dwarf goats, however, the findings of the present study was in close agreement with the range of 14 to 20.2 IU/L reported by Tibbo *et al.* (2008) for indigenous goat breeds of Ethiopia. The mean ALT values were lower in this study than those obtained for ewes in Saudi Arabia (Osman and Al-Busadah, 2003).

5.1.3.2 AST

Serum AST activity in this study were within the range of 72.89 to 88.84 IU/L which is not in accordance with that reported by Kaneko *et al.* (1997), Osman and Al-Busadah (2003), Daramola *et al.* (2005) and Tibbo *et al.* (2008). A significantly wider range of AST activity from 167 to 513 IU/L was reported for goats (Kaneko *et al.*, 1997). The findings of the present study were also lower than that of 141.6 IU/L reported by Osman and Al-Busadah (2003) for Saudi Arabian ewes. In contrast, Daramola *et al.* (2005) and Tibbo *et al.* (2008) reported a significantly lower range of AST activity for West African Dwarf goats (12 to 38 IU/L) and indigenous goat breeds of Ethiopia (43.2 to 49.3 IU/L), respectively. However, the results of the present study supported the findings of Kahn (2005), who reported an AST activity of 66 to 220 IU/L for goats, even though the values of the present study is towards the lower margin.

5.1.3.3 ALP

The ALP values obtained for female crossbred goats in the present study was 131.71 to 220.13 IU/L which was within the range of 61 to 283 IU/L established by Kahn (2005), but the values are in a narrow range in the present study. The values reported by Osman and Al-Busadah (2003) and Tibbo *et al.* (2008) showed a slightly lower ALP values for goats. The observed ALP values were 112.4 IU/L for ewes in Saudi Arabia (Osman and Al-Busadah, 2003) and 93.36 IU/L for indigenous goat breeds of Ethiopia (Tibbo *et al.*, 2008). Significantly lower ALP values of 1.4 to 25.7 IU/L were observed by Daramola *et*

al. (2005) for West African Dwarf goats. Tibbo *et al.* (2008) also reported significantly higher ALP activities in young animals (119.36 IU/L) as compared to adult (93.36 IU/L). In contradiction to the present findings Daramola *et al.* (2005) reported a significantly lower ALP values in young goats (9.91 IU/L) as compared to adult (11.7 IU/L).

5.1.3.4 GGT

The 31.41 to 39.13 IU/L of GGT activity observed in the present study fell within the range of 20 to 56 IU/L reported by Kaneko *et al.* (1997) and 20 to 50 IU/L reported by Kahn (2005) for adult goats. The findings of the present study were within a narrow range as compared to the range observed by Kaneko *et al.* (1997) and Kahn (2005).

5.1.4 Dog

The study results showed a mean serum ALT, AST, ALP and GGT activities of 33.56 ± 3.38, 35.83 ± 2.49, 92.9 ± 7.53 and 4.0 ± 0.15 with a reference range of 25.75 to 41.36, 30.35 to 41.31, 75.87 to 109.93 and 3.66 to 4.34 IU/L, respectively in adult healthy dogs of age 1.5 to 3 years, irrespective of breed and sex.

5.1.4.1 ALT

The values obtained in this study were consistent with earlier reports for tropical dogs (Kaneko *et al.*, 1997 and Kahn, 2005). The narrow reference interval of ALT activity, 25.15 to 41.36 IU/L obtained in the present study was within the reference range 21 to102 established by Kaneko *et al.* (1997) and 8.2 to 57 IU/L reported by Kahn (2005). The values obtained for ALT in the present study were higher compared to those reported for Mongrel and pure bred dogs, 3 to 20 IU/L (Lumsden *et al.*, 1979), 3.7 to10.1 IU/L for Mexican hairless dogs

(Kimura *et al.*, 1992) and 11.8 to 17.4 IU/L and 12.6 to 17.2 IU/L for Alsatian and local dog breeds, respectively (Ariyibi *et al.,* 2002). However, a higher ALT activity of 60 IU/L was reported by Caisey and king (1980). The results showed no significant difference in mean serum ALT activities between different breeds and within each breed and this were in agreement with the reports of Lumsden *et al.* (1979), Kimura *et al.* (1992) and Ariyibi *et al.* (2002).

5.1.4.2 AST

The refernce range obtained for serum AST activities in adult healthy dogs in the present study were 30.35 to 41.31 IU/L. The findings were in close agreement with 32 IU/L, reported by Caisey and King (1980). The values were within the reference range of 23 to 66 IU/L reported by Kaneko *et al.* (1997) and 8.9 to 49 IU/L reported by Kahn (2005). However, a significantly lower AST value of 7 to18 IU/L was observed for mongrel and pure bred dogs (Lumsden *et al.,* 1979), 8.2 to 10.1 IU/L for Mexican hairless dogs (Kimura *et al.,* 1992) and 4 to 21.2 IU/L for Alsatian dogs and 10.2 to 20 IU/L for local dogs (Ariyibi *et al.,* 2002). The present study showed no significant difference in the concentrations of AST between different dog breeds.

