J Anim Diagn Med (2011) 1: 7-14
Research Article

Exponential Increases in Serum Liver Enzymes in Fulminant Rabbit Hemorrhagic Disease

Sheng-Yi Chen[1], Chi-Chung Chou[1,2], Ming-Tsung Lai[3], Jui-Hung Shien[1,2], and Chau-Loong Tsang [1,2]*

[1]Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan
[2]Veterinary Teaching Hospital, College of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan
[3]Department of Medicine, China Medical University, Taichung, Taiwan

Received: January 21, 2011 / Revised: March 24, 2011 / Accepted: April 27, 2011

ABSTRACT

The kinetics of serum liver enzymes in New Zealand White rabbits experimentally infected with rabbit hemorrhagic disease virus (RHDV) were compared to establish monitoring criteria for infected rabbits. Blood samples of rabbits individually inoculated with one 50% rabbit lethal dose RHDV, were collected at 0 hour post inoculation (HPI), 18 HPI and every 6 hours until 60 HPI. After RHDV inoculation, highly significant elevations in serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase, γ-glutamyltransferase and alkaline phosphatase ($p<0.0001$) were noted. All the infected rabbits showed AST values over 44.8 IU/L at 24 HPI and ALT values over 86.9 IU/L at 30 HPI, respectively. An elevation of AST in individual paired serum sampling of an interval of over 18 hours from inoculation could also be used to indicate the possibility of RHDV infection. Although the creatine kinase elevated significantly ($p=0.0023$), but most of the values were under 2000 IU/L, and its' change was not correlated with the 5 mentioned enzymes above. Thus skeletal muscle damage induced ALT and AST elevations could be ruled out. Histopathological findings of the liver from the dead rabbits correlated well chronologically with the release of the liver enzymes into the serum. Our results suggested that exponential increases in AST and ALT would be a strong prediction of the fulminant consequence in RHDV infection and the profound changes in serum liver enzymes, particularly AST and ALT, may be used as useful parameters in monitoring the progression of RHDV infection. This is the first demonstration that compared and described the increasing patterns of 6 liver related serum enzymes in fulminant RHDV infection. © JADM 2009. All rights reserved.

Keywords: *Alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, lactate dehydrogenase, γ-glutamyltransferase, rabbit hemorrhagic disease*

INTRODUCTION

Rabbit hemorrhagic disease (RHD) was first reported in China in 1984 (Liu et al., 1984) and had spread worldwide since then. This lagomorph disease, caused by the highly contagious Calicivirus (Ohlinger et al., 1990), is designated by the World Organization for Animal Health (OIE) as a reported disease. It is considered to be of socio-economic importance in the international trade of rabbits and its products. This disease was first reported in Taiwan in 1994 by Lu et al. (cited in Shien et al., 1998). Importations of domestic rabbits or contaminated rabbit foods were presumed to be responsible for the introduction of RHD into Taiwan. Therefore, the development of a rapid, effective monitoring test is important for quarantine purposes.

RHD infection has been characterized as an acute fatal necrotic viral hepatitis with mortality over 85% in adult rabbits (Tunon et al., 2003). The vial particle has been characterized by viral protein analysis and RT-PCR (Ohlinger et al., 1990; Shien et al., 2000), electron microscopy (Shien et al., 1998) and hemagglutination test (Shien et al., 1998; Mizoguchi et al., 2003). The viral particles, single strand and non-enveloped RNA virus (Ohlinger et al., 1990), were mainly accumulated in the

* Corresponding author: Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University, 250 Kuo Kuang Road, Taichung 40228, Taiwan
E-mail address: cltsang@mail.vm.nchu.edu.tw

membrane-bound cisternae and scattered around the vacuoles in the cytoplasm of many necrotic hepatocytes (Park et al., 1992). Hence, tests of serum liver enzymes could be efficiently used for monitoring fulminant RHD. In addition, younger rabbits usually survive and become RHD virus (RHDV) carriers (Xu et al., 1989). RHD infections in these rabbits are sub-clinic. Therefore, the application of tests of serum liver enzymes would be of great benefit in monitoring the inner progression in younger rabbits. In this study, we experimentally infected New Zealand White rabbits with RHDV to obtain and compare the profiles in 6 serum liver enzymes and correlating them with the histopathological changes at different period after inoculation to describe the increase pattern in fulminant RHD and choose better suitable parameters for monitoring the progression of RHD in rabbits.

