Pathological Studies On Rabbit Hemorrhagic Disease In Sharkia Provence

Mohamed M.M. Metwally

Dept. of Pathology, Fac. of Vet. Med., Zagazig University

ABSTRACT

Rabbit hemorrhagic disease (RHD) was diagnosed in *Chinchilla* rabbits on three rabbit farms in Sharkia Province, Egypt, in June, 2008. The clinical signs of this outbreak included depression, anorexia, fever, paddling, convulsions, and sudden death. The morbidity rate was 100% and the mortality rate was 90% in rabbits more than 8 week old. The pathological changes were consistent with severe generalized circulatory disturbances (hyperemia and hemorrhage), marked degeneration of parenchymatous organs, and pronounced serous-hemorrhagic pneumonia and extensive disruption of the reticulo-lymphoid tissues. In liver specimens, obtained from dead and diseased rabbits, RHD viral antigen was detected via hemagglutination assay and Enzyme-Linked Immunosorbent Assay.

The objectives of this study were to describe the gross and histopathological findings for this outbreak and identify the causative agent.

INTRODUCTION

Rabbit hemorrhagic disease (RHD) is an acute, highly infectious, and usually fatal. It affects domestic, farmed and wild rabbits of the species *Oryctolagus cuniculus* ***(1)***. The disease was first reported in China in 1980 in Angora rabbits imported from West Germany ***(2)***. Since its emergence in 1980, RHD has resulted in the deaths of nearly a quarter billion free-living and domestic rabbits in more than 40 countries from Asia, Africa, Europe and America ***(3,4)***. The highly contagious and fatal nature of RHD has had profound economic effects over wide areas which coupled with implications for conservational aspects have led to intensive international efforts to understand and control the disease ***(5)***. Also, there is an increasing interest to study animal hemorrhagic fevers because they could represent models for human hemorrhagic diseases caused by viruses ***(6)***. The etiological agent of RHD is the rabbit hemorrhagic disease virus (RHDV), a member of the family *Caliciviridae* genus *lagovirus* ***(7)***. RHDV is a non-enveloped RNA virus with a diameter of approximately 36-40 nm. The virons are composed of a positive sense single-stranded RNA, 7.5 kb in length and a single major protein capsid with a molecular mass of 60-70 kDa ***(8,9)***. RHD can be confirmed by detection of the virus or the viral antigen, in tissues from rabbits that have died of the disease. The liver contains the highest viral titers and semi-purified or crude extracts of homogenized liver provide the basic material for diagnostic tests ***(5)***. RHDV is environmentally stable, highly infectious, and transmissible by close contact or by contact with fomites. Rabbits can acquire the disease through the oral, nasal or conjunctival routes. Indirect arthropod vectors, including blow flies or flesh flies, have also been implicated in the spread of RHDV ***(10)***. The virus usually affects rabbits over two months of age. Younger rabbits might be protected by passive immunity transferred through the colostrum ***(2)***. They also lack the viral receptors in the epithelium of the respiratory and digestive systems ***(11)***. The incubation period ranges from several hours to one or two days in natural cases ***(12)***. The morbidity rate is 70-80 % with up to 100% mortality in the affected rabbits ***(13)***. Outbreaks in different regions showed many similarities and may be divided into three categories: peracute, acute and subacute forms ***(3,14)***. The peracute RHD is usually seen when the disease is first introduced and characterized by animals found dead without premonitory signs. Occasionally, a hemorrhagic foamy discharge from the nostrils and vaginal discharge are observed ***(12,15)***. The acute form of RHD predominates in areas where the disease has become enzootic and characterized by animals dying

