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Molecular, Clinical and Pathological Studies on Viral Rabbit Hemorrhagic Disease

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Key words

Rabbit hemorrhagic disease (RHD), Histopathology, Hemagglutination assay, Bacteriological assay, RT-PCR.

ABSTRACT:

Rabbit hemorrhagic disease (RHD), is worldwide highly infectious disease of rabbits species *Oryctolagus cuniculus*. RHD is one of the major viral diseases which threaten rabbit population, caused by a *calicivirus* (genus *Lagovirus*). Multidisciplinary approach study on natural infection with rabbit hemorrhagic disease virus (RHDV) in foreign rabbit breeds from some provinces in Egypt was carried out. The postmortem and histopathology investigations in naturally infected rabbits showed hemorrhagic rhinitis with submucosal hemorrhages in trachea, bronchi and bronchioles. The lungs showed characteristic scattered hemorrhages, liver was congested friable with reticular pattern of necrosis. Focal hemorrhages were seen in kidneys and visceral organs. Bacteriological examination showed negative results of *Pasteurella multocida* isolation. Hemagglutination assay (HA) was performed on supernatants from liver homogenates resulted in similar positive hemagglutination by human O type and sheep blood. Reverse Transcriptase PCR was applied using specific primers for genomic region encoding the capsid protein VP60. The amplified cDNA was given size of approximately 540bp. Finally, our findings describe the pathological and molecular detection of RHD. Exclusion of pasteurellosis reinforced the results and hemagglutination assay with erythrocytes from mammalian and human O type was essential for identification of RHDV. RT-PCR proved to be confirmatory for the diagnosis of the RHDV.

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1. INTRODUCTION

Rabbit haemorrhagic disease (RHD), is highly contagious and acute fatal disease of rabbits of species *Oryctolaguscuniculus*. The causative agent of RHD is calcivirus, also known as rabbit calicivirus disease (RCD) a positive-sense single stranded RNA virus. RHD outbreaks were first reported in China (Lui *et al.*, 1984), the disease was introduced into Egypt in 1992 (Ghanem and Ismail, 1992) and (Salem and El-Ballal, 1992). Subsequently, outbreaks were recorded in several governorates (El-Mongy, 1998 and Mostafa, 2001). RHD is a destructive agent for industry through economic losses in rabbit production in Egypt due to high morbidity and mortality (Mohamed 2009; Fahmy *et al.* 2010).

RHD is characterized by high morbidity and mortality. Infection occurs in rabbits of all ages but clinical disease is only observed in adults and young animals older than 40-50 days (Capucci et al., 1991). RHDV threatens rabbit industry through economic losses in rabbit production in Egypt due to high morbidity and mortality (Mohamed 2009; Fahmy *et al.*, 2010).

The disease outbreak was reported in most of Egyptian provinces as Sharkia in1991 with drastic losses (Ghanem and Ismail 1992). Subsequent disease occurred in Kaluobia province (Sharawi 1992), in Assuit Province during winter of 1992 (Salem and El Ballal 1992). In 1993, the disease was recorded in Minia and Sohag provinces with high mortality rate (El Zanaty 1994). Abd El Ghafar *et al.* (2000) recorded incoordination, convulsion and epistaxis with vaginal bloody discharge of impact rabbits.

For two decades, the problem of RHD was regarded endemic disease in Egypt. Infection was taken place despite of different vaccination regimes were followed. Outbreaks were recorded in vaccinated rabbit colonies (Metwally and Madbouly2005); (Abd El Lateff 2006); (Ewees 2007) and (El Sissi and Gafer 2008). Although the causative virus was HA positive but it may be of different strain. Also, a variant strain of RHDV lacking the ability of hemagglutination was

recorded to induce outbreaks in different provinces in Egypt (El Sissi and Gafer 2008).