MATERIALS AND METHODS

Animals

Twenty (10 males and 10 females) 15 week-old clinically healthy New Zealand White rabbits, weighing 2.69± 0.16 Kg, were obtained from National Animal Health Research Institute in Taiwan. The rabbits were kept isolated in sanitized wired cages and sepa- rated by sex. All were fed commercial dry rabbit feed (Fwu-Sow Industry Co., Taichung, Taiwan) containing 19% crude protein, 10% crude fiber and 2500 Kcal/kg. None of the rabbits were previously vaccinated with RHD-killed vaccine. The rabbits were kept and handled according to the regulations laid down by Institutional Animal Care and Use Committee of National Chung-Hsing University.

Virus and inoculation

The RHDV was produced from homogenized liver specimens of a field case. After isolation (Shien et al., 1998) and virus titration, the experimental subjects were then intramuscularly inoculated with 1 rabbit LD_{50} in 0.5 ml RHDV suspension.

Pathologic examination

Six infected rabbits were randomly selected rabbits to be sacrificed for liver pathological examination, 2 at 18 hours post inoculation (HPI), 2 at 24 HPI, 1 at 30 HPI and 36 HPI, respectively. All liver specimens of the sacrificed and died rabbits were fixed in 10% buffered formalin for 24 hours, dehydrated and embedded in paraffin. Sections of 4-μm thin were cut and stained with hematoxylin and eosin for light microscopic examination.

Blood sample collection

About 2.0 ml of blood from auricular vein was obtained from rabbits after 8 hours of fasting as base line (0 HPI). After virus inoculation, blood samples were collected from 18 HPI and every 6 hours repeatedly until the animal died or been sacrificed. Blood samples collected in syringes without anticoagulant were left at room temperature for 30 minutes and then at 4℃ for 30 minutes for serum collection. The crude sera were then centrifuged at 1000 x g for 10 minutes and the sera were used for serum biochemistry tests.

Serum tests

The Liver related serum enzymes including aspartate aminotransferase (AST), alanine amino- transferase (ALT), lactate dehydrogenase (LDH), γ-glutamyltransferase (γ-GT) and alkaline phosphatase (ALP) were measured. Since the rabbits died after muscular convulsions and coma, creatine kinase (CPK) was also measured. Serum concentrations of the above parameters were measured with an autoanalyzer (model 7150, Hitachi Co., Japan) using spectrophotometic method.

Data analysis

Data from both sexes at different HPI were analyzed by Statistic Analysis System (SAS 9.1). The means and standard deviations were calculated by Means Procedure on sex and time. Least squares means of General Linear Model (GLM) procedure were applied for the analysis of the differences on the effect of time and sex. P values less than 0.05 were considered significant and <0.01 were considered highly significant. The analysis for frequency distribution was applied for the interpretation of the inner changing tendency. The strength of the relationship that exists between the parameters were defined by the statistics of the Pearson correlation coefficient (r) and its' significance by Correlation Procedure. For interpreting the strength of correlation, R squared, which can be interpreted as the proportion of variation in the variables, were applied for the strength of correlation. The simple, quadratic and logistic linear regression procedures were applied for regression analysis.