within 12-48 hour after signs of convulsions, ataxia, posterior paresis, opisthotonus, rapid respiration and elevated body temperature *(3,15)*. The course of acute RHD is between 12-36 hour *(13)*. The subacute form of RHD is uncommon and occurs in the later stages of an epizootic. The affected rabbits exhibit depression, anorexia and fever. The clinical signs last for 2-3 day and the majority of animals survive. Such animals are resistant to re-infection *(14,16)*. The pathological changes of RHD have been described in the respiratory tract, liver, kidneys, central nervous system and lymphoid tissues *(14)*.The striking characteristic lesion is extensive disseminated intravascular coagulation (DIC) and focal to massive hepatic necrosis with little inflammatory response *(17,18)*. The gallbladder is usually distended with bile. Petechial hemorrhages and focal coagulative necrosis may be observed in its mucosa *(19)*. Pulmonary hyperemia, hemorrhages and edema with tracheal hemorrhages and serous-hemorrhagic pneumonia are common features. The inflammatory cells, infiltrating the airways and alveoli were predominantly macrophages, lymphocytes and neutrophils *(5,20,21)*. Extensive congestion and focal hemorrhages have been consistently described as the major renal changes in RHD *(22)*. Nephritis and membranous glomerulonephritis have been reported infrequently *(14)*. Lymphoid necrosis, in the spleen and lymph nodes, is another characteristic lesion of RHD *(23)*. Thrombi may be found in small blood vessels in many tissues, including the central nervous system. Non-suppurative encephalomyelitis has also been reported *(15)*. Changes reported in other tissues included catarrhal gastritis, adrenal cortical necrosis, and severe endometrial congestion with hemorrhages into the uterine lumen *(12,20)*.

MATERIAL AND METHODS

An outbreak occurred in three *Chinchilla* rabbit commercial farms during June, 2008 over an approximately two week period. The rabbit farms were located on approximately 4 acres of land in a rural setting in San Elhagar City, Sharkia Province, Egypt. These farms were separated by a distance of approximately 200 meters among them. At the time of the outbreak, the entire rabbit population (suckling, young and adults), in theses farms, were approximately 502, 821, and 1315 respectively. The case history, clinical signs, general health condition, and description of all lesions were reported. Postmortem examination was done for all the dead and some diseased rabbits. Specimens were collected from the liver, kidneys, heart, spleen, lymph nodes, brain, lungs, trachea, muscles, and intestine were taken and immediately fixed in 10% buffered neutral formalin solution. Five-micron thick paraffin sections were prepared, stained by Hematoxyline and Eosin and examined microscopically *(24)*.

Detection of RHDV antigens

The liver specimens, which were collected from the dead and diseased rabbits of the three farms where RHD outbreaks were suspected, were also assessed for RHD viral antigen detection by hemagglutination assay and Enzyme-Linked Immunosorbent Assay. None of these rabbits received commercial RHD killed vaccine. Each liver specimen was diluted 1:4 in PBS containing 2% sucrose, pH 7.2, mechanically homogenized, filtered through cheesecloth and clarified by centrifugation at 5000 g for 5 minutes as described by *(25)*.

Hemagglutination test

Hemagglutination (HA) tests were carried out in U-bottomed microdilution plates (Nunc) with equal volume of a serial 2-fold dilution of the filtered liver homogenates, and 0.5% suspensions of group O human erythrocytes in 0.05 MNaH2PO4: Na2HPO4–0.15 M NaCl (pH 6.5) at 24°C *(7)*. The end point titer was defined as the greatest dilution at which hemagglutination occurred. Hemagglutination assay end point titers ≥ 1:80 were considered positive.

Enzyme-Linked Immunosorbent Assay (ELISA)

Three steps sandwich-ELISA was carried out *(26)*. 100 µl/well of chicken-anti RHDV-

serum (prepared in Veterinary Serum and Vaccine Research Institute, Alabasya, Egypt) (diluted 1:1000) were coated overnight at 37°C onto 96-well Nunc Maxisorp immunoplate. Then the plates were washed three times with PBS containing 0.2 per cent Tween 20 (PBS-Tween) and the unattached sites were blocked by incubation for two hours in blocking buffer (1.75% bovine serum albumin in PBS-Tween (Fluka). 100 µl of the supernatant was diluted 1:5 in PBS-Tween and incubated for one hour at 37°C in two adjacent wells of coated plates. a positive rabbit hyperimmune serum produced by immunization with inactivated RHDV (Veterinary Serum and Vaccine Research Institute, Alabasya, Egypt) and a negative rabbit serum (Sigma) were added respectively into the adjacent wells and incubated for one hour at 37°C. After three washes with PBS-Tween 100 µl of horseradish peroxidase (HRPO)-conjugated goat anti-rabbit antiserum (Sigma) was added to each well and incubated for one hour at 37°C. The wells were washed three times with PBS-Tween followed by the addition of orthophenylene diamine substrate (Fluka). After incubation for 30 minutes at 37°C, the absorbance optical density (OD) was measured on an ELISA reader (Behring) at 405 nm. Positive and negative control livers were included in each set of samples; PBS-Tween was used instead of a liver sample in the wells serving as blanks. The results of each sample were analyzed in relation to the positive and negative controls with the following formula *(27)*:

$$\text{S/P ratio (Sample to positive control ratio)} = \frac{\text{OD sample} - \text{OD negative control}}{\text{OD positive control} - \text{OD negative control}}$$

A liver sample was considered positive when the ratio was more than 1-100.

