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Immunological and Virological Studies on Rabbit Hemorrhagic Disease Virus

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Abstract: Studies regarding natural infection with rabbit hemorrhagic disease virus (RHDV) in native and foreign rabbit breeds from different geographic locations in Egypt were undertaken in order to detect the possible presence of RHDV and to evaluate various diagnostic techniques. The virus was isolated from 10% liver homogenates of 20 freshly dead rabbits (100-200,day-old). Confirmation of the isolated virus as RHDV was based on hemagglutination assay (HA), hemagglutination inhibition (HI), propagation *in vitro* cell line designated DJRK cell line (RK-13) and electron microscopy (EM). HA test performed on supernatants from liver homogenates resulted in 85% of the samples being positive by human ″O″ type whereas the positivity of the tested samples by sheep, pigeon and chicken erythrocytes were 90%, 80% and 45%, respectively. Variable HI log titers were obtained with the examined liver homogenates and culture supernatants. RK-13 cell culture investigated to determine the causative agent has provoked striking cytopathic changes on both first and second passages. Furthermore, electron microscopy emphasized the morphogenesis of RHDV in infected RK-13 cell cultures which proved uniform, isometric, unenveloped viral particles about 40 nm in diameter, characteristic to calicivirus family. Collectively, our above mentioned findings describe quick, easy and available technical procedure concerning hemagglutination assays with erythrocytes from other mammalian and avian species rather than human ″O″ type essential to the identification of RHDV. Finally, the successful adaptation of RHDV to cultured RK-13 cell line together with electron microscopy will provide the possibility of large-scale production of cell-cultured calicivirus vaccine which is important for functional analysis and better vaccine development in the future.

Key words: Rabbit Hemorrhagic Disease Virus · Calicivirus · Hemagglutination assays · RK-13 · Electron microscopy

INTRODUCTION

Rabbit hemorrhagic disease virus (RHDV) infection is an extremely contagious and often fatal viral disease of domesticated and wild rabbits. The first observation of a new rabbit hemorrhagic disease virus, causing severe losses in rabbits was made in China in 1984 [1]. In few years, the disease expanded and became endemic in most European countries [2]. Recent outbreaks have been recorded in North Africa, America, Asia, Australia, Egypt and New Zealand [1, 3-6]. Nowadays, the disease has a world wide geographic distribution, often associated with liver necrosis, hemorrhages and high mortality [6].

RHDV is highly lethal with mortality rates in adult animals that vary from 60 to 90% whereas infected rabbits below the age of 2 month usually survive [7, 8]. Death is the result of a widespread circulation dysfunction associated with disseminated intravascular coagulation and necrotizing hepatitis lesions [8, 9]. Large quantities of virus particles are found in several organs, especially the liver, which is considered the major site of virus replication [7, 8, 10, 11]. Yet very little is known about the pathogenesis of naturally occurring RHDV infections and identification of the cellular receptor(s) used by the virus to establish infection would lead to a better understanding of the pathogenesis of RHDV [12].

The etiological agent was designated as a single-stranded positive-sense RNA virus of the family *caliciviridae* named rabbit hemorrhagic disease virus [13]. The entire genome of RHDV contains two open reading frames (ORFs); the longer one (ORF1) encodes a polyprotein which produces several non-structural proteins including a helicase, a protease, a polymerase and the capsid protein (VP60) at its C- terminus after

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proteolytic cleavage, while the shorter (ORF2) encodes a structural protein (VP10), in fact the main antigenic determinants of RHDV are located on the C- terminal end of the VP60 [14,17].

RHVD isolates detected in different continents, despite non hemagglutinating variants [18, 19] showed a high degree of similarity with regard to their morphology, pathogenicity, immunity and antigenic properties and displayed low level of genetic variation [20,22].

RHDV core-like particles (CLP) are found in liver and spleen but not in blood stream in some cases when the subacute/chronic RHDV is followed by death, or more often, by recovery, [23, 24]. The liver, which contains the highest viral titers, is the tissue of choice for viral identification in suspected cases of RHD. The spleen and serum, which are also rich in virus, can serve as alternative diagnostic materials [25].