RESULTS

Pathologic findings

Most of the rabbits died between 26 and 45 HPI with a total mortality of 79 % (11/14). Gross and histological changes in liver were corresponded well with the elevation in values of AST and ALT (Fig. 1). The more extensive the hepatic lesion was, the higher the enzyme value reached. Rabbits with high AST and ALT values showed gross and microscopic focal spotty coagulated hepatic necrosis in the liver (Fig. 1A) at 18 HPI. Hepatic necrosis predominantly spread from the portal trial,

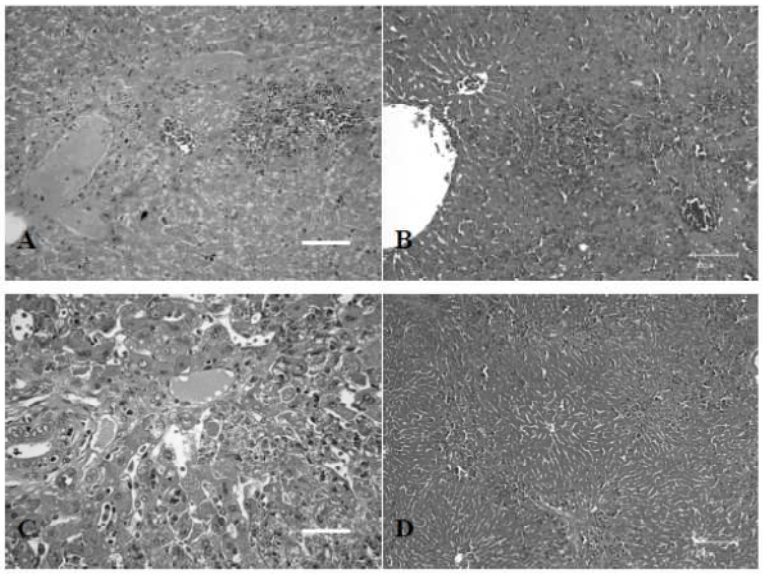

Fig. 1 Changes of the microscopic findings on liver specimens at different hours post inoculation. (A) Spotty necrosis. Liver specimen of rabbit that sacrificed at 18 HPI with AST=29.5 IU/L and ALT=56.3 IU/L. 200X. H&E stain. Bar=50μm. (B) Portal area necrosis. Liver specimen of rabbit that sacrificed at 30 HPI with AST=769 IU/L and ALT=829.1 IU/L. 100X. H&E stain. Bar=100μm. (C) Portal area necrosis. Liver specimen of rabbit that sacrificed at 24 HPI with AST=766 IU/L and ALT=1093.2 IU/L. 400X. H&E stain. Bar=25μm. (D) Fibrosis. Liver specimen of rabbit that died at 45HPI with AST=1671 IU/L and ALT=1105 IU/L. 50X. H&E stain. Bar=200μm.