5.1.4.3 ALP

The 75.87 to 109.93 IU/L of serum ALP activity obtained for adult healthy dogs of mixed breeds belonging to an age group of 1.5 to 3 years was found to be in partial accordance with the reference range of 10.6 to 101 IU/L reported for adult dogs (Kahn, 2005). The lower limits of the present study supports the mean ALP activity of 57.6 to 76.8 IU/L reported for Alsatian dogs by Ariyibi *et al.,* (2002). According to the results of Kimura *et al.,* (1992) a significantly lower value of 24 to 42.6 IU/L was found in case of Mexican hairless dogs as compared to the present study. The results were contrast to the findings of Caisey and King (1980), who reported an ALP activity of 173 IU/L in adult dogs. Although ALP

level can be influenced by pregnancy, age and diseases, the animals in this study were apparently healthy, non pregnant and these parameters could not have been influenced by these factors.

5.1.4.4 GGT

The mean values of GGT ranging from 3.66 to 4.34 IU/L in adult healthy dogs is within the range of 1.2 to 6.4 IU/L for adult dogs reported by Kaneko *et al.* (1997) and 1 to 9.7 IU/L reported by Kahn (2005). The values were in accordance with the results of less than 10 IU/L reported for Mexican hairless dogs (Kimura *et al.,* 1992).

5.1.5 Comparison of normal hepatobiliary enzyme activities between different species

5.1.5.1 ALT

In the present study, highest serum ALT activity was observed in buffaloes (50 ± 3.53 IU/L) followed by dogs (33.56 ± 3.38 IU/L). The ALT activity obtained in cattle was comparable to that in goats and significant differences were not found in the serum ALT activity between the two species. Similar results were reported by Kaneko *et al. (*1997) who suggested a reference value of 3 to 23 IU/L and 6 to 19 IU/L for cattle and goat ALT activity which were also comparable to each other. The serum ALT activity depends upon its concentration in liver, so high serum ALT activity in dogs and buffaloes obtained in the present study indicate increased liver ALT concentration in these species. Lower ALT activity in cattle and goat serum indicates decreased liver ALT concentration in these species. Thus, from the present study it can be inferred that ALT is liver specific in dogs and buffaloes whereas nonspecific in cattle and goats for the diagnosis of hepatic damage. The findings are in agreement with those of earlier workers (Kaneko *et al.,* 1997; Ettinger and Feldman, 2000 and Latimer *et*

al., 2003) who reported ALT as a liver specific enzyme in dogs and increased serum ALT activity was observed in acute hepatocellular necrosis and inflammation in these species whereas, low liver ALT activity was reported for horses, pigs, ruminants and birds. The present results are also supported by low ALT activity reported by Bartholomew *et al.* (1987) in cattle. However, in contrast to the low liver ALT activity reported for ruminants (Kaneko *et al.,* 1997 and Latimer *et al.,* 2003), the study showed a significantly higher ALT activity in buffaloes as compared to those observed for cattle and goat. Similar findings in buffaloes were also reported by earlier workers (Terzano *et al.,* 2005 and Grasso *et al*., 2004). Therefore, ALT estimation can be recommended for diagnosis of hepatic damage in buffaloes also.

5.1.5.2 AST

Significant differences were noticed in the mean serum AST values between cattle, buffalo, goat and dog. Between the species highest AST activity was observed in buffaloes and the lowest level in dogs. Compared to cattle, the serum enzyme activity was found to be higher in goats. Increased serum AST concentration is suggestive of the use of this enzyme to diagnose hepatic disorders in all the four species studied. The findings of the present investigation are supported by the studies of Kaneko *et al.* (1997), Ettinger and Feldman (2000) and Radostits *et al.* (2000). They suggested AST as an effective marker for assessing hepatic disorders in large and small animals. Since, increased AST level in serum is also associated with muscular damage its measurement in serum alone cannot be used as a specific hepatocellular damage marker (Benjamin, 2001).

5.1.5.3 ALP

Observations made in the present study showed highly variable values for mean serum ALP activity in all the four species studied. The presence of many

isoenzymes may be the probable cause of variability. Serum ALP level was found to be within a wider range as compared to other hepatobiliary enzymes studied in the present experiment. Between species, highest ALP activity is observed in buffaloes and lowest in dogs. The results also showed no significant difference between the ALP values of dogs and cattle and they fall some what in a similar range. But the values observed in goats were significantly higher than that of cattle and dog. Laker (1996) reported physiological increase in ALP activity during active bone growth and pregnancy period and pathological increase in hepatobiliary and bone diseases. Importance of ALP in assessing cholestasis supports the findings of Schwartz (1973), Kaneko *et al.* (1997) and Radostits *et al.* (2000). Since, the animals selected for the present study were adult, non pregnant and apparently healthy, the values obtained serve as the normal reference level for future use in clinical veterinary practice for the assessment of hepatobiliary diseases in all the four species studied.

5.1.5.4 GGT

Reference levels suggested for GGT were found to be almost similar for cattle and buffalo serum. No significant difference was found between the two species. These findings were in accordance with the reports of Kaneko *et al.* (1997) and Hilali *et al.* (2008) who reported almost similar GGT reference ranges for adult cattle and buffaloes. Highest level of GGT was observed in goats whereas, lowest activity in dogs. The relatively higher GGT activity observed in cattle and goats and the lower values in dogs was supported by the findings of Braun *et al.* (1983) and Kaneko *et al.* (1997). However, the values obtained in the present investigation were under a narrow range in all the four species as compared to other hepatobiliary enzymes. The present findings suggest, GGT could be used as the most specific marker for hepatobiliary diseases in all the four species.