Pathological characterization of RHD was neglected against molecular identification despite its strength in differentiation with bacterial septicemia caused by *Pasteurella multocida*. Clinical signs of RHD were confusingly differentiated with *P.multocida* infection in Rabbits. Depression, anorexia, pyrexia, rapid respiration, coughing (snuffles) and epistaxis were commonly seen. Septicemia and congestion and ulceration of nasal mucosa, hemorrhages in lungs beside frothy exudates in trachea, multiple abscesses in lungs and congested brain were recorded (Eid and Ibraheem 2006; Suelam and Abdel 2011; Awad, 2013). Other pathological changes were confusingly with RHD like hemorrhagic exudates in the thoracic cavity, pneumonia, congestion of liver and subcutaneous abscesses were also observed in pasteurellosis infected rabbits. Pathological investigation plays an important role in identification of viral infection in Rabbit diseases (Hamed *et al.*, 2013).

The application of the reverse transcription (RT)-PCR to the detection of RHDV-specific nucleic acid has been described by several authors (Gould et al., 1997; Guittre et al., 1995). On account of less sequence variation among RHDV isolates and the high sensitivity of PCR, RT-PCR represents an ideal rapid diagnostic test for RHD and represented 10^4 fold more sensitive than ELISA (Guittre *et al.*, 1995).

The currently study aims to (i) investigate the antigenic and molecular diagnostic tool that allow facilitated detection of circulating field RHDV during 2015. (ii) Providing pathological characterization of naturally infected viral rabbit hemorrhagic disease.

2. MATERIALS AND METHODS

2.1 Samples

Thirty freshly dead foreign rabbit-breeds (100-200 days old) suspected to encounter RHD infection were collected from local farms in Alexandria, Giza and Kaliobia Provinces.

2.2 Bacterial isolation

Thirty nasal swabs and internal organs (heart, liver, lung and intestine) were collected from animals and preserved at -20°C for bacterial isolation. Isolation and Identification of *P. multocida, E .coli* was done according to standard methods (Lee and Arp 1998). All the collected samples were pre-enriched in buffered peptone water (Oxoid Ltd, UK) and after incubation at 37°C for 24 hours they were inoculated simultaneously on Dextrose starch agar, sheep blood agar and MacConkey agar (Oxoid Ltd,UK), then all plates were incubated aerobically at 37°C for 24 hours. Thereafter, selected colonies were identified morphologically, microscopically for observing bipolar rod and biochemically according to the standard protocols(Quinn et al. 1994).

2.3 Histopathological examination

Samples of liver, heart, trachea, lungs and kidneys were collected and fixed in 10% buffered neutral formalin. The fixed tissues were embedded in paraffin, sectioned at 4 µm thick and stained with haematoxylin and eosin H&E. The slides were examined under light microscope.

2.4 Sample preparation for hemagglutination

A suspension of liver homogenate was prepared in 10% phosphate buffered saline (PBS) at pH 7.2–7.4, filtered and clarified by centrifugation at 5000xg for 20 minute according to the method described by (OIE, 2010). At this stage, the supernatant can be directly examined by the hemagglutination HA test and or stored at -20°C for further investigations. Samples with the highest HA titer will be used for RT-PCR.

2.5 Erythrocyte suspension

Blood samples from human O type (purchased from Vaccera) and sheep were mixed with an anticoagulant 4% sodium citrate (one part to three parts blood) and transferred slowly to a large, conical centrifuge tubes for washing. An equal amount of PBS at pH 7.0-7.2 was added and the suspension was centrifuged as 500 xg for 5 min. The supernatant was poured off and 20-30 volumes of PBS were added to the packed cells. The cells were resuspended gently to be ready for use.

2.6 Hemagglutination test

Hemagglutination was performed to identify the virus according to Chasey et al.1995 in micro titration multiwell-plate with 96 V-shaped wells. Briefly, 50 µl of PBS (pH 7.0 - 7.2) was dispensed into each well and then 50 µl of the supernatant was added in the first well. A two fold dilutions of volume of the supernatant were made then 50 µl of the prepared 0.5% suspension of erythrocytes was dispensed. The plate was incubated at 4°C. After 1hr of incubation, the hemagglutination titer was taken as the reciprocal the highest dilution producing complete agglutination of erythrocytes.