The Diagnosis of RHD is based on clinical symptoms, post-mortum examination, histological changes, hemagglutination (HA) and hemagglutination inhibition (HI) tests [1, 26-32] and the immunoelectron microscope [15, 33, 34].

Hemagglutination titers have been detected in liver tissue of infected rabbits as early as 2 h post infection and observed to rise rapidly, reaching a maximum (as high as 10×2^{14} to 10×2^{16}) at death [35, 36]. The HA test, using either human ″O″ type or guinea pig erythrocytes, is used for routine laboratory diagnosis of RHD. In this test, agglutination at an end-point dilution of greater than 1/160 is considered positive. Moreover, positive results are also confirmed by ELISA, electron microscope, or immunostaining [25].

Since 1984, a number of attempts have been made to cultivate RHDV in various cell culture systems and primary rabbit cells (kidney, liver, lung and testis) as well as cell lines (PK-15, BHK-21, MA-104, IBRS-2, HeLa and VERO) have been used to cultivate RHDV [28, 37, 38], none of them, however, has been successful in the adaptation of RHDV to cell cultures. Recently, an epithelial type cell line, named Du and Ji Rabbit Kidney (DJRK) was established from primary rabbit kidney cells upon serial passages indicates that RHDV is adaptable to DJRK cells [39].The detection and identification of RHDV can be accomplished on the basis of viral morphology by immunoelectron microscopy-serotyping directly on the EM specimen grid-type-specific identification and can serve as an important aid for rapid virus diagnosis [15, 33, 34, 40,44]. RHD virion also exhibits characteristic cup-shaped depressions on the surface with T = 3 icosahedral symmetry, which is revealed by negative staining electron microscopy [45].

Because of the sudden onset and rapid progression of RHD, whereas the importation of live rabbits or rabbit products from RHD endemic areas remains a potential source for virus introduction although prevention, eradication and vaccination (or a combination) are available for managing this disease, it seems that virological or serologic investigation in both domestic and free ranging rabbits to confirm disease existence is still required. Consequently, this study aimed at verifying rapid and advanced laboratory diagnostic techniques to identify viral isolates of RHDV in supernatants from liver tissues of infected rabbits arising from a natural field infection using hemagglutination and hemagglutination inhibition assays. Moreover, subsequent investigation of the mechanisms of RHDV replication and pathogenesis *in vitro* culture system (RK-13 cell line) and the negative staining electron microscope were undertaken.

MATERIALS AND METHODS

Sampling: Freshly dead native and foreign rabbit-breeds (100-200day-old) suspected to encounter RHD infection were collected from local farms in Giza and Kaliobia Provinces-Egypt during years of 2010 & 2011. Necropsy findings showed characteristic pathological lesions including pale, fragile liver, often with accentuation of the lobular markings and interspersed with hemorrhages, an enlarged, congested spleen, reddish speckled kidneys and lungs with hemorrhagic lesions of different degrees. Twenty liver samples collected from diseased animals were preserved at -20°C for diagnostic investigations.

Sample Preparation for Virus Isolation: A 10 per cent suspension of liver homogenate was prepared in phosphate buffered saline (PBS) at pH 7.2–7.4, filtered through cheesecloth and clarified by centrifugation at 500g for 20 minute and 6000g for 60 min according to the method described by OIE [47]. At this stage, the supernatant can be directly examined by the HA, HI tests and or stored at -20°C for further investigations.

Erythrocyte Suspension: Freshly collected blood samples from human ″O″ type, chicken, sheep and pigeon were mixed with an anticoagulant 4% sodium citrate (one part to four 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 and

the centrifugation step repeated once more, again pouring off the supernatant. The cells were then used to prepare 0.5% suspension (0.5 ml packed cells to 100 ml PBS at pH 7.0-7.2) [48].

Hemagglutination Test: Hemagglutination was performed to identify the virus according to Chasey *et al*. [18] 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 1 hr of incubation, the hemagglutination titer was taken as the reciprocal of the highest dilution producing complete agglutination of erythrocytes.