5.2 ASSESSMENT OF STORAGE STABILITY OF HEPATOBILIARY ENZYMES

Many investigations have been undertaken on the stability of enzymes, *in vitro*, but the results are widely divergent and most of them showed great instability during preservation. Different treatment of the blood before analysis like conditions of preservation, centrifugation, haemolysis and bacterial growth could account for the variations in the results and partially because of the varying specificities of individual methods of analysis. In veterinary medicine, to date not much studies have been published on the stability of biochemical markers especially serum enzymes. At present, as there are conflicting data regarding the effect of different temperatures and durations of storage on the stability of the activities of hepatobiliary enzymes, which are routinely analysed for clinical diagnostic use, it is of primary importance to reexamine the storage stability of these enzymes. Besides, data on this line in the hot humid tropical conditions are very meagre. Therefore, the present study is on the effects of storage time and temperature on the measured activities of the hepatobiliary enzymes like ALT, AST, ALP and GGT in the sera samples of cattle, buffalo, goat and dog under various storage conditions *viz*, at room temperature, 4 ºC and -20 ºC for a period of two weeks.

5.2.1 Cattle

5.2.1.1 ALT

According to the results, it has been observed that, ALT was sufficiently stable for a period of two weeks at 4 ºC, but with variations in the percentage of activity. In contrast, the enzyme was found to be unstable at -20 ºC, where it showed marked increase in enzyme activities through out the investigational period. A nonsignificant change was observed after 24 h storage period at -20 ºC. A similar study was reported by Davy *et al.* (1984) in marmoset plasma, where

ALT was found to be highly unstable when kept at -15 to -20 ºC for a period of 48 h and stable at 4 ºC for the same period. But in this study, the enzyme was found to be stable only for one day, at room temperature, after which a marked increase in enzyme activities was observed up to five days followed by a gradual decline in activity till the end of experimental period. These findings were in accordance with the increased ALT activity at room temperature and freezer for cattle serum (Benjamin, 2001). The present study suggest that ALT assay in cattle should be carried out in fresh sera samples to get more accurate result.

5.2.1.2 AST

The stability of AST activities in cattle serum revealed a steady decrease over the entire experimental period. Serum samples lost appreciable AST activity at room temperature, with only less than 50 % of the initial activity remaining on 14 th day and the enzyme was found to be totally unstable at room temperature. So if transportation of specimens is required, AST assay should be done within 24 h of venipuncture in case of cattle, otherwise proper storage conditions should be provided. At refrigerator and freezer, AST was seen to be highly stable up to two weeks. The results of the AST stability in freezer supports the study of Benjamin (2001) in bovine serum, who reported that AST was stable for as long as 38 days in freezer. Contrary to the findings of the present investigation, Benjamin (2001) also reported five days stability at 4 ºC and increased activity at room temperature. However, Heins *et al.* (1995) reported a decreased AST activity in human serum samples stored at room temperature. To preserve sera samples of cattle for AST estimation, the present study recommends either 4 ºC or -20 ºC.

5.2.1.3 ALP

Serum ALP activity showed great variations under the three storage conditions. Even though, significant differences were not found in ALP activities at room temperature, considering the great fluctuations in the percentage change

in initial activity, it was considered to be unstable at room temperature. Compared to -20 ºC, ALP was more stable at 4 ºC. In both the conditions, an increased ALP activity was observed with a marked increase by more than 60 % of initial activity at -20 ºC within 24 h of blood collection and up to 70 % increase after two weeks. At 4 ºC, the enzyme showed significant increase in activity from the second day of storage onwards with a maximum increase of 34.71 % after two weeks. Alkaline phosphatase in human serum demonstrates a linear increase in activity depending upon the increase in temperature and time (Kaplan and Pesce, 1989). The increased ALP activities at 4 ºC and -20 ºC might be due to occurrence of several isozymes for ALP which differ in their sensitivity to temperature or due to inter individual variations or the enzyme may became more concentrated at lower temperatures. Since, cattle serum ALP activity increased significantly with decrease in storage temperature, it is suggested that the assay should be done on the day of blood collection.

5.2.1.4 GGT

Gamma glutamyl transferase was found to be stable for a period of one day at room temperature, 11 days at 4 ºC and 8 days at -20 ºC in cattle serum. Significant increase in GGT activity was observed after one day at room temperature and after 14 days, more than 80 % increase in initial activity was noticed. The GGT activity increased significantly by about 38 % at 4 ºC after 11 days of storage. At -20 ºC, the enzyme was stable for as long as 8 days after which the enzyme showed marked increase in the activity and on 14^{th} day about 50 % increase in initial activity was observed. In human serum, GGT was reported to be stable for 2 days at room temperature, one week at 4 ºC and one month at -25 ºC (Kaplan and Pesce, 1989). In the present study, it has been found that GGT activity in cattle serum was stable up to one day at room temperature, 11 days at 4 ºC and 8 days at -20 ºC.