RESULTS

The owners of the rabbit farms reported that there was no history of disease in the farms, and the rabbits were not vaccinated against RHD.The clinical signs were those of the acute form of RHD. They appeared suddenly, only on rabbits over eight week of age. These signs included fever (the rectal temperature ranged from 38.3 to 41 C°), anorexia, rapid and shallow respiration, dyspnea, and epistaxis (Fig. 1). Lateral recumbency, paddling, ataxia, opisthotonus and terminally frenetic behavior were seen at the terminal stage of the disease. Sometimes squealing and decrease in the body temperature were noticed. Rabbits under eight week of age survived the infection without the presentation of clinical signs, although they are suspected to carry the infection.

The morbidity and mortality rates (in rabbits over 8 week of age), were nearly the same in the three farms. The morbidity rate was 100% and the mortality rate was 90%. One percent of the surviving rabbits developed jaundice, with weight-loss and three rabbits died after 3 week from the onset of the clinical jaundice.

The postmortem examination revealed that most of the dead rabbits were in good bodily condition and with full stomach. The inner surface of the skin was markedly congested (Fig. 2). The liver was friable, pale and showed multiple coalescing necrotic areas, with severely distended gallbladder (Fig. 3). The spleen was black and engorged with blood and showed rounded edges. The alimentary tract showed no gross lesions. The kidneys were enlarged and showed dark-reddish-brown spots. The trachea was severely congested and contained frothy blood stained mucus and clotted blood (Fig. 4). The lungs were severely congested. They presented focal petechial hemorrhages, besides frothy blood stained mucus in the bronchi (Fig. 5). The brain showed congested meninges (Fig. 6).

Microscopically, the primary lesion was acute diffuse hepatic coagulative necrosis. Intralobular focal hemorrhages with minimal leukocytic infiltration were seen. The necrotic areas were characterized by disassociated hepatic cells. The latter were hyereosinophilic, swollen, and vacuolated. Their nuclei showed pyknosis, karyorrehexis and karyolysis (Figs 7&8). The portal areas showed severe congestion accompanied with few lymphocytic infiltrations (Fig. 9). Some bile ducts showed

cholestasis, represented by eosinophilic material in their lumens, accompanied with mild lymphocytic infiltrations to their walls (Fig. 10). The spleen showed diffuse congestion with lymphoid necrosis (Fig. 11). Lymphoid necrosis was detected also in the saccular rotundus, appendix and Peyer's patches (Fig. 12). The kidneys revealed multiple hemorrhages, congestion and coagulative necrosis of the tubular epithelium (Fig. 13). Hemorrhages, in the glomerular tufts with focal replacement of the renal tubules with exudate, containing round cells were also observed (Fig. 14).The renal medulla showed multiple hemorrhages, besides numerous hyaline casts in the tubular lumens (Fig. 15). The trachea revealed focal hemorrhages, with severe congestion and necrosis of the epithelial lining (Fig. 16). The lungs showed extensive hemorrhages and edema, characterized by flooding of the alveolar lumens with serous fluid, erythrocytes or a mixture of both. This was accompanied with pulmonary emphysema of the adjacent alveoli (Figs. 17&18). Congestion of the pulmonary blood vessels, besides mild lymphocytic hyperplasia were detected (Fig. 19). The myocardium was markedly congested and revealed focal coagulative necrosis. The brain showed marked congestion with mild perivascular lymphocytic cuffing and activated pericytes (Fig. 20). No significant histopathologic lesions were detected in the other tissues examined.

All suspensions of the liver specimens yielded strong positive HA results, indicating the presence of RHD viral antigen (titers ranged from 1:10 to 1:40). Also the ELISA detected RHDV antigen in all rabbit liver specimens (titers ranged from 1:1,000 to 1:2,000).

