Vet. Med. J., Giza. Vol. 44, No. 3. (1996):601-611.

STUDIES ON RAPID DIAGNOSTIC METHODS FOR RABBIT HAEMORRHAGIC VIRAL DISEASE (RHVD)

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SUMMARY

Rabbit haemorrhagic disease virus (local isolate) was successfully propagated in MDBK cell line culture. The RHVD was detected in organs of naturally infected rabbits using AGP, HA, ELISA, IP and IFA tests.

Infected liver homogenate gave the highest absorbance value (2.439) than other organs. Immune peroxidase staining and indirect immunofluorescence showed the specific RHVD reaction more intense in liver cells. AGP test was considered less sensitive in the detection of the RHVD in infected organ homogenates. ELISA, IP and IFA were rapid sensitive, specific tests for diagnosis of RHVD directly from naturally infected rabbits.

INTRODUCTION

Infection with rabbit haemorrhagic disease virus (RHDV)is characterized by high morbidity and mortality rates. The infected animals show high fever, respiratory distress, bloody nasal discharge, viremia and sudden death. The first outbreak of RHVD was recorded in China 1984 (Liu et al., 1984). Several reports have been published later in various countries. Rodak et al. (1990a) demonstrated antibodies against RHVD in stored rabbit's sera since 1987 in Czechoslovakia. Xu and Chen (1989) reported the occurrence of an acute, higbly infections disease in rabbets in many regions of China caused by the same virus. Subsequently the disease was reported in severall European countries. In November 1990, sudden death occurred in 2-18 month old rabbits in Israel caused by RHVD (Kuttin et al., 1991). Occurrence of outbreak of RHVD in domestic breed of rabbits in Egypt was reported by Ghanem and Ismail (1991) and by Salem and Ballal (1992). Based on the research effort to study the physical and morphological characteristics of the virus demonstrated that the virus was included inthe calicivirus family (Capucci et al ., 1990; Ohlinger et al., 1990 and Rodak et al., 1990a). RHVD - infected animals showed typical pathological lesions which were repoprted as acute hepatic necrosis with small intranuclear inclusion bodies (Xu and Chen, 1989), haemorrhage and oedema of lungs and trachea (Marcato et al., 1992). Diagnosis of RHVD can be made on the bases of the epidemiological features, clinical signs,

pathological findings and electron microscopy (Xu and Chen, 1989), haemagglutination test with human erythrocyte type O (Zhao et al., 1988), immunoenzyme staining, dot-enzyme linked immunosorbent assay and indirect fluorescent staining (Liu et al., 1992). The aim of the present work was to describe rapid detection of RHVD using prepared positive immune serum in chicken and mice, against propagated positive RHVD in MDBK cell line culture. Agar gel precipitation, immunosorbent assay, immunoenzyme staining and indirect fluorescent staining tests were applied for the rapid diagnosis of natural infection in rabbits.

MATERIAL AND METHODS:

Animals:

Ten improved balady pregnant rabbits died suddenly within a week were collected from small private farm. Four males, four females and eight young foreign breed white rabbits were collected from the same farm. The case history and the clinical symptoms were recorded according to the owner observation and the dead rabbits were subjected to postmortem examination.

Propagation of RHDV in MDBK cell line:

The isolate of RHDV was obtained from the collection of the Fac. of Vet. Med. Moshtohor and the homogenate of the collected organs were passed separately through a 0.22 µm filter and propaggated in cell culture. Examination of the inoculated cells compared to uninoculated control ones was done daily, and three successive passages were done before harvesting the virus.