5.2.2 Buffalo

5.2.2.1 ALT

At room temperature serum ALT activity was unstable. It showed significant decrease up to the end of the experimental period and lost about 70 % of the initial activity after two weeks. At 4 ºC, the enzyme was considered to be quite stable for 14 days and did not result in any changes in enzyme activity that could be considered clinically significant. As compared to stability at 4 ºC, it was equally stable at -20 ºC also. From these results, it was found that ALT activity in buffalo serum decreased significantly after 24 h of venipuncture at room temperature and was stable for 14 days at 4 ºC and -20 ºC. The observations for serum ALT stability were consistent with the study of Boyanton and Blick (2000) in human plasma. They observed a 20 % decrease in ALT activity at 48 h and 56 h at room temperature and the cited reason was due to the increased lactate concentration as a result of bacterial contamination. This study recommends either 4 ºC or -20 ºC for preservation of buffalo sera samples for ALT assay.

5.2.2.2 AST

At room temperature, AST was stable for as long as 8 days but after which the activity decreased to a point of significance at 11^{th} and 14^{th} day of storage, with about 60 % of the original activity on 14^{th} day. The enzyme was more stable at 4 ºC when compared to -20 ºC. There was no significant change in AST activity in serum when stored at 4 ºC for up to 11 days whereas, samples at -20 ºC revealed a storage stability of only 2 days after which a statistically significant decline in activity was observed up to 14^{th} day in both conditions. The AST activity under various storage conditions suggested for human serum was 3 days at room temperature, one week at 4 ºC and one month -25 ºC (Kaplan and Pesce, 1989). Due to significant decrease in AST activity at -20 ºC, the present study suggests 4 ºC as the better storage condition for buffalo sera samples.

5.2.2.3 ALP

In the present study, ALP was found to be highly unstable under the three storage conditions *viz*, room temperature, 4 ºC and -20 ºC. The samples showed a stability period of one day at room temperature and were unstable after storage at 4 ºC and -20 ºC from 1st day onwards. A significant decline to below base line values was observed in all the storage conditions. These results were contradictory to the reports of Kaplan and Pesce (1989) in human sera samples where ALP activity increased with increase in temperature. The present results suggest the instability of buffalo ALP enzyme during preservation of sera samples 4 ºC and -20 ºC and the assay should be performed on the day of blood collection.

5.2.2.4 GGT

Gamma glutamyl transferase showed no appreciable change in activity over a period of two weeks under both refrigerator and frozen state. About 80 % of the initial activity was retained in both storage conditions up to the end of the experiment. The results were in accordance with the study of Donnley *et al.* (1995) on human serum and they stated that GGT to be highly stable at 4 ºC (14 days) and -20 ºC (4 months). They reported a stability of 48 h at room temperature, which in the present study was 24 h. The increase in serum GGT activity at room temperature may be due to bacterial contamination. A similar finding was reported by Lazaroni *et al.* (1958) who stated that, bacterial contamination can cause either an increase or decrease in the enzyme activity in human serum maintained at room temperature. The present study suggests -20 ºC as the most suitable storage condition for GGT assay in buffalo sera samples.

5.2.3 Goat

5.2.3.1 ALT

Alanine aminotransferase activity in the present study was found to be highly stable at room temperature and -20 ºC through out the study period as compared to 4 ºC. Even though there are differences in the percentage change in initial activity between the two storage conditions, a statistically significant difference was not found between both conditions. However, the stability of ALT at -20 ºC observed in the present study were contrary to the findings of Davy *et al.* (1984) who observed ALT to be highly unstable in marmoset plasma at -20 ºC. This reveals species specific differences in the stability characteristics of ALT. At 4 ºC, the enzyme was stable up to 8 days of preservation and a marked significant increase in ALT activity was noticed thereafter. A similar result was observed in case of camel serum stored at 4 to 5 ºC (Saeed *et al.,* 1995). The results suggest -20 ºC as the ideal storage condition for the preservation of goat sera samples for ALT assay.

5.2.3.2 AST

In goat serum, AST activity was found to be stable for a period of 2 days at room temperature, 11 days at 4 ºC and 14 days at -20 ºC. At room temperature, the samples showed only less than 1 % change in activity up to 2 days post collection and a gradual decline in activity was found from 5^{th} to the end of the experiment. The decline in activity may be due to increased degradation of enzyme active site with increased temperature. These observations were consistent with similar studies conducted by Lazaroni *et al.* (1958); Heins *et al.* (1995) and Saeed *et al.* (1995) on human, marmoset and camel serum, respectively. At 4 ºC the enzyme was found to be sufficiently stable for a period of 11 days and the activity increased significantly by about 20 % after 11 days. In serum samples stored at -20 ºC, AST activities were sufficiently stable through out the study period of two weeks. So, -20 ºC is recommended for the preservation of goat sera samples for AST assay.

5.2.3.3 ALP

The stability of ALP in goat serum also showed wide variation over the storage period. At room temperature the enzyme did not show any statistically significant difference in activity after 24 h of storage. So the enzyme was considered to be totally unstable at this storage condition. Alkaline phosphatase activities were not influenced by storage at 4 ºC up to two days after which, the values showed an increase in activity which was significant after 8 days. However, at -20 ºC, ALP showed decrease in activity and a statistically significant decrease was noticed from 8th day onwards. So it is revealed that ALP was quite unstable at room temperature where it lost activity within 24 h of blood collection. At 4 ºC and -20 ºC, the enzyme can not be preserved for more than 5 days.