Hemagglutination Inhibition Test: Hemagglutination inhibition technique was applied for the serological diagnosis of RHDV according to OIE [47] in micro titration multi-well plate with 96 V-shaped wells at room temperature. Liver homogenates and culture supernatants after the 2nd viral passage were tested and titrated against rabbit hyperimmune serum obtained from experimentally vaccinated rabbits. 25 µl of PBS (pH 7.0 - 7.2) was dispensed into each well then 25 µl of supernatant was added in the first well and a two fold dilutions of the supernatant were made. Rabbit hyper immune serum was prepared against inactivated rabbit hemorrhagic virus vaccine (purchased from VET.SER & Vacc. RES. INST, Cairo, Egypt). 25 µl of rabbit hyperimmune serum was added to each well and the plate was incubated at 25°C for 30-60 min then 25 µl of human ″O″ type at 0.5% concentration was added to each well and incubation was made at 25°C for 30–60 min. Rabbit hyperimmune serum was titrated with each test to ensure that 8 HA/ 25 µl were used and included positive and negative serum controls. The serum titer is the end-point dilution showing inhibition of HA.

Cell Culture: Established cell line of rabbit kidney cell (RK-13) [purchased from tissue culture unit in the holding company of biological products and vaccines (VACERA)], in minimal essential medium (MEM) containing 5% fetal calf serum; 100 IU/ml penicillin; 100 µg/ml streptomycin; 100 µg/ml gentamicin; and 5 µg/ml amphotericin (fungizone) was used. The inoculum consisted of the supernatants from ten selected liver homogenates designated [2, 3, 6-9, 14, 16, 18, 20] processed for virus isolation according to OIE standard protocol [47], in MEM with 2% calf serum supplemented with antibiotics which had been filtered through sterile syringe filter sized 0.45um. Cells ($5x10^5$ cells/well) were seeded in tissue culture flasks (Nunc and Life Technologies) and grown to 80% confluence. The supernatants were freeze-thawed twice to ensure release of virus from cells and centrifuged at 600 xg for 10 min. Tissue culture flasks showing confluent cell growth were inoculated with 0•5-ml volumes of the filtered supernatants [49]. These culture flasks were incubated for 24 h at 37°C and observed under inverted cell culture microscope for the cytopathic effect (CPE).

Preparation of RHD-Virions for Electron Microscopy Examination: The procedure was performed as mentioned by Angelika *et al*. [50]. Virions were isolated from the medium of infected RK-13 cells 24–72 hr post-inoculation (p.i.). The medium was centrifuged at 800 xg for 10 min and viral particles of infected RK-13 cells were filtrated through syringe filter of 0.45um pore diameter. For electron microscopy, aliquots of virus suspension (~3uL) were absorbed onto glow-discharged, carbon-coated copper grids, negatively stained with 1% uranyl acetate and air-dried. Specimens were transferred in a Jeol electron microscope (Model JEM-1230 EM attached with CD camera) operated at 100 kV. Images were recorded at a nominal magnification of 50000 times at 2um under focus, the data of 2um under focus were used to calculate the initial centre and orientation of each virus particle image.

RESULTS

Hemagglutination Test: The hemagglutinating activity of rabbit haemorrhagic disease virus isolated from liver homogenates with human ″O″ type and the other species was illustrated in table (1), which revealed that 85% of the samples were found positive by human ″O″ type whereas the results of the tested samples performed on sheep, pigeon and chicken erythrocytes were 90%, 80% and 45%, respectively. Table (2) showed results of HA regarding positivity with mammalian and avian erythrocytes as follow; human ″O″ type, seventeen out of twenty samples were positive, of the seventy positive samples, 3 (17.6%) were positive with HA titers 8 up to 10 $\log_2$, 6 (35.2%) 4 up to 6 $\log_2$ and 7 (41.1%) remained positive 1 up to 3 $\log_2$.

Table 1: HA titers of liver homogenate samples of rabbits from RHD field infection against different mammalian and avian erythrocytes.