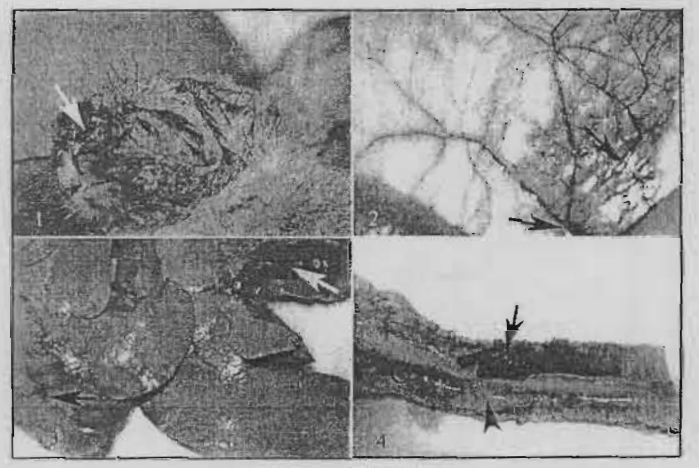

Fig. 1-4.
1- Rabbit showing epistaxis (arrow).
2- Skin showing congested inner surface (arrows).
3- Liver showing multiple coalescing necrotic areas (black arrow), anddistended gall bladder (white arrow).
4- Trachea showing severe congestion (C), and frothy blood stained mucus (arrow-head) and clotted blood (arrow).

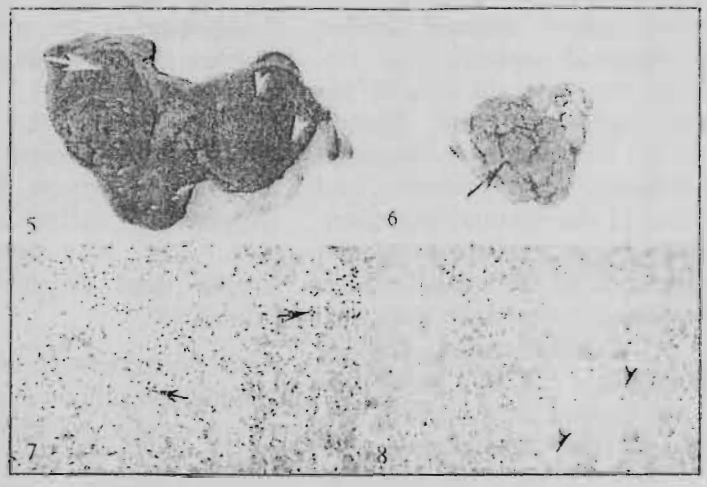

Fig. 5-8.
5- Lung showing congestion (arrow) and hemorrhages (arrowheads).
6- Brain showing congested meninges (arrow).
7- Liver showing diffuse coagulative necrosis with pyknosis and karyorrhexis (arrows), besides loss of the cellular organization., H&E., X1200.
8- Liver showing acute diffuse coagulative necrosis with cellular swelling, cytoplasmic vacuolation (arrowhead) and individualization of the necrotic cells. , H&E., X1200.

Fig. 9-12.
9- Liver showing congested portal veins (arrow), accompanied with few lymphocytic infiltration of the portal area (arrowhead). , H&E., X1200.
10- Liver showing cholestasis (arrow) accompained with mild lymphocytic infiltration (arrowheads). , H&E., X300.
11- Spleen showing congestion (arrow-heads) and lymphoid necrosis (arrow)., H&E., X300.
12- Peyer's patches showing lymphoid necrosis (arrowheads)., H&E., X120. H&E., X300.