5.2.3.4 GGT

Gamma glutamyltransferase in goat serum was found to be highly stable at room temperature, 4 ºC and -20 ºC for as long as two weeks. The changes were only negligible under the three storage conditions which are insignificant. The results were in accordance with the study of Donnley *et al.* (1995) on human serum and they stated that GGT to be highly stable at 4 ºC for 2 weeks and up to 4 months at -20 ºC.

5.2.4 Dog

5.2.4.1 ALT

In case of dog serum, ALT showed a stability of 14 days at refrigerator, one day at -20 ºC and unstable at room temperature. At room temperature and -20 ºC the enzyme showed significant increase in activity which affected the stability of enzyme markedly whereas, at 4 ºC, more than 90 % of the original activity retained after two weeks. These findings were supported by the report of

Kaplan and Pesce (1989) in human sera samples, suggested that ALT was unstable at -20 ºC, stable for 5 days at 4 ºC and 2 days at room temperature. The present study suggests, 4 ºC as the ideal storage condition for preserving dog sera samples meant for ALT assay as compared to room temperature and -20 ºC.

5.2.4.2 AST

In the present study, a significant increase in AST activity was noticed at room temperature after 24 h of storage and the enzyme was found to be totally unstable at this condition. However, the enzyme was found to be highly stable at 4 ºC and -20 ºC up to 14 days and only negligible variations were noticed in these samples. In human sera samples Kaplan and Pesce (1989) reported AST stability for a period of one month at -25 ºC. In the present study both refrigerator and freezer could be considered as the suitable storage conditions for AST assay in dog serum.

5.2.4.3 ALP

During the entire experimental period ALP activity showed significant variation at room temperature. At room temperature, refrigerator and freezer the enzyme showed a general tendency of decrease in activity but the variations were more prominent in sera samples stored at room temperature. The samples at 4 ºC showed decrease in activity after one day and after 5 days for samples at -20 ºC. From the present study, it has been found that -20 ºC as the suitable storage condition for dog sera samples for ALP estimation as compared to refrigerator even though prolonged storage is not recommended.

5.2.4.4 GGT

The enzyme was found to be highly stable at room temperature, 4 ºC and -20 ºC during the entire experimental period and the changes were only less than

12 %. Kaplan and Pesce (1989) reported a stability of one week at 4 ºC and one month at -25 ºC for the GGT activity in human sera.

5.3 COMPARISON OF STORAGE STABILITY OF HEPATOBILIARY ENZYME ACTIVITIES BETWEEN DIFFERENT SPECIES

5.3.1 ALT

Storage stability of ALT was different for the four species studied. From the results of the present study it was found that, at room temperature serum ALT activity was more stable in goat as compared to cattle, buffalo and dog. The enzyme was found to be stable for a period of 14 days whereas that of cattle, serum was one day and buffalo and dog serum was unstable at room temperature. In goat serum there may be some components that may protect the enzyme activity. The findings were supported by the stability of ALT for 6 days at room temperature for camel serum (Saeed *et al.,* 1995). Contrary to the findings of Saeed *et al.* (1995), earlier studies on human serum reported ALT to be highly unstable at room temperature (Jull *et al.,* 1967 and Ono *et al.,* 1981) which is in agreement with the present findings in cattle, buffalo and dog serum.

Compared to room temperature, ALT was less stable at 4 ºC in goat serum and it was found to be stable for only 8 days at refrigerator. In contrast, all other species showed marked stability at the same condition. A result which is similar to ALT activity in goat serum was reported by Jull *et al.* (1967) and Saeed *et al.* (1995) and they observed stability for 8 days for human serum and 7 days for camel serum, respectively at 4 ºC. A 48 h storage stability at 4 ºC was observed in human serum (Ono *et al.*, 1981) and marmoset serum (Davy *et al.,* 1984).

Results of the stability study at -20 ºC revealed that ALT to be stable up to 14 days for buffalo and goat serum whereas it was unstable for cattle and dog serum. The maximum stability observed for dog serum was one day at -20 ºC.

These findings were in agreement with the following studies, Jull *et al.* (1967) and Tornquist *et al.* (2000) who reported 8 and 7 days, respectively for human ALT activity at -20 ºC. Hartman *et al.* (1981) reported that ALT to be stable for 55 weeks at -15 to -20 ºC in ethyl glycol stabilized serum. Contrary to the findings, Davy *et al.* (1984) reported ALT to be highly unstable at -15 to -30 ºC in marmoset plasma, which is similar to our findings in cattle and dog.

5.3.2 AST

The enzyme was comparatively more stable at refrigerator for up to two weeks in all the species studied except for goat and buffalo where a stability of 11 days was observed. At -20 ºC the enzyme was stable up to two weeks in cattle, goat and dog serum whereas only 2 days for buffalo. The enzyme was unstable at room temperature for all the species studied except buffalo serum where it remained stable up to 8 days. The results of the present study suggest 4 ºC and -20 ºC as the ideal storage condition for estimating AST activity in cattle and dog serum. For goat serum, it was found to be more stable at -20 ºC (14 days) than 4 ºC (11 days) recommending -20 ºC as the suitable storage condition for this species as compared to refrigerator whereas, in buffalo, the enzyme was found to be more stable at refrigerator (11 days) than at freezer (2 days). Since, AST activity showed variation after 2 days, a prolonged storage of buffalo serum samples in freezer for evaluating AST activity is not recommended. Even though, buffalo serum samples showed a greater stability of 8 days at room temperature it can not be considered as a suitable storage condition.