Preparation of antisera against RHDV:

The propagated virus in T. C. cells was harvested after freezing and thawing three times, centrifuged and mixed with an equal volume of complete Freund's adjuvant, inoculated in foot pads of mice, chicken and rabbits in 0.2 ml, 0.2 ml and 1 ml, respectively. After four weeks blood samples were collected from inoculated ones and second dose of T. C. propagated virus was mixed equaly with incomplete Freund's adjuvant and inoculated into the foot pads of mice and chickens with the same previously mentioned dose, while rabbits were inoculated with heat inactivated virus at 70°C for 30 minutes as 1 ml, mixed with incomplete adjuvant. After 4 weeks, blood was collected and sera were separated, inactivated at 56°C for 30 minutes and stored till use.

Agar gel diffusion test:

Ouchterlony double immunodiffusion analysis of tissue suspension of different organs and polyclonal chicken antisera against RHDV were performed in 0.9 % agarose solution prepared in phosphate buffered saline (PBS) pH 7.4 containing 0.8 % of NaCl as described by Azad et al. (1985).

Virus-neutralization test (VNT):

It was done according to Saifuddin and Colin (1990). Each of the collected organ suspension

propagated in MDBK tissue culture cell line was tested against mice anti-RHDV serum in 96-well flat bottom tissue culture plates. Serum was diluted in two fold steps from 1:10-to 1:480, and each dilution reacted with approximately 100 TCIC 50 of each of the collected sample. The test was prepared in duplicate and the neutralization titre was taken as the reciprocal of the highest dilution of serum which inhibits completely the cytopathic effects of the suspected virus in monolayers of MDBK cell line.

Haemagglutination test:

Haemagglutination test was applied on homogenated organ samples by adding human erythrocyte according to the method of Fitzner et al., (1992).

Antigen-captured enzyme-linked immunosorbent assay for detection of RHVD:

Organ suspensions were tested for RHDV antigen by ELISA test according to the method described by Capucci et al. (1991). A standard volume of 100 µl / well was used for all reagents. Ninety six-well polystyrene plates were coated with the captured antibody, which was immune chicken anti - RHDV serum diluted 1:100 in carbonate coating buffer (pH 9.6) and then incubated at 37°C for 1 hour and 4°C over night. The plates were rinsed twice in PBS with 0.1% tween - 20 (KPL), the infected and noninfected organ suspensions diluted separately with diluting buffer and 100 µl amount was added. The plates were rinsed in washing buffer, mice anti-RHDV diluted 1:100 in diluting buffer was added, and the plates were incubated at 37°C for one hour. After treatment in washing solution mice anti-IgG peroxidase-labeled diluted 1:100 in diluting buffer (PBS - tween - BSA 2%) was added, and the plates were incubated at 37°C for one hour. The plates were washed twice followed by sterile distilled water, and the substrate 2, 2-Azino - bis (3-ethybenz - thiazotine - 6 sulphonic - acid) (ABTS of KPL ELISA Kit) was added, plaltes were incubated at 37°C for 15 - 30 minutes. Colour development was read at 410 nm when positive control well which contain RHDV pooling of organ suspensions infected with positive RHDV, reaches an abosorbance value of approximately 1. 0. Sample was considered positive if it produced an absorbance reading above the absorbance of control - ve.

Immunohistochemical studies:

Using immunohistochemical test as described by Park and Itakura (1992) and avidine-biotine peroxidase complex, ABC kits (Vectastain ABC Kit). Sections were depara finized and hydrated through xylene and graded alcohol series then rinsed for 5 minutes in distilled water. The sections were incubated for 30 minutes in 0.3% H_2O_2 in methanol, washed in methanol, washed in buffer for 20 minutes, incubated for 20 minutes with diluted normal mice serum and re-incubated with mice anti-RHDV serum diluted in buffer 1/10. Sections were then washed for 10 minutes in buffer and incubated for 30 minutes with diluted biotinylated antibody (anti mice solution). The sections were washed for 10 minutes in buffer and incubated for 2-7 minutes in DAB substrate solution (3,3 diaminobenzidine)

cleaned, mounted and examined. IP staining was scored depending on intensity and extent of staining as +, ++, +++ for positive staining of less than 25%, 25-50% to 75% and over of cells in the section, respectively.