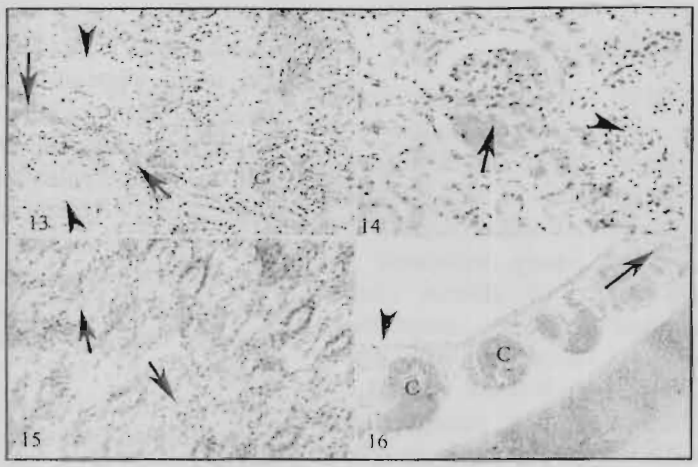

Fig. 13-16.
13- Kidney showing multiple hemorrhages (arrows), and congestion congestion of the renal blood vessels (C) with coagulative necrosis of the tubular epithelium (arrowheads)., H&E., X300.
14- Kidney showing hemorrhage within the glomerular tufts (arrow) and focal replacment of the renal tubules by exudate, containing round cells (arrowhead). , H&E., X1200.
15- Kidney showing numerous hyaline casts in the tubular lumen (arrows). , H&E., X300.
16- Trachea showing hemorrhages (arrow) besides severe congestion (C) and necrosis of the tracheal epithelial lining (arrowhead)., H&E., X300.

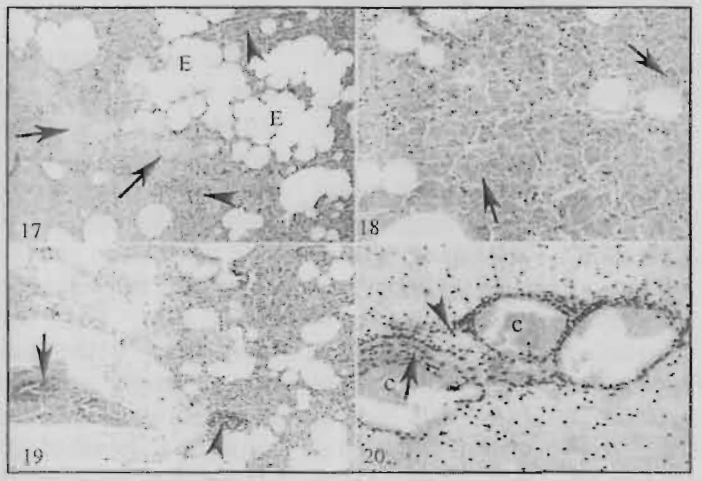

Fig. 17-20.
17- Lung showing focal hemorrhages (arrowheads) and edema (arrows) accompanied with pulmonary emphysema of the adjacent alveoli (E). , H&E., X300.
18- Lung showing flooding of the alveolar lumens with erythrocytes (arrows). , H&E., X1200.
19- Lung showing congestion (arrow). besides mild lymphocytic hyperplasia (arrowhead)., H&E., X300.
20- Brain showing marked congestion (c) with mild perivascular lymphocytic cuffing (arrowhead) and activated pericytes (arrow)., H&E., X1200.

DISCUSSION

Outbreaks of RHD have been reported in many parts of the world. Usually the epizetiological features, signs and lesions are diagnostic, but confirmation of the diagnosis depends on laboratory examination *(2,28)*. The results of the present study indicated the sudden onset of signs of illness (fever, anorexia, rapid and shallow respiration, dyspnea, epistaxis, and various nervous signs) and death of rabbits over 8-week age in the three rabbit farms. Similar results were reported *(2,16)*. The hyperthermia, anorexia, rapid respiration and dyspnea were related to the viremia induced by the virus. The epistaxis could be related to the disseminated intravascular coagulation (DIC). The nervous signs might be related to the meningial congestion and massive hepatic necrosis, caused by the virus. The sudden deaths are the result of a widespread circulatory disturbances and massive hepatic necrosis *(28,29)*. The lack of mortality and clinical signs of RHD in young rabbits (less than 8-weeks-old) was due to absence of viral receptors in the epithelium of the respiratory and digestive systems of young animals *(11,30)*. The ability of rabbit hemorrhagic disease virus to agglutinate human erythrocytes and to attach to rabbit epithelial cells of the upper respiratory and digestive tracts depend on the presence of ABH blood group antigens that are presented in epithelial cells of the upper respiratory and digestive tracts of adult rabbits *(11,30)*. Young rabbit tissues were almost devoid of these viral receptors. Consequently, only very weak binding of virus particles could be obtained on these tissues and this difference between adult and young animals could explain the resistance of young rabbits to RHD *(11,30)*.