Heins *et al.* (1995) reported a decreased AST stability at room temperature in case of human serum samples which is similar to our findings in cattle, goat and dog sera samples kept at room temperature. Saeed *et al.* (1995) also supported these findings and reported a stability of 3 days for camel serum kept at room temperature. Comparatively higher AST stability was observed in human (Jull *et al.,* 1967) and camel serum (Saeed *et al.,* 1995) at 4 ºC and the reported

AST stability were 8 days and 9 days, respectively. The results of the present study is also supported by studies of Jull *et al.* (1967) and Hartman *et al.* (1987) in human serum and they reported a greater stability of AST at -15 to -20 ºC as compared to room temperature and 4 ºC.

5.3.3 ALP

In the present study, ALP showed wide variation upon storage. Significant differences were found in the storage stability between different species and different storage conditions. A more likely explanation was the presence of isozymes, small sample size, inter individual variation, as well as inter assay and intra assay variability. No specific storage condition could be suggested for ALP assay. However, in the present study the enzyme showed a stability period of 14 days for cattle serum kept at room temperature. In buffaloes, the enzyme was stable up to one day and unstable for goat and dog serum at the same temperature. Even though, cattle serum kept at room temperature showed nonsignificant changes up to 14 days, the enzyme was considered to be unstable at this temperature because of the greater variations in the percentage of original activity retained over the entire experimental period.

At 4 ºC, goat serum showed comparatively greater stability for ALP activity (8 days) whereas, it was one day for dog and was unstable for cattle and buffalo sera. Compared to 4 ºC, the enzyme was found to be less stable at -20 ºC. A maximum stability of 5 days was noticed in case of goat and dog serum maintained at -20 ºC. For cattle and buffalo serum kept at -20 ºC, the enzyme was found to unstable within 24 h of storage. The over all results of the present study recommends ALP assay on the day of venipuncture itself in all the species studied. The results of the present study were supported by studies on marmoset plasma (Davy *et al.*, 1984) who also reported wide variation in serum ALP activity upon storage. Jull *et al.* (1967) in their studies on human serum also found ALP to be highly unstable and they reported a maximum of one week

stability for ALP regardless of temperature. Isozyme proportion of ALP will vary according to the health status of the animals and individual isozymes may not react identically (Davy *et al.*, 1984). Saeed *et al.* (1995) in their studies on camel serum, observed a stability of 7 days for ALP at 4 to 5 ºC which supports our findings in goat serum maintained at 4 ºC (8 days). At room temperature, the enzyme was stable up to 8 days in camel serum (Saeed *et al.*, 1995) which is also similar to the findings of ALP activity for cattle serum in the present study.

5.3.4 GGT

Among the hepatobiliary enzymes studied GGT was found to be highly stable over the experimental period. However, Species specific differences were observed regarding the storage stability. At room temperature the enzyme was stable during the entire experimental period for goat and dog. But for cattle and buffalo serum, the enzyme showed marked variation in stability within one day.

At 4 ºC, the enzyme was found to be stable for 14 days in buffalo, goat and dog whereas it was 11 days for cattle. At -20 ºC, the enzyme was comparatively more stable in all the species studied except cattle. In cattle, the enzyme was found to be stable only for 8 days. According to the present results, room temperature, 4 ºC and -20 ºC could be considered as the suitable storage condition for GGT assay in goat and dog whereas, in buffalo 4 ºC and -20 ºC is found to be suitable. For cattle serum, refrigerator is recommended to be more suitable as compared to -20 ºC. The greater stability of GGT upon storage was supported by the findings of Donnely *et al.* (1995) who reported a stability of 48 h at room temperature, 14 days at 4 ºC and 4 months at -20 ºC for human serum samples. The comparatively lower GGT stability in cattle serum at room temperature in the present study was supported by the studies of Ehsani *et al.* (2008) who observed a stability of 24 h for GGT in bovine serum samples kept at room temperature. Davy *et al.* (1984) also suggested lesser stability of GGT at room temperature as compared to 4 ºC and -20 ºC for marmoset plasma.

Summary

6. SUMMARY

The present study was undertaken with the objectives of estimating the normal serum activities of hepatobiliary enzymes such as, ALT, AST, ALP and GGT for ruminants and dogs reared under the hot humid climatic conditions and also to identify the ideal storage condition for each enzyme in these species by keeping sera samples at room temperature, 4 ºC and -20 ºC up to 14 days. The study was carried out during the period of April to October 2008 in adult healthy female crossbred cattle, Murrah buffalo and crossbred goats maintained at University Livestock Farms, College of Veterinary and Animal sciences, Mannuthy. The dogs selected were of mixed breeds maintained at nearby kennels.