2.7 RT- PCR

RHDV RNAs were extracted from liver samples (highest HA titer) suspension with RNeasy (QIAGEN, Germany) and A single-step RT-PCR with the following primers specific for the PV60 gene: [forward: 5'-GAG-CTC-GAG-CGA-CAA-CAG-GC-3]', [reverse: 5'- CAA-ACA-CCT-GAC-CCG-CGA-AC-3'] (Guittre *et al.*, 1995) produced by Metabion® Company, Germany. cDNA synthesis was performed with RT–enzyme Access Quick RT-PCR system kit (Cat No. #A1850, Promega®) in a 25µl reaction mixture containing 12.5µl of kit-supplied mix and 20pmol of specific RHDV primers, 0.1µl from Access quick RT- Enzyme , 4.5µ DEPC water and 5µl of each RNA and control RNAs were amplified using thermal cycler ABI2720. The thermal program started with RT step at 50^0C for 30min. Followed by initial denaturation at 95^0C for 1min, then 35 cycles of (95^0C for 1 min, 56^0C for 1 min, and 72^0C for 2 min), a final extension step at 72^0C for 10 minutes on thermocycler. The amplified PCR reaction mixture is loaded into 1.5% agarose gel (molecular biology grade) electrophoresis on, visualized by ultraviolet transillumination stained with ethidium bromide

3. RESULTS

3.1 Clinical signs

The clinical signs of disease were characterized by depression, anorexia, pyrexia, rapid respiration and epistaxis. Hemorrhagic foamy discharge from the nostrils and vagina were observed in some cases. The mortality rate ranged from 50-60%. It was common that infected RHDV rabbits died suddenly without any clinical manifestations.

3.2 Postmortem lesions

The postmortem findings in naturally infected rabbits included hemorrhagic rhinitis with muffle of bloody stained and frothy exudates in trachea, bronchi and bronchioles. The lungs showed characteristic punctuate hemorrhages and blood tinged fluids were seen in thoracic cavity. Liver was congested friable with reticular pattern of necrosis. Minute hemorrhages were seen in visceral organs and intestinal tract were seen congested and hemorrhagic (Fig. 1a, b).

3.3 Histopathology

Histopathology findings in naturally infected rabbits included hemorrhagic tracheitis, submucosal hemorrhagic sacs in trachea and sloughing of mucosal epithelium (Fig. 1c, d). Lungs showed characteristic scattered hemorrhages, congested pulmonary blood capillaries, alveolar sacs were severely obstructed with esinophilic exudates filled with mononuclear cells and compensatory giant alveolar sacs were observed (Fig. 1e). Heart showed hemorrhagic myocarditis characterized by streaks of red blood cells diffusely distributed among myocardium (Fig. 1f). Liver showed multifocal hemorrhages scattered in hepatic parenchyma (Fig. 1g). Multifocal mononuclear cells aggregations associated with focal coagulative necrotic foci were observed (Fig. 1h). Kidneys showed massive hemorrhages, with mononuclear cells diffusely infiltrated in renal cortex (Fig. 1i). Glomerulonephritis characterized by proliferative mesangial cells were observed (Fig. 1j).

3.4 Bacterial isolation

Bacteriological samples of nasal swabs and internal organs to detect the possible infection of pasteurellosis were negative results.

3.5 Hemagglutination test (HA)

The end point HA titer expressed as $\log_2$which ranged from 2^4 to 2^7 in examined samples. All samples were positive HA but affinity of hemagglutination to human blood type O was higher than sheep erythrocytes (Table 1).

Table 1: HA titers of liver homogenate samples of rabbits from RHD field infection against human type O and sheep erythrocytes.

province	No. of samples	No of samples / (HA titer to human O type $\log_2$)	No of samples / (HA titer to sheep RBCs $\log_2$)
Alexandria	10	4/(5)	
		3/(6)	7/(4)
		3/(7)	3/(6)
Giza	10	3/(5)	
		2/(6)	5/(5)
		5/(7)	5/(6)
Kaliobia	10	1/(4)	
		3/(5)	4/(4)
		5/(6)	6/(5)

1/(7)

3. RT-PCR

The amplified PCR product of purified RNA extracted from liver of infected rabbits with RHDV by RT-PCR was visualized by UV in agarose gel electrophoresis revealed a size of 540 bp with marker 100bp DNA ladder. No amplification was shown in negative control (Fig. 2).