Immunofluorescence technique:

Using the method adopted by Liu et al. (1992), sections were deparaffinized and hydrated through xylene, methanol, ethanol bathes. The samples were washed with PBS for 20 minutes and incubated for 30 minutes with a 1:10 dilution of mice anti-RHDV serum in a humid chamber at room temperature. After 10 minutes in PBS, slides were incubated for 30 minutes with anti-mouse IgG conjugated with fluorescein isothiocyanate at 1:250 (Sigma Chemical Co., St. Louis, Missouri). Slides were washed with PBS for 10 minutes, a cover slip was mounted wih 9 part PBS and one part glycerol mixture and slides were viewed through and ultraviolet microscope at 20x. Slides were considered positive FA staining depending on intensity and extent of staining as +, ++, +++ as with immunoperoxidase test. Control for both the immunofluorescence and immuno peroxidase tests were tissues from uninfected rabbits stained with positive sera. Infected tissues were tested with normal mice serum and also tested with anti-mouse not conjugated with immunoperoxidase and fluorescein isothiocyanate.

Histopathological study:

Specimens for histopathological examination were taken from all visceral organs and brain fixed in 10% neutral buffered formalin, processed and stained with haemtoxylin and eosin (H and E).

RESULTS

Clinical symptoms:

The disease was hyperacute with mortality up to 90%, it was accompanied by convulsion, respiratory distress, bloody nasal discharge and fever just before death. Young rabbits aged 25 - 30 days refused to eat, developed diarrhoea, emaciation, abdominal distension, recumbency, convulsions, epistaxis and death.

Postmortem lesions:

Gross pathological findings of the infected rabbits revealed congestion and peticheal haemorrhage on most of the organs and on the serous surfaces of abdominal and thoracic cavities. Oedema and haemorrhage of the lungs were observed in many cases. Paleness of the liver was very prominent, some of which showed focal areas of haemorrhage and / or necrotic foci (white spots).

Serological tests:

Agar gel precipitation test:

The tissue homogenates of livers, spleens, kidneys and lungs of infected rabbits gave line of precipitation against prepared chicken serum, while suspensions from intestine, heart, brain and organs of normal negative control rabbit gave no line of precipitation.

Table (1): Mean of absorbance vaues for sera against RHDV using ELISA technique.

Species	Absorbance value		
	Positive	Negative control	
Mice	0.543*	1.253**	0.130
Chicken	0.452	0.976	0.137
Rabbit	0.326	0.712	0.145

* Four weeks post inoculation of the first dose.
** Four weeks post inoculation of the second dose.

Table (2): Comparison of viral antigen detection using agar gel precipitation, haemagglutination, ELISA, immunoperoxidase and immunofluorescence tests in different infected organs.

Organ	AGP	HA	ELISA	IP	FA
Liver	+ve	1/640	2.439	+++ve	+++ve
Spleen	+ve	1/160	2.102	++ve	+++ve
Kidney	+ve	1/20	1.873	+ve	++ve
Thymus	-ve	1/20	1.754	+ve	++ve
Lung	+ve	1/80	1.974	+ve	+++ve
Intestine	-ve	1/20	1.102	+ve	+ve
Heart	-ve	1/20	1.534	+ve	+ve
Brain	-ve	1/40	0.964	+ve	+ve