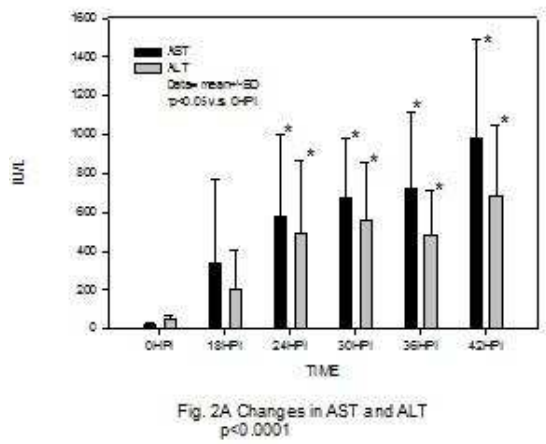

Fig. 2A Changes in AST and ALT
p<0.0001

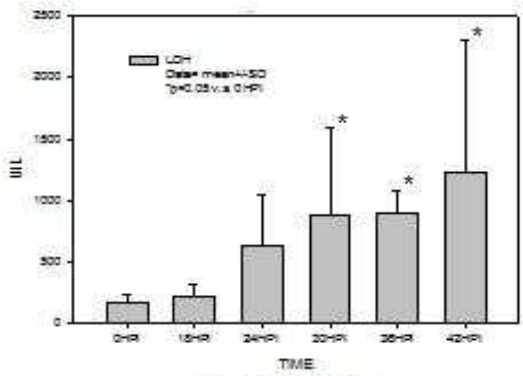

Fig. 2B Change in LDH
p=0.0012

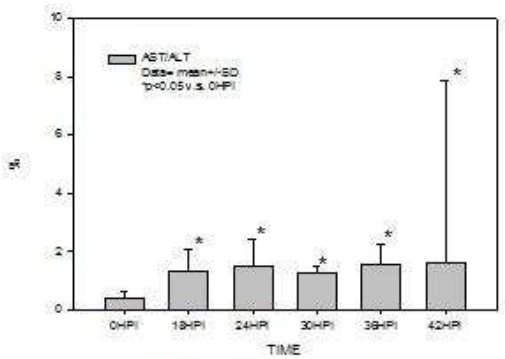

Fig. 2C Change in AST/ALT ratio
p<0.0001

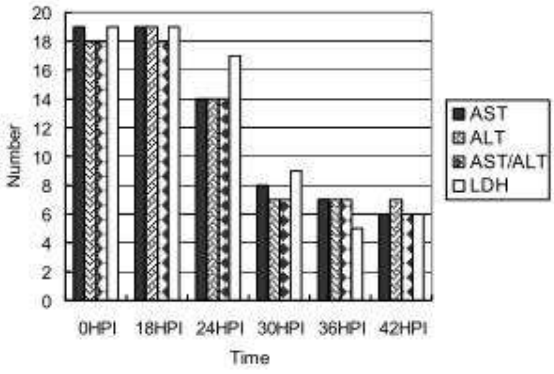

Fig. 2D The sample size (N) of serum AST, ALT, AST/ALT and LDH at different period of time in RHD.

Fig. 2 Changes in serum AST, ALT, AST/ALT and LDH in RHD at different period of time.