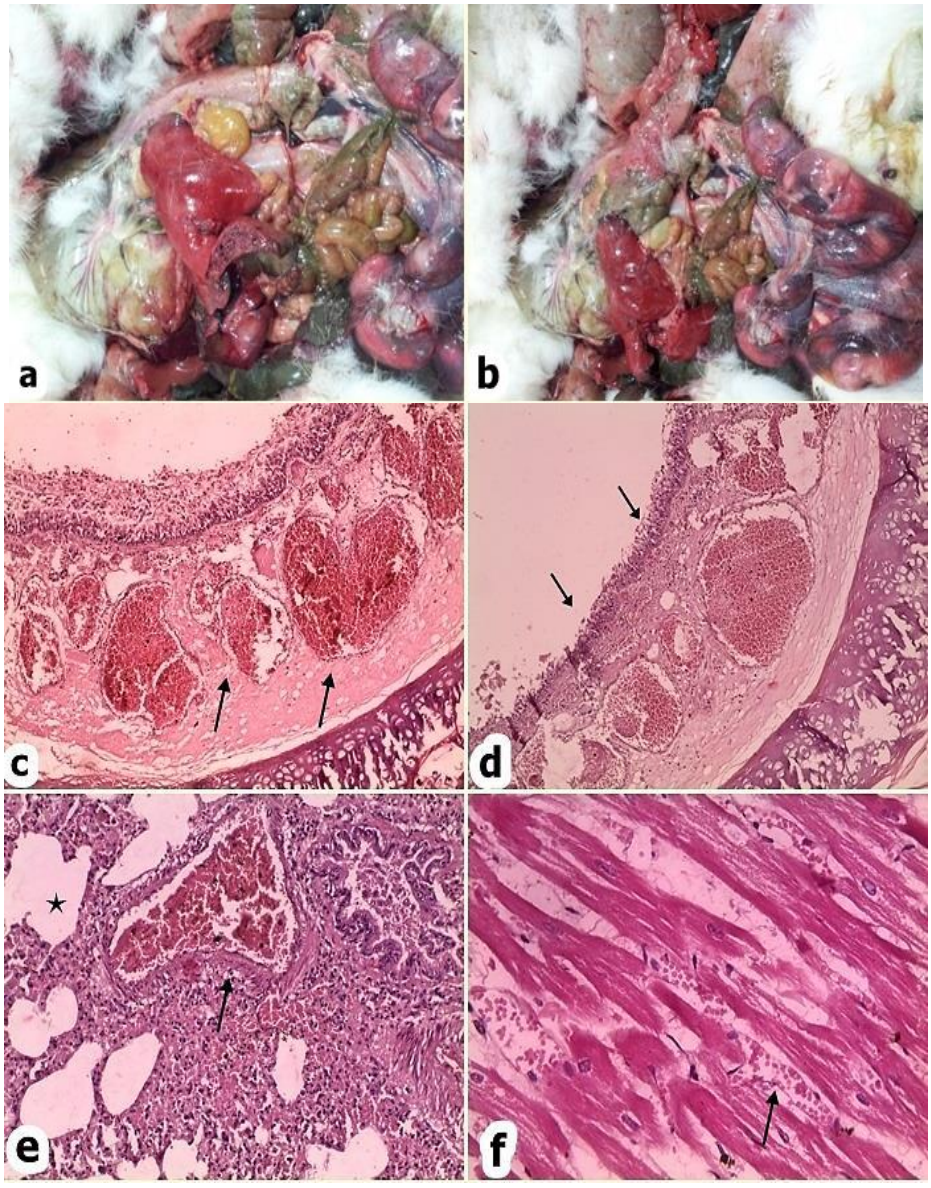

Figure 1: Gross and histopathology of RHD: (a, b) Gross appearance of RHD naturally infected rabbits showing severe congested visceral organs. (C) Trachea is showing submucosal hemorrhages (arrows, x200). (d) Trachea is showing sloughed mucosal epithelium (x200). (e) Lung is showing severe interstitial pneumonia, hemorrhages (arrow) compensatory alveoli (star, x200). (f) Heart is showing hemorrhagic myocarditis (x200). (g) Liver is showing dilated sinusoids diffuse hemorrhages, focal hemorrhages (arrow, x100).