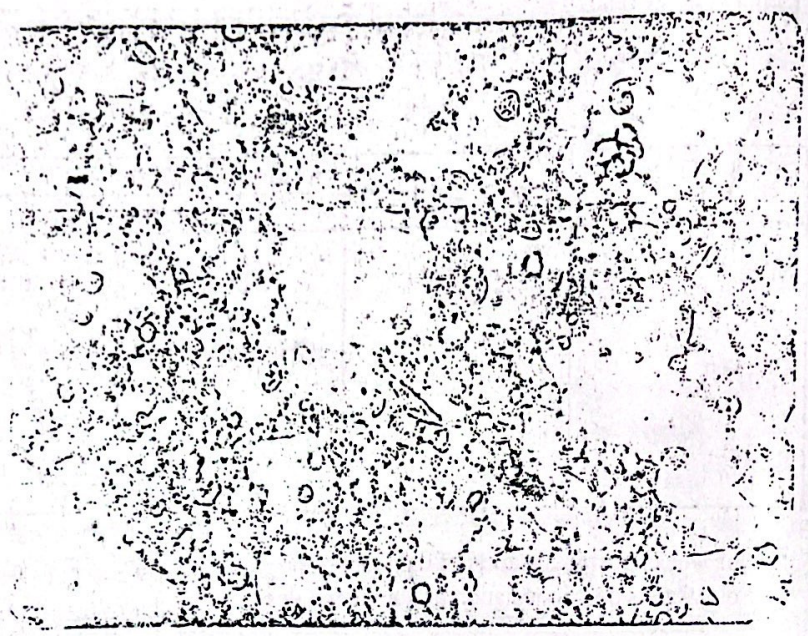

Fig. (1): MDBK monocellular layer showing CPE 3 days post inoculation (x 400).

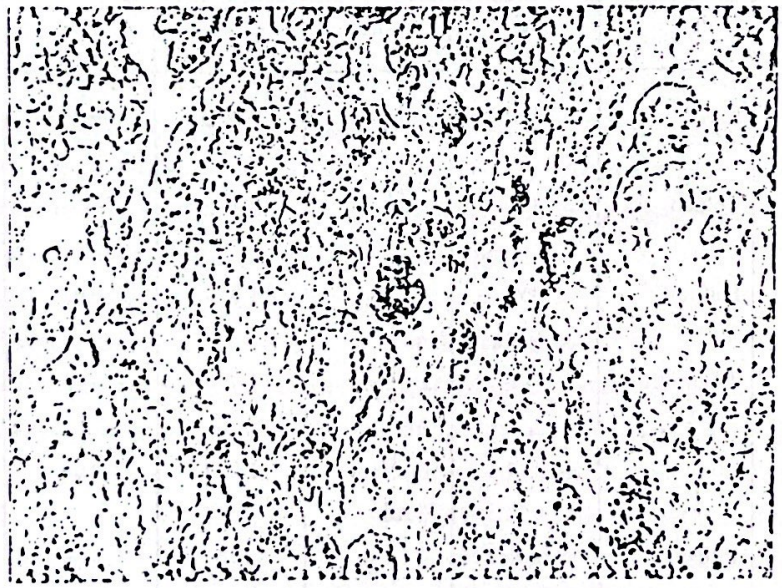

Fig. (2): Immunoperoxidase staining demonstrated the RHDV antigen in hepatocyte cytoplasm (x 400).