The enzyme activity was analysed in fresh sera samples as per the standard procedures. The research findings showed comparatively lower ALT activities in cattle and goat serum and the observed mean ALT values were 19.46 ± 1.54 and 15.94 ± 0.84 IU/L, with a range of 16.11 to 22.81 and 14.17 to 17.72 IU/L, respectively. There was no significant difference in ALT activities between these two species. The highest ALT activity was found in buffalo (50.00 ± 3.53 IU/L) followed by dog (33.56 ± 3.38 IU/L) with a range of 42.02 to 57.98 and 25.15 to 41.36, respectively. Among the four species studied, due to significantly higher ALT activity in buffalo and dog serum, the enzyme is considered to be more suitable for assessing hepatocellular damage in these two species as compared to cattle and goat.

All the four species studied showed significant differences in their mean AST activities. The enzyme showed significantly higher activity for buffalo serum (113.53 to 146.49 IU/L) and lowest values was observed for dogs (30.53 to 41.31 IU/L) with a mean value of 130.00 ± 7.29 and 35.83 ± 2.49, respectively. The AST concentration observed for cattle and goat sera samples were 68.67 ± 2.29 IU/L (63.61 to 73.72) and 80.87 ± 3.71 IU/L (72.89 to 88.84), respectively. Even though,

AST level may increase in muscle damage, it is also be considered as a marker of hepatocellular damage in large and small animals because of its high concentration in liver cells.

Among the hepatobiliary enzymes assessed the activity of ALP showed marked variation between different species and also within a species. The results were comparatively in a wider range for all the four species studied as compared to other hepatobiliary enzymes. The highest ALP activity was found in buffaloes with a mean value of 323.6 ± 32.09 IU/L ranging from 251.00 to 396.19 IU/L. The ALP activities for cattle and dog were 113.7 ± 7.59 IU/L (96.53 to 130.87) and 92.9 ± 7.53 IU/L (75.87 to 109.93) and no significant difference was found in these values between the two species. But ALP values obtained for goat was 175.92 ± 20.09 IU/L with a range of 131.71 to 220.13 IU/L. The study suggests ALP to be a sensitive marker for detecting hepatobiliary disease in all the four species studied even though, its levels may be affected by physiological changes like growing period, pregnancy and pathological changes like bone diseases in addition to cholestasis.

Gamma glutamyl transferase was found to be highly liver specific and its levels were within a narrow range in all the four species studied. The highest serum GGT activity was found in goats (32.57 ± 1.73 IU/L) and the lowest value was observed in dogs (4.0 ± 0.15 IU/L) with a range of 31.41 to 39.13 and 3.66 to 4.34, respectively. The serum GGT activity of cattle and buffalo were found to be some what similar (13.15 ± 0.78 and 10.11 ± 1.28 IU/L, respectively) with a range of 11.45 to 14.86 and 7.15 to 13.07, respectively and no significant differences were noticed between these two species. Even though, GGT is found in liver and kidney, the serum enzyme activity is particularly of bile duct origin, so the enzyme is recommended as the most specific marker of cholestasis in all the four species studied when compared to ALP.

The study on the storage stability of enzymes reveals significant differences between the storage stability of each enzyme in each species at room temperature, 4 ºC and -20 ºC. According to the results, it was found that ALT to be sufficiently stable up to the study period of 14 days at 4 ºC in case of cattle, buffalo and dog serum where as that of goat serum was 8 days. But at -20 ºC and room temperature, goat serum showed a marked stability for as long as 14 days. In contrast, cattle, buffalo and dog serum was unstable for ALT activity after preservation at room temperature. The enzyme was also unstable at -20 ºC in case of cattle and dog serum where as buffalo serum showed 14 days stability.

The serum AST activity was found to be comparatively more stable at 4 ºC and -20 ºC in all the species studied. The enzyme was stable up to a period of 14 days in cattle and dog serum and 11 days for buffalo and goat serum samples stored at 4 ºC. At -20 ºC, AST activity was found to be sufficiently stable through out the study period in all the species except buffaloes where the enzyme was stable only 2 days. At room temperature, AST activity showed comparatively more stability for buffalo serum, with a stability of 8 days, whereas, that of cattle and goat samples was 1 and 2 days, respectively and dog serum was unstable.

Alkaline phosphatase showed highest variation upon storage in all the four species as compared to other hepatobiliary enzymes. So the enzyme was found to be comparatively unstable in all the storage condition. A maximum stability of 8 days was observed for goat serum stored at 4 ºC. At freezer, goat and dog sera samples showed a stability period of 5 days whereas, in cattle and buffalo ALP activity showed significant variation on the first day of storage itself.

Among the four hepatobiliary enzymes studied, GGT was found to be more stable through out the study period under the three storage conditions. Buffalo, goat and dog sera samples showed comparatively higher GGT stability up to 14 days at both 4 ºC and -20 ºC whereas, cattle serum showed a lesser stability for GGT activity at the same temperature and the observed stability period was 11 days at 4 ºC and 8 days at -20 ºC. The present investigation also showed marked GGT stability at room temperature up to the end of the study period for goat and dog serum samples.