Sample number	Human "O" type (log_2)	Chicken RBC's (log_2)	Sheep RBC's(log_2)	Pigeon RBC's (log_2)
1	2	0	1	2
2	4	2	5	6
3	4	0	2	2
4	2	0	2	0
5	3	0	2	3
6	8	2	1	3
7	10	0	0	2
8	6	1	1	2
9	8	6	8	9
10	2	0	2	1
11	3	2	0	2
12	3	0	0	1
13	0	0	0	0
14	4	0	2	1
15	0	1	1	1
16	5	5	4	7
17	0	0	6	5
18	4	1	7	6
19	1	0	3	1
20	3	2	2	1

Table 2: Percentages of positive HA titers of liver homogenate samples of rabbits from RHD field infection against mammalian and avian erythrocytes

Species/Erythrocytes	% of Positivity of 20 tested samples	Number and % of Positive samples	HA titres (log_2)
Human "O" type	85%	3 (17.6%)	8 up to10 log_2
		6(35.2%)	4 up to 6 log_2
		7(41.1%)	1 up to 3 log_2
Sheep	90%	2(11.1%)	7 up to 9 log_2
		3(16.6%)	5 up to 6 log_2
		13(2.3%)	1 up to 3 log_2
Pigeon	80%	3(18.7%)	6 up to 8 log_2
		3(18.7%)	3 up to 5 log_2
		10(62.5%)	1 up to 2 log_2
Chicken	45%	2(22,2%)	5 up to 6 log_2
		7(77.7%)	1 up to 2 log_2

Table 3: HI titers of the investigated 20 liver homogenates of rabbits from RHD field infection against human "O" type

Sample number	Human "O" type(log_2)
3 &,15	1
5	2
1, 2, 6, 8,13 & 17	3
4, 7, 12,14 & 20	4
11,16 & 19	5
10 & 18	6
9	7

Table 4: HI titers of ten selected culture supernatants after the 2nd passage of rabbits from RHD field infection

Sample number	Log2
2	More than log12
3	More than log12
6	More than log12
7	More than log12
8	Log8
9	Log8
14	Log8
16	Log8
18	More than log12
20	More than log12

Table 5: Results of RHDV-isolates trial on RK-13 cell line from ten selected liver homogenates of rabbits from RHD field infection

Sample number	CPE effect on 1st passage 24-72hr post inoculation	CPE effect on 2nd passage 12-24hr post inoculation
7	positive (+)	Positive (+ + +)
9	Positive (+)	Positive (+ + +)
14	Positive (+)	Positive (+ + +)
20	Positive (+)	Positive (+ + +)
2	negative	negative
3	negative	negative
6	negative	negative
8	negative	negative
16	Positive(+)	Positive(+)
18	Positive(+)	Positive(+)
Normal control RK-13	negative	negative

HA titers with sheep erythrocytes resulted in eighteen positive samples which could be summarized as: 2 (11.1%) 7 up to 9 log2, 3 (16.6%) 5 up to 6 log_2 and 13(2.3%) 1 up to 3 log_2. Moreover, 16 out of the 20 tested samples using pigeon erythrocytes expressed positive HA titer; 3 (18.7%) 6 up to 8 log_2, 3(18.7%), 3 up to 5 log_2 and 10(62.5%) 1 up to 2 log_2, while chicken erythrocytes recorded only nine positive samples with HA titers as 2 (22.2%) 5 up to 6 log_2 and 7 (77.7%) 1 up to 2 log_2, respectively.

Hemagglutination Inhibition Test: Liver homogenates tested by HI test produced HI titer ranged from 1-7 log (Table 3). Culture supernatants (after the 2nd passage) revealed that six samples had HI titer more than log^{12} while four samples expressed HI titer equal to Log^8 (Table 4).