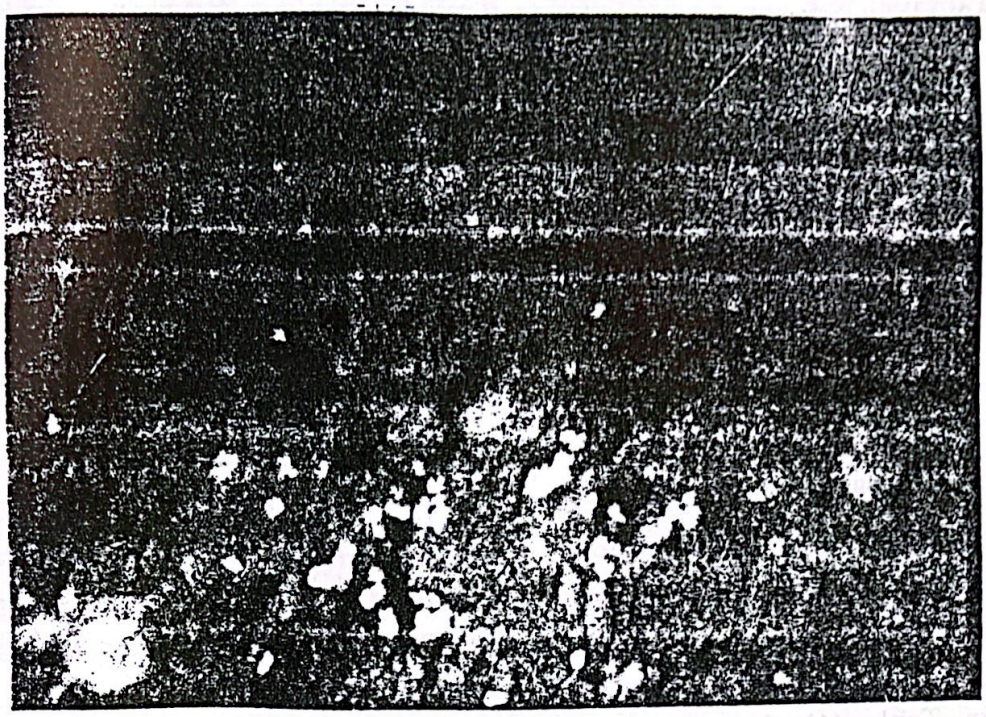

Fig. (3): Liver showing specific RHDV fluorescence reaction (x 400).

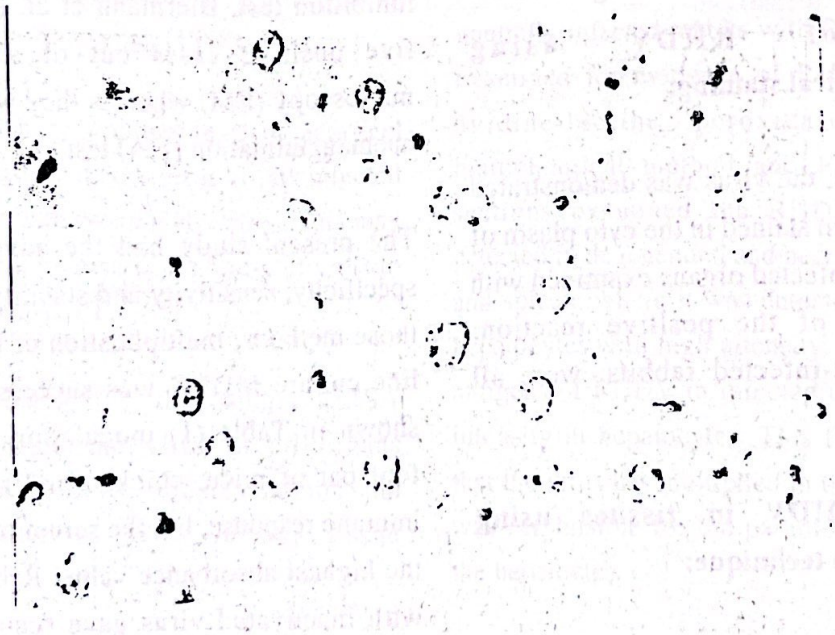

Fig. (4): Intranuclear inclusions in the infected liver cells (Phloxine Tertrazine) (x 630).

Serum neutralization test:

The prepared chicken serum diluted 1/10 completelly inhibited the cytopathic effect of isolated virus from the infected organs propagated in MDBK cell line culture.

Haemagglutination:

As shown in Table (2), the tissue homogenates of collected infected organs agglutinated the human erythrocytes with varying titres.

Antigen-captured ELISA:

As shown in Table (1) the coated mice anti-RHDV caught the virus in the organ suspension and the absorbance value varied in different organs.

Detection of RHDV using immunohistochemical staining:

As shown in Fig. 2, the virus was demonstrated as pale to dark brown stained in the cyto plasm of infected cell in all infected organs examined with varying intensity of the positive reaction. Sections from non-infected rabbits. were all negative.