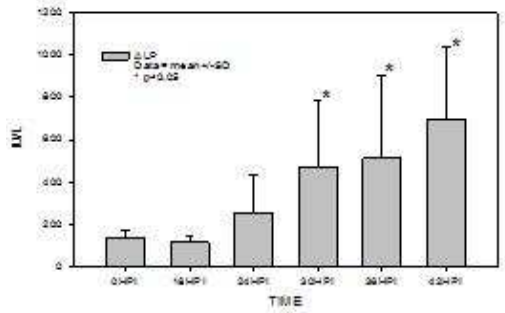

Fig. 3A Change in ALP
p<0.0001

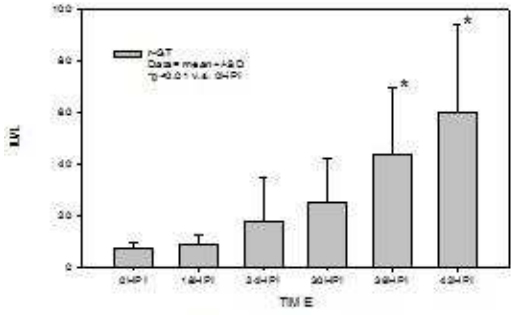

Fig. 3B Change in r-GT
p<0.0001

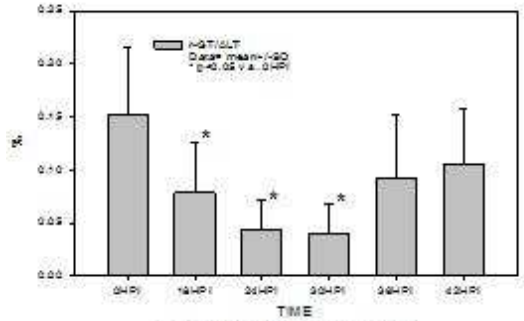

Fig. 3C Change in r-GT/ ALT ratio
p=0.036

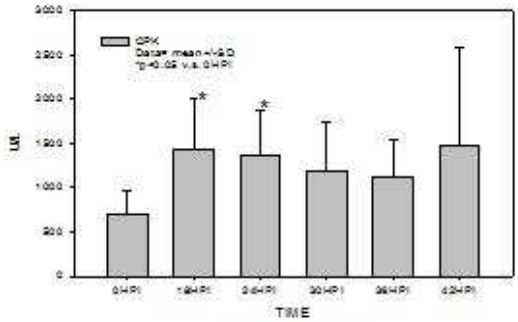

Fig. 3D Change in CPK
p=0.0023

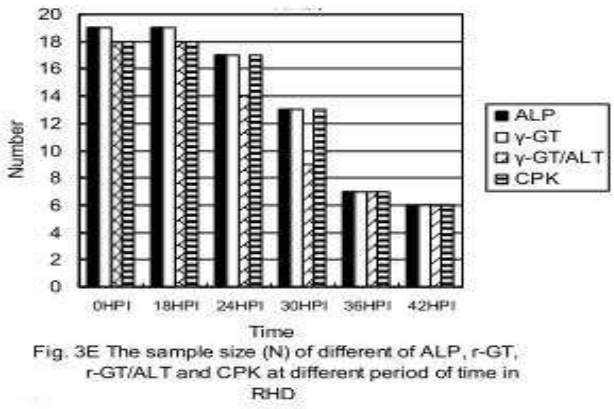

Fig. 3E The sample size (N) of different of ALP, r-GT, r-GT/ALT and CPK at different period of time in RHD

Fig. 3 Changes of serum ALP, γ-GT, γ-GT/ALT and CPK in RHD at different period of time.

while massive or sub-massive hepatic necrosis was seen in all liver areas, mainly at the periphery of the lobules (Fig. 1B-C). Pan-necrosis was observed in one of the rabbit that died at 37 HPI. Following the disease progression, fibrosis appeared at 45 HPI (Fig. 1D).

Serum Parameter Examination

Because 79% of the rabbits died within 45 hours, the number of the rabbits still alive was less than 4 from 48 HPI. Thus the significant analysis of variance in serum parameters was carried out only with data obtained before 42 HPI (n>3).

Changes in AST, ALT and LDH

The AST, ALT, LDH and AST/ALT ratio significantly elevated without sex difference. Elevation of AST was more marked than in ALT and LDH (Fig. 2A-B). In paired serum comparisons with individual values at 0 HPI, 95% of the rabbits showed immediately elevations of AST at 18 HPI (Fig. 4). The mean values of the enzymes peaked at 42 HPI, with maximal increment of 47 folds, 11 folds and 8 folds high of the means at 0 HPI for AST, ALT and LDH, respectively. The upper limit of 0 HPI, as normal values, for AST, ALT and LDH were 44.8 IU/L, 86.9 IU/L and 274 U/L, respectively. The timing at which 100% rabbits with values over the normal values were 24 HPI, 30HPI and 36 HPI for AST, ALT and LDH, respectively (Fig. 5). The earliest significant elevation was at 18 HPI for AST/ALT ratios when compared with values at 0 HPI with mean values over 1.20 (Fig. 2C).

Changes in ALP and γ-GT

The ALP and γ-GT were not markedly elevated at 18 HPI immediately (Fig. 3A and 3B). Significant elevation at 30 HPI and 36 HPI for ALP and γ-GT were observed, respectively. The mean values for ALP and γ-GT both peaked at 42 HPI, with about 5 folds and 8 folds increments, respectively. The γ-GT/ALT ratio (Fig. 3C)

tended to decrease from 0 HPI to 30 HPI and then increased from 36 HPI to 42 HPI, but all mean ratios after infection were lower than the baseline. No sexual difference was observed.

Change in CPK

Serum CPK was significantly elevated and peaked at 18 HPI (Fig. 3D). After that, most of the mean values hovered between 1295 U/L and 2000 U/L until 42 HPI. Male rabbits had higher values than the female.

Regression analysis

The ALT, AST and LDH values versus time fit the logarithmic curve significantly ($p<0.0001$) (Table 1). For ALP and γ-GT, the quadratic regression line fit both of them better (Table 1).

Correlation coefficient analysis among parameters

After RHDV inoculation, AST, LDH, ALP and γ-GT were significantly and positively correlated with ALT (Table 2). The LDH and AST correlated with ALT better than with γ-GT or ALP. The γ-GT correlated strongest with ALP. Serum CPK was the only parameter that correlated neither with ALT and AST nor with γ-GT and ALP.