Cytopathic Effect in RK-13 Cell Line: The propagation of the causative agent of RHD attempted in RK13 cells using the homogenated livers which gave positive HA titers against human ″O″ type resulted in cytopathic effect which was more clear at 12-24hr on the 2nd passage than the 1st passage (Table 5). The cytopathic effect on the 1st passage after 72 hr of incubation appeared as rounding and aggregation of some shrunken epitheloid cells (Figure 1). However; on the 2nd passage, the cytopathic effects of affected cells showed marked necrosis characterized by vacuolation, rounding of ruptured cell membranes and partial detachment of cells (Figure 2). Cellular debris could still be detected in necrotic cells, destruction and disassociation of cell components, loss of cellular organization that were also numerous and evident at the 2nd passage (Figure 3). Later, all cells were affected and the cell monolayer was detaches completely (Figure 4) when compared with that of the non-infected normal RK13 (Figure 5).

Electron Microscopy Examination of RHD Virus: Virus particles with icosahedral symmetry and 40 nm diameter were evident by electron microscopy from purified supernatant of infected RK-13 cell line. These virions displayed a structural surface consisting of regularly arranged cup-shaped depressions (Figure 6). Size and morphology were consistent with classical calciviruses. No virus particles were observed in a matching fraction from uninfected RK13-cell line.

Fig. 1: RK-13 (rabbit kidney) cells infected with RHDV (early CPE on 1st passage) showing rounding and aggregation of some shrunken epitheloid cells (X 400).

Fig. 2: RK-13 (rabbit kidney) cells infected with RHDV (CPE on 2nd passage) showing marked necrosis and partial detachment of cells (X 400).

Fig. 3: Cellular debris disassociation of cell components and loss of cellular organization (CPE on 2nd passage) (X 400).

Fig. 4: Complete detachment of the cell monolayer (CPE on 2nd passage) (X 400).

Fig. 5: Epitheliod-shaped cells form a dense confluent monolayer of normal rabbit kidney cells (X 400).

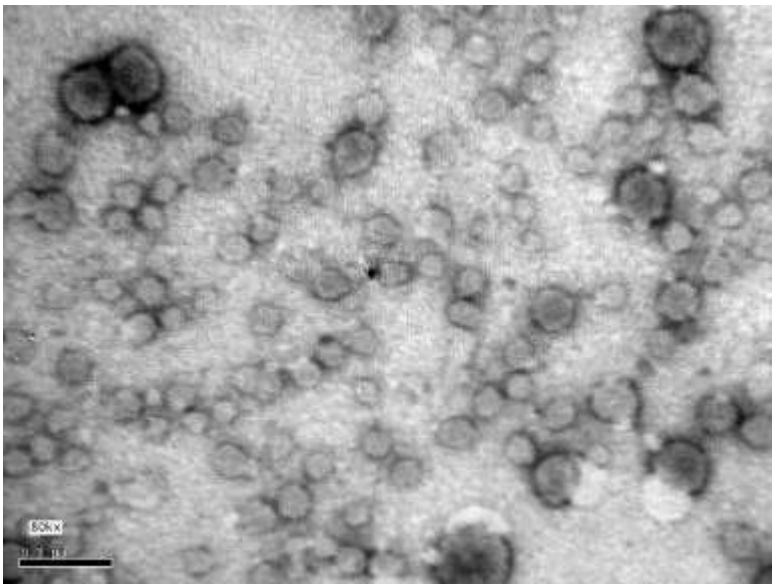

Fig. 6: Electron micrograph of RHDV viral particles characterized by cup-shaped surface depressions arranged in icosahedral symmetry (X 50000).

DISCUSSION

Rabbit viral hemorrhagic disease is a highly contagious, highly fatal, peracute and acute viral disease of both wild and domestic rabbits caused by rabbit hemorrhagic disease virus (RHDV). Viruses, exploit 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 [51]. Tissue obtained from infected rabbits, mainly the liver, which contains the highest concentration of the viral particles is the only source of viral antigen used in laboratory diagnostics and vaccine production, thus testing for hemagglutination activity in processed liver samples is one of the cornerstones for rapid diagnosis of RHDV outbreaks in rabbits [52].