The postmortem examination revealed marked congestion of the inner surface of the skin, generalized congestion and hemorrhages. The liver was friable, pale and showed multiple necrotic areas, with severely distended gallbladder. The spleen was black and engorged with blood. The kidneys were enlarged with dark-reddish-brown spots. The trachea was severely congested and contained frothy blood stained mucus and clotted blood. The lungs were severely congested with focal petechial hemorrhages. Similar results were reported by several investigators *(2,3,16,28)*. The severe hepatic damage was due to the replication of RHDV in the liver as RHDV has shown special tropism for liver cells *(6,31)*. The generalized congestion and hemorrhages was due to the DIC induced by the virus *(18)*. Hepatic diseases leading to severe tissue necrosis as RHD, stimulate fibrinogen synthesis and release massive amounts of tissue thromboplastins *(17,32)*. The defective clearance of the activated clotting factors by the damaged liver, combined with decreased levels of coagulation inhibitors in the plasma, trigger DIC.The latter aggravates hepatic necrosis, producing a vicious circle. The hemorrhagic lesions in lungs, trachea, spleen and kidneys are more likely attributable to DIC than to viral replication in these organs *(18)*.

The histopathological examination revealed that the primary lesion was acute diffuse hepatic necrosis and intralobular foci of hemorrhage with minimal leukocytic infiltration. Similar results were previously reported by several investigators *(3,5,16,17)*. The replication of RHDV in the liver resulted in severe liver necrosis and terminated with DIC *(4)*. The spleen showed diffuse congestion with marked lymphoid necrosis. These results are consistent with previously reported data *(16,21)*.The kidneys revealed multiple hemorrhages, congestions and coagulative necrosis of the tubular epithelium, besides focal replacement of the renal tubules with exudate containing round cells. Similar results were mentioned in several reviews *(2,28)*. The hemorrhages and congestions could be related to the DIC, while the necrosis of the renal epithelium might be related to the viremia. Multiple focal hemorrhages and severe congestion, besides mild lymphocytic aggregation were detected in the trachea and lungs. These results are in agreement with those obtained in previously reported works *(5,21)*. The striking pulmonary congestions and hemorrhages are the result of DIC *(18)*.

The diagnosis of RHD is usually confirmed by HA and ELISA tests, performed on the rabbit liver *(7,31)*. The liver samples were selected as the liver contains the highest viral titers *(5)*. The HA test is useful for preliminary identification of RHDV antigen in tissue suspensions from infected animals because RHDV can agglutinate type O human erythrocytes, *(13)*. Positive reactions could be detected even when the tissue suspensions were highly diluted *(33)*. All suspensions of livers from the rabbits yielded strong positive HA results, indicating the presence of RHD viral antigen and The ELISA detected RHDV antigen in all rabbit liver specimens.

It could be concluded that the cause of this outbreak was rabbit hemorrhagic disease virus. The morbidity and mortality rates were 100% and 90% respectively, among rabbits more than 8 week old, while rabbits less than 8 week old were resistant.

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الملخص العربى

دراسات باثولوجية على مرض الأرانب النزفى بمحافظة الشرقية

محمد متولى محمد متولى

قسم الباثولوجيا- كلية الطب البيطرى- جامعة الزقازيق

أجريت هذه الدراسة خلال شهر يونيو 2008 فى 3 مزارع لانتاج أرانب الشنشيلا بمدينة صان الحجر بمحافظة الشرقية وذلك لتشخيص فاشية مميتة فى هذه الأرانب حيث تم تسجيل الأعراض المرضية (خمود، وفقد الشهية، وحمى، وأعراض عصبية، وموت مفاجىء) وكان معدل المراضة 100% ومعدل الوفيات 90% فى الأرانب التى تجاوزت أعمارها 8 اسابيع. وكانت الآفات العيانية متمثلة باحتقانات وأنزفة حادة فى معظم الأعضاء وكذلك تنكس ونخر فى الاعضاء المتنية والأعضاء الليمفاوية. تم أخذ عينات من الكبد و الرئتان والقصبة الهوائية والكليتان و الطحال والمخ والقلب والامعاء والعقد الليمفاوية وذلك لعمل قطاعات بارافينية للفحص المجهرى لمعرفة الآفات المرضية. وتم أخذ عينات من الكبد وفحصت بواسطة اختبار التراص الدموى ومقايسة الممتز المناعى المرتبط بالانزيم وأظهرت النتائج وجود مستضدات فيروس مرض الأرانب النزفى فى كل العينات المفحوصة.