DISCUSSION

Cytoplasmic enzymes are usually soluble, easily released, and readily pass through the cell membrane. Even though when they appeared microscopically intact (Meyer et al., 1992), their changes in the serum could be observed earlier than pathological changes. In our study, all 5 liver enzymes significantly elevated after virus inoculation. The marked elevations of AST, ALT and LDH at 18 HPI were 8 hours earlier than the first death observed. This result was concurred with the detections done by the reverse transcription-polymerase chain reaction (RT-PCR) (Guittre et al., 1996; Shien et al., 2000) and ELISA with monoclonal antibodies (Collins et al., 1996). Thus, monitoring these enzymes might give some early warning of RHD. Among the enzymes tested above, the change in AST was the earliest and sharpest for an immediately elevation at 18 HPI and a peaked increment of 47-fold at 42 HPI. If we define the normal maximum values of 44.8 IU/L for AST, 86.9 IU/L for ALT and 274 U/L, then 100% of the infected rabbits could be screened out at 24 HPI by AST was earliest. Furthermore, in individual paired serum comparison with values at 0 HPI, 95% rabbits had marked AST elevation at 18 HPI, and 100% at 24 HPI. Hence, although ALT is a specific and routinely measured enzyme for hepatic function in dogs, cats and primates (Bush, 1991; Meyer et al., 1992), our findings strongly suggested that AST is very sensitive

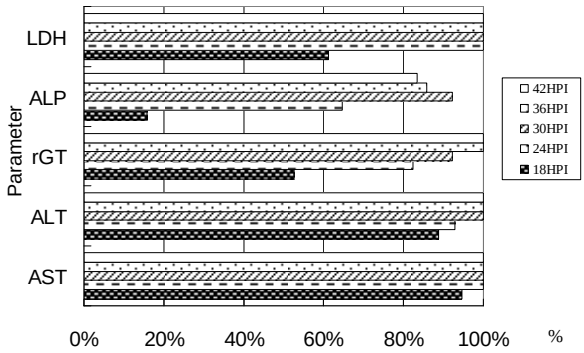

Fig. 4 The frequency distribution of rabbit with individual paired serum comparisons positive (value > 0 HPI) in AST, ALT, *r*-GT and LDH at different hour pot inoculation.

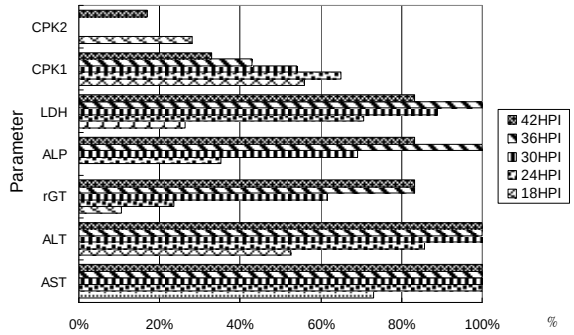

Fig. 5 The frequency distribution of rabbit with value higher than normal value (0 HPI) at different hour pot inoculation. The normal values were AST=44.8 IU/L, ALT=86.9 IU/L, *r*-GT= 14.4 IU/L, ALP=219 IU/L, LDH= 274 IU/L, CPK1=1295 U/L and CPK2>2000 U/L.

and suitable for monitoring the infection of RHD. Since the regression analysis of AST, ALT and LDH fit the logarithmic curve, it indicated that the damage of membrane permeability increased dramatically in the liver at acute phase which offered useful index for the prediction of the outcome of fulminant hepatitis. The AST/ALT ratio was suggested to be a prognostic index in severe acute viral hepatitis in human with values of 0.31 to 0.63 for survivors and 1.20 to 2.26 for nonsurvivors (Gitlin, 1982). In the present study, the AST/ALT ratio was significantly elevated from 18 HPI through 42 HPI with mean values over 1.20 which implicated bad prognosis future of these rabbits and was proved by the high mortality of 79 % within 45 hours after infection.