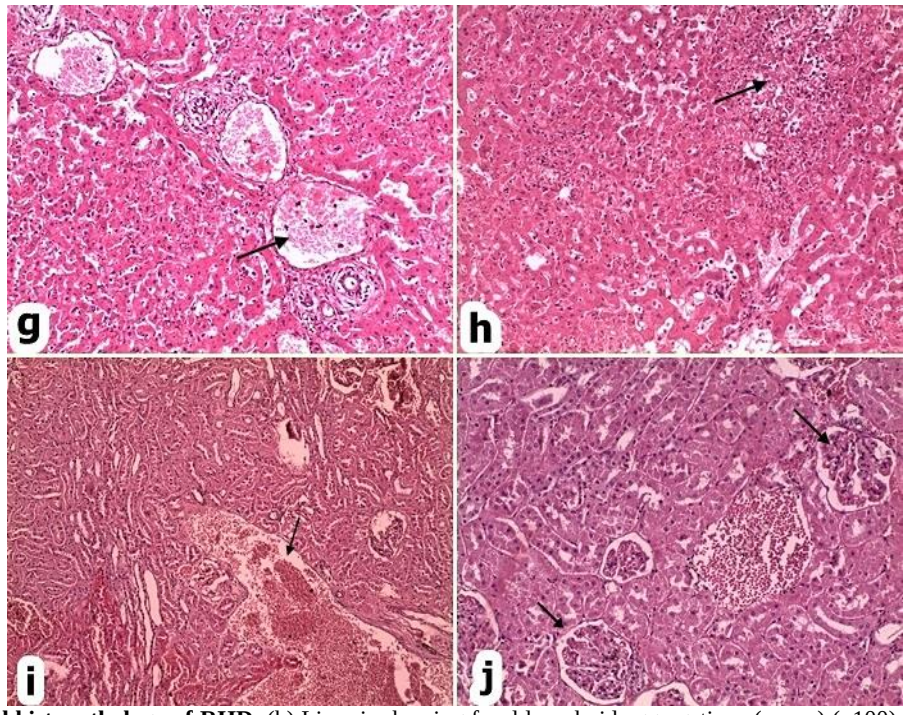

Figure 1: Gross and histopathology of RHD: (h) Liver is showing focal lymphoid aggregations (arrow) (x100). Kidney is showing focal hemorrhages (arrow) (x100). (J) Kidney is showing glomerulonephritis (arrows, x200)

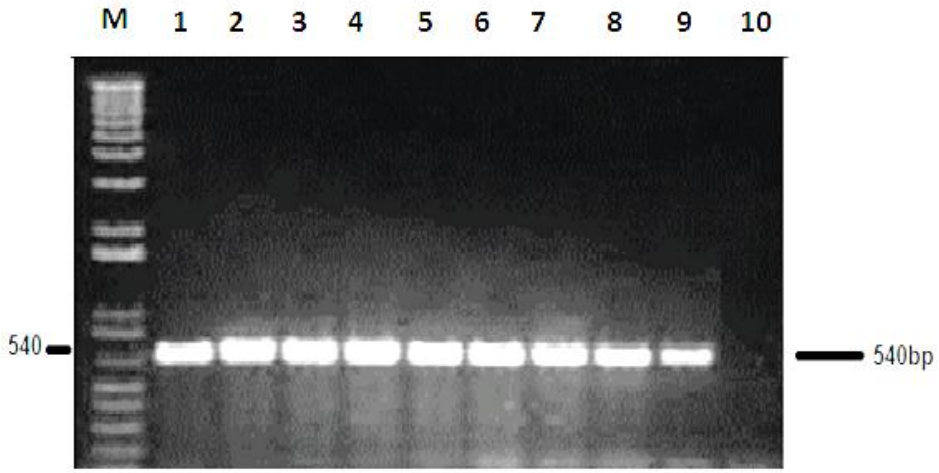

Figure 2: RT/PCR products of 540 bp fragment of VP60 gene using 1% agarose stained with ethidium bromide electrophoresis Lane M: 100bp DNA ladder marker, Lane 1 to lane 9 positive amplicon of expected molecular weight 540 bp , Lane 10: negative control (normal liver).