Thus the present study reveals specific reference values for each serum hepatobiliary enzyme in cattle, buffalo, goat and dogs of hot humid tropics. The study also suggests variations in the storage stability of these enzymes between the four species and the following conditions are recommended for the preservation of sera samples. It is suggested that ALP estimation should be performed in fresh serum samples in all the four species studied. In cattle, the ideal condition recommended for serum preservation for ALT and GGT assay are at 4 ºC and for AST estimation either -20 ºC or 4 ºC. For buffalo, the serum can be preserved at 4 ºC or -20 ºC for ALT and GGT assay and for AST, the ideal condition is at 4 ºC. The suitable condition of storage of goat serum for ALT, AST and GGT estimation is at -20 ºC and for GGT it can also be preserved at 4 ºC. The dog serum can be preserved at 4 ºC or -20 ºC for AST and GGT assay and for ALT, the suitable condition is 4 ºC. Preservation at -20 ºC is not recommended for sera samples of cattle and dog for the ALT assay and also for AST assay in buffalo. Room temperature is not recommended for preserving sera samples for enzyme assay in all the four species except for GGT estimation in goat and dog serum where the enzyme was found to be stable up to the end of the study period. It is therefore advisable to consider stability of serum hepatobiliary enzyme activity of each species separately for a more valid and reliable result.

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COMPARATIVE STUDY AND STORAGE STABILITY OF HEPATOBILIARY ENZYMES IN RUMINANTS AND DOGS OF HUMID-TROPICS

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ABSTRACT

The present research was designed and conducted with the objectives of assessing the normal serum activity of hepatobiliary enzymes such as, ALT, AST, ALP and GGT for different animal species reared under the hot humid climatic conditions and to identify the ideal storage condition for each enzyme in these species by keeping sera samples at room temperature, 4 ºC and -20 ºC up to 14 days. The study was performed in adult healthy female crossbred cattle, Murrah buffalo and crossbred goats maintained at University Livestock Farms, College of Veterinary and Animal sciences, Mannuthy and dogs of mixed breeds maintained at nearby kennels during the period of April to October 2008.

The research findings showed highest ALT activity in buffalo (50.00 ± 3.53 IU/L) followed by dog (33.56 ± 3.38 IU/L) and comparatively lower ALT activity in cattle and goat serum where the values were 19.46 ± 1.54 and 15.94 ± 0.84 IU/L, respectively. The reference range obtained for ALT activity in cattle, buffalo, goat and dog were of 16.11 to 22.81, 42.02 to 57.98, 14.17 to 17.72 and 25.15 to 41.36 IU/L, respectively. These results recommend the use of ALT assay particularly in dogs and buffalo for the diagnosis of hepatic damage. All the four species showed significant differences in the mean AST activities with highest activity for buffalo serum (113.53 to 146.49 IU/L) and lowest for dog (30.53 to 41.31 IU/L) with a reference range of 113.51 to 146.49 and 30.35 to 41.31 IU/L, respectively. For cattle and goat sera samples, the AST activity observed were 68.67 ± 2.29 IU/L (63.61 to 73.72) and 80.87 ± 3.71 IU/L (72.89 to 88.84), respectively.

The analysis showed comparatively wider range for ALP activity in all the four species studied as referred to other hepatobiliary enzymes. The highest ALP activity was found in buffalo (323.60 ± 32.09 IU/L) ranging from 251.00 to 396.19 IU/L. The mean serum ALP activities for cattle and dog were 113.70 ± 7.59 IU/L

(96.53 to 130.87) and 92.90 ± 7.53 IU/L (75.87 to 109.93) and were non significant. The ALP values obtained for goat was 175.92 ± 20.09 IU/L (131.71 to 220.13 IU/L). GGT levels obtained in the present study were within a narrow range in all the animals studied. The highest serum GGT activity was found in goats (32.57 ± 1.73 IU/L) and the lowest value in dogs (4.00 ± 0.15 IU/L) with a range of 31.41 to 39.13 and 3.66 to 4.34, respectively. The serum GGT activity of cattle and buffalo were found to be some what similar (13.15 ± 0.78 and 10.11 ± 1.28 IU/L, respectively). The corresponding reference ranges were 11.45 to 14.86 and 7.15 to 13.07, respectively. The results suggest GGT as the most suitable hepatobiliary enzyme than ALP to detect cholestasis in all the four species studied.

The present study also reveals significant differences in the storage stability characteristics of the hepatobiliary enzyme among the four species. The investigation recommends 4 ºC as the ideal storage condition for ALT assay for cattle and dog serum samples, whereas, -20 ºC for goat serum and both conditions for buffalo serum. Both 4 ºC and -20 ºC was suitable for AST assay for cattle and dog serum, -20 ºC is recommended for goat whereas, buffalo serum showed maximum AST stability at 4 ºC. The present study suggests that ALP assay should be performed on the day of venipuncture itself in all four species studied as the enzyme showed wide variation upon storage. Both 4 ºC and -20 ºC was found to be as the ideal storage conditions for GGT assay in buffalo, goat and dog sera samples whereas, 4 ºC is recommended for cattle serum where the observed stability period was 11 days. Room temperature is not recommended for preserving sera samples for enzyme assay in all the four species except for GGT estimation in goat and dog serum where the enzyme was found to be stable up to the end of the study period. These differences in enzyme stability should be considered while preserving sera samples to get an accurate result.