Our observation showed that the LDH mildly elevated at 18 HPI and significantly increased later at 30 HPI concurred with the report by Tunon et al. (2003). LDH is an enzyme that could be found in many tissues, specially in the muscle. However in our study, despite no evidence of muscular damage in pathologic examination coupled with insignificant changes in CPK change, a sharp elevation in LDH in paired serum comparison at an interval of over 30 hours was observed. Thus LDH should also be considered as a parameter to be monitored in

Table 1 Regression analysis among serum liver enzymes in RHDV infected New Zealand White rabbits in 42 hours post inoculation

Parameters	Regression	p value	R^2	Function
AST	logarithmic	<0.0001	0.6869	$\log_e(AST)=0.10062(TIME)+3.12718$
	quadratic	<0.0001	0.4097	
ALT	logarithmic	<0.0001	0.5731	$\log_e(ALT)=0.06419(TIME)+3.95352$
	quadratic	<0.0001	0.3779	
LDH	logarithmic	<0.0001	0.4507	$\log_e(LDH)=0.04571(TIME)+4.93371$
	quadratic	<0.0001	0.3603	
ALP	logarithmic	<0.0001	0.3401	
	quadratic	<0.0001	0.4829	$Y(ALP)=0.57(TIME)^2-8.10265(TIME)+126.6379$
γ-GT	logarithmic	<0.0001	0.4206	
	quadratic	<0.0001	0.5080	$Y(\gamma\text{-GT})=0.04919(TIME)^2-0.81508(TIME)+7.61635$

R^2: R Square Y: value TIME: HPI value

Table 2 Correlation analysis among serum parameters in RHDV infected New Zealand White rabbits in 42 hours post inoculation

AST	R^2	0.4501	0.3162	0.2627	0.4538	
	r	0.6811	0.5725	0.5147	0.6645	N.S.
	p	<.0001	<.0001	<.0001	<.0001	
		ALT	**γ-GT**	**ALP**	**LDH**	**CPK**
ALT	R^2		0.3008	0.2739	0.5615	
	r	1	0.5583	0.5224	0.7512	N.S.
	p		<.0001	<.0001	<.0001	
		ALT	**γ-GT**	**ALP**	**LDH**	**CPK**
γ-GT	R^2	0.3008		0.8088	0.6178	
	r	0.5583	1	0.8723	0.7739	N.S.
	p	<.0001		<.0001	<.0001	
		ALT	**γ-GT**	**ALP**	**LDH**	**CPK**
ALP	R^2	0.2739	0.8088		0.5394	
	r	0.5224	0.8723	1	0.7436	N.S.
	p	<.0001	<.0001		<.0001	
		ALT	**γ-GT**	**ALP**	**LDH**	**CPK**
LDH	R^2	0.5615	0.6178	0.5394		
	r	0.7512	0.7739	0.7436	1	N.S.
	p	<.0001	<.0001	<.0001		

R^2:R-squared p: probability N.S.: not ignificant

r: pearson correlation coefficients

RHDV infection. ALP and γ-GT are indicators of cholestasis. The mild and later significant elevation in ALP and γ-GT at 30 HPI and 36 HPI, respectively, indicated that choleatasis is a sequential change following severe hepatic necrosis in the first 42 hours of RHD. It was only late until 36 HPI that all the live rabbits have ALP values over the normal value (219 IU/L). For γ-GT, 100% increment in individual paired serum comparison with values at 0 HPI was late until 36 HPI. Since high γ-GT/ALT ratio has been used to distinguish biliary tract disease from hepatocellular disease (Bush, 1991), the decreasing γ-GT/ALT ratios till 30 HPI indicated that the early elevation of serum enzymes were caused mainly by hepatocellular disease rather than biliary tract disease

The magnitude of increases in serum CPK generally correlate with the extent of muscle injury, but exceptions do occur. Only marked (>10,000 IU/L) or moderate but persistent increases (>2000 IU/L) in enzymatic activity are considered to be clinically significant (Bain, 2003). In our study, although the CPK elevated significantly (p=0.0023), most of the values were under 2000 IU/L, and its' change was not correlated with the 5 mentioned enzymes above. Thus skeletal muscle damage induced ALT and AST elevations could be ruled out. Its moderate increase from 18 to 42 HPI might result from the intramuscular virus injection. The higher values of CPK in the male rabbits were similar to those observed in dog (Bush, 1991). Analysis of normal values (lab data, not shown), also showed that the male had higher values. Hence, sexual difference in CPK value was predominantly caused by

physical difference between both sexes but due to RHDV infection.