4. DISCUSSION

RHDV exhibited clinical manifestation including symptoms as pyrexia and dullness were prominent but, respiratory signs as dyspnea and frothy bloody nasal discharge were observed in late stage. The most severely affected organs were trachea, lungs, heart, kidneys and liver. Liver was brownish, red and fragile, often with a marked lobular pattern (Okerman, 1994). Lungs were severely congested, with pronounced cellular exudates mainly mononuclear cells giving the typical picture of RHDV infections. Heart were congested with characteristic hemorrhagic myocarditis also, Kidneys showed severe hemorrhages with widespread hypercellularity caused by both infiltrating mononuclear inflammatory cells and proliferation of endothelial and mesangial cells which forms focal proliferative glomerulonephritiswhich were characteristic to RHDV infections. The presence of the virus was evidenced by HA, histopathology, Electron

microscope on liver homogenates and RTPCR then, concluded that the RHDV adversely affects the liver, kidneys, spleen and lungs, leading to severe mortalities (Hamed *et al*.,2013).

Clinical signs and histopathological examination exhibited the picture of RHD, and exclusion of probable *Pasteurella multocida* co-infection by bacterial isolation proved that the RHDV infection was the initial pathogen. The justification of absence of *E.coli* and *P. multocida* isolation might be attributed to misuse of broad spectrum antibiotics applied by the owners.

RHDV utilized host cell- surface hemagglutinins as receptors for cell attachment and tissue colonization and a large number of pathogenic species depend on these interactions for infection, their glycan partners on mammalian cell surfaces (receptors) and insights into the molecular interactions that take place. Viruses express an enormous number of glycan-binding proteins or lectins. Many of these microbial lectins were originally detected based on their ability to induce hemagglutination (Varki et al., 2009). Tissue obtained from infected rabbits, mainly the liver, which is the best organ of choice for viral identification in which it contains the highest viral titre. The most severe lesions are detected in liver, trachea and lung.

The first test developed for detecting RHDV was the hemagglutination test (Pu et al., 1985), which is based on the ability of RHDV to agglutinate human O type erythrocytes (Liu et al., 1984) and other mammalian erythrocytes as sheep blood cells (Sahar et al., 2011). This test was widely used by Chinese and European scientists as a screening method (Calvete et al., 2002). However, the sensitivity and specificity of this method appear to be unsatisfactory. Investigations estimated the specificity of the test to be 92%, and its sensitivity to be in the order of 80 to 90% (Capucci et al., 1991).

The most sensitive and specific method for detection of RHDV is the use of the reverse transcriptase polymerase chain reaction (RT-PCR) to detect viral RNA (Lenghaus et al., 1994) examined liver samples of rabbits positive with RT-PCR at dilutions up to 10^{10}. RT-PCR also has been used successfully to detect viral RNA in rabbit serum (Moss et al., 2002). Moreover RT-PCR test demonstrates about 98.7% homology in N-terminal portion of the capsid protein which is the highly conserved portion of RHDV (Guittre et al., 1995).

5. CONCLUSIONS

For diagnosis of RHD virus, it is respectable to mention, that Histopathology is doing a strong role in proper diagnosis and concluded that the RHDV adversely affects the lungs, heart, liver and kidneys leading to severe mortalities. RHDV hemagglutination of sheep blood was less sensitive than human O type. Molecular detection of RHDV by RT-PCR proved to be highly sensitive and confirmatory in diagnosis.

6. ACKNOWLEDGEMENT

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