Detection of RHDV in tissues using immunoflurescence technique:

The virus was detected in the cytoplasm of all examined sections of infected organs (Fig. 3) whereas non-specific staining reactions were presented in tissues of uninfected control rabbits.

Histopathology results:

Liver:

Intranuclear inclusion bodies were detected in the intact hepatocytes (Fig. 4).

DISCUSSION

Outbreaks of RHVD in populations of domestic rabbits were recorded in Egypt (Ghanem and Ismail, 1991; Salem and Ballal, 1992). The disease occurs in Israel (Kuttin et al. 1991). Continuing sporadic outbreaks diagnosed in Egypt, may be attributed to the subclinical infected breeding stock.

The diagnosis of the disease is based on the clinical and pathological manifestations, with confirmation provided by the haemagglutination inhibition test. Biermann et al. (1992) recorded five positive cases out of six by electron microscopy (EM) whereas they were negative by haemagglutination (HA) test .

The present study had the advantage of high specificity, sensitivity and stability. In addition to those methods, multiplication of the virus in cell line culture MDBK was successfully done. As shown in Table (1) inoculation of RHDV into foot pat of mice, chicken and rabbits gave an immune response, but the serum of mice recorded the highest absorbance value. Rabbits inoculated with inactivated virus gave secondary immune response less than those recorded in mice and chicken which were inoculated with living virus. Our results indicated that using agar gel precipitation test as a preliminary test is valuable

for rapid diagnosis of RHDV in infected organs especially liver homogenate. This may be due to the high titre of virus in hepatocytes. The RHDV antigen was demonstratel in the hepatocytes sugaesting that RHDV has replicated in these cells.

Our results were in agreement with those obtained by Olinger et al. (1989), whereas Abd-El Motelib (1993) failed to produce any line of precipitation using the same technique.

Atempts to isolate the virus in cell line culture was successful as shown in Fig. (1). Mizak et al. (1991) failed to induce virus replication in a ceil culture. While Xu (1991) passed the virus in diploid rabbit kidney cells with cytopathic effect.

As shown in Table (2) the liver suspension gave the highest titre of HA using human erythrocyte followed by spleen suspension. Similar results were obtained by Zhao et al. (1988).

The test was more applicable than agar gel precipitation test to detect virus in all infected organs used but with varying HA titres. This may be due to the requirement of high titre of virus to be precipitated in AGPT.

As shown in Table (2), the capture ELISA technique detected the virus in all organs collected from naturally infected rabbits and caught any titre of virus in any tissue homogenate.

The results of ELISA on various tissues indicated that the virus titre was higher in liver and spleen suspension.

The present assay may be useful for rapid diagnosis of outbreaks of RHVD. These results are in agreement with the findings of Capucci et al. (1991) that the ELISA in superior to HA and immune elecronic-microscopy for detection of the RHDV.

Intranuclear inclusions were demonstrated in hepatocytes which supported the findings of Gregg et al. (1991) who described small single or multiple intranuclear inclusion bodies in degenerated hepatocytes of infected rabbits . On the other hand, electron microscopical study of the hepatic cells reported by Salem and Ballal (1992) revealed the presence of viral particles in the cytoplasm of the hepatocytes.

In this study formalin-fixed sections from different organs were obtained from suspected nautrally infected rabbits with RHVD and were examined for evidence of RHDV antigen by avidine-biotine peroxidase immuno - histochemical method and FA tests. In all sections examined the RHDV antigen was detected in degenerated and necrotic cells of liver and spleen, where it was detected in degenrated hepatocytes with high nitensity. FA test detected antigens of RHDV in infected organs with high intensity in hepatocytes. This finding indicated that the virus has multiplied in the liver cells and was responsible for the pathological damage of the hepatoctes.

It is concluded that the use of ELISA, IP and FA techniques are capable of rapid detection of the

RHDV infection in rabbits with high degree of efficiency which excludes the need for conventional virus isolation and identification techniques.

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