Serum parameters have been popularly used to diagnose, monitor and prognosticate the progression of human disease for many years. By measuring the concentrations of certain parameters in a small amount of blood sample, we could monitor the subtle changes when animals were still alive. Thus measurement of serum liver enzymes can help to screen out the RHD infected rabbits. Our study showed that the elevations of leakage enzymes could be seen as early at 18 HPI, which is 8 hours earlier than the first death. Serum AST was the most sensitive parameter among the enzymes in RHD and followed by ALT, which could be helpful monitors for the progression of RHD. The exponential increases in serum AST and ALT would be a strong prediction of the fulminant consequence. This is the first demonstration that compared and described the increasing patterns of 6 liver related serum enzymes in fulminant RHD.

Acknowledgements

This work was partially supported in grant-in-aid of ATU projected grant to NCHU by the Ministry of Education, Taiwan

REFERENCES

Bain PJ. 2003. Liver. In: Latimer KS, Mahaffey EA, Prasse KW, (ed). Veterinary laboratory medicine: clinical pathology. 4th ed. Harcourt Brace Jovanovich. pp. 193-214.

Bush BM. 1991. Plasma biochemistry. In: Interpretation of Laboratory results for small animal clinicians. Blackwell Scientific Publications. pp. 224-340.

Collins BJ, White JR, Lenghaus C, Morrissy CJ, Westbury HA. 1996. Presence of rabbit haemorrhagic disease virus antigen in rabbit tissues as revealed by a monoclonal antibody dependent capture ELISA. J Virol Methods. 58: 145-154.

Guittre C, Ruvoen-Clouet N, Barraud L, Cherel Y, Baginski I, Prave M, Gamiere JP, Trepo C, Cova L. 1996. Early stages of rabbit haemorrhagic disease virus infection monitored by polymerase chain reaction. Xentraibl. Vet Med B. 43: 109-118.

Gitlin N. 1982. The serum glutamic oxaloacetic transaminase /serum glutamic pyruvic transaminase ratio as a prognostic index in severe acute viral hepatitis. Am J Gastroenterol. 77: 2-4.

Liu SJ, Xue HP, Pu BQ, Quian NH. 1984. A new viral disease in rabbits. Anim Husb Vet Med. 16: 253-255.

Meyer DJ, Coles EH., Rich LJ. 1992. Hepatic test abnormalities. In: Veterinary laboratory medicine: interpretation and diagnosis. Harcourt Brace Jovanovich. pp. 55-70.

Mizoguchi T, Itou KI, Sakurai M. 2003. Hemagglutination and antigenic comparison of rabbit hemorrhagic disease virus. J Vet Med Sci. 65: 95-97.

Ohlinger VF, Haas B, Meyers G, Weiland F, Thiel HJ. 1990. Identification and characterization of the virus causing rabbit hemorrhagic disease. J Viol. 64: 3331-3336.

Shien JH, Shieh HK, Lee LH. 1998. Characterization of rabbit hemorrhagic disease field isolates in Taiwan. J Viol Med. 71: 27-33.

Tunon MJ, Sonia SC, Javier GF, Marcelino A, Francisco J, Javier G G. 2003. Rabbit hemorrhagic viral disease: characterization of a new animal model of fulminant liver failure. J Lab Clin Med. 141: 272-278.

Xu EJ, Chen WX. 1989. Viral haemorrhagic disease in rabbits: a review. Vet Res Commun. 13: 205-212.