Many investigators reported that RHD virus has a great ability to agglutinate human ″O″ type erythrocytes [1, 53-55], such a distinctive characteristic of RHD virus to agglutinate human ″O″ type erythrocytes depends on the presence of a histo-blood group antigens (ABH antigens) which was explained by Ruvëon *et al*. [55] who stated that RHD virus hemagglutinin receptor on human red blood cells corresponds to a developmental antigen which is not

expressed on fetal cells and is mainly carried by poly glycosylceramides and the glycolipid nature of the receptor on human red blood cells suggests that the carbohydrate moiety could be recognized by the virus capsid protein. However, carbohydrate antigens of the histo-blood group family are developmental antigens that can be shared among various mammal species [56,58].

As shown in tables (1,2), the liver suspension containing virus strongly agglutinated both human ″O″ type and sheep erythrocytes with percentages of 85% and 90% of total tested samples at room temperature, respectively. In addition, we tested whether the isolated RHD virus would bind to avian erythrocyte as strongly as they did with mammalian ones. In tables (1,2), it was observed that weak hemagglutinating reactions were seen with chicken erythrocyte with a percentage of 45% of total tested samples indicating that their cell receptors did express much smaller amounts of ABH antigens than human ″O″ type and sheep erythrocytes. In contrast, pigeon erythrocytes performed stronger HA positivity giving a percentage of 80% of total tested samples and almost possessed similar strong HA activity as mammalian erythrocytes. It should be noted that these findings are mainly related to the presence of such antigens for accomplishing hemagglutination ability which have been clearly demonstrated by Ruvëon-cluet *et al*. [12] as well.

With regard to the remained samples that were devoid of the hemagglutinating capability (i.e non-hemagglutinating) observed in this work among different host species, this was explained by Granzow *et al.* [59] who assumed that HA- negative RHDV isolate is a small particle named core like particles that arises from a truncated RHDV genome or due to defective expression. Moreover, Capucci *et al*. [27] and Barbieri *et al.* [23] mentioned that samples of normal HA- RHDV sometimes fail to hemagglutinate erythrocytes and this is related to proteolytic action and degradative processes on the RHDV capsid protein as consequence of appearance of anti-RHDV IgM and physiological clearance of the RHDV-IgM immune-complex formed in large amount at the beginning of the humoral response which occurs mainly in animals with subacute or chronic disease.

Hemagglutination inhibition test has been well documented to detect and confirm virus infection of rabbit hemorrhagic disease [16, 18, 47, 53, 60, 61]. In the current study, we pointed out that culture supernatants (after the 2nd passage) induced higher HI log titers when compared to liver homogenates. In our speculation it appears that viral propagation and multiplication on RK-13 cell line could participate in the increase of virus concentration and these observations warrant further study.

Various cell cultures, including primary rabbit cells (kidney, liver, lung and testis), as well as cell lines (PK-15, BHK-21, MA-104, IBRS-2, HeLa and VERO) have been used to cultivate RHD virus [62-64]. Indeed, the RK cell line is a mutant of the rabbit kidney epithelial cell which is highly viable, metabolically active and rapidly dividing, is suitable for the replication of viral hemorrhagic disease virus [65]. In the present study, a successful propagation of the isolated RHD virus on RK-13 cell line has been observed by the evident cytopathic effects which became more prominent after 12-24hr on the second passage. These results agree with those previously reported in literatures concerning the morphological features and morphogenesis of RHD virus as major criteria for the classification and in understanding the replication and assembly sites of the virus in infected cells of viral hemorrhagic disease [50, 65].

The results of the morphogenesis of VHDV with the localization and development of the viral antigens in cell culture indicating that the early stages of viral replication take place in the nucleus have led some research workers to conclude that RHD virus is a calicivirus [66, 67]. Contradictory opinions to our results have also been reported on the RHDV as many other caliciviruses cannot be propagated *in vitro* [52, 68, 69].

Rapid diagnosis of viruses by EM is based on the visualization and morphological identification of virus particles in samples of diseased tissues or organic fluids [44, 70, 71]. In particular, negative staining procedures are developed for visualization of thin sections and particulate samples that provides a suitable and very fast way of revealing virus particles in EM and is thus extensively used for diagnosis [72]. Our observations on results involving EM investigation pointed out that viral particles isolated from liver of RHD virus-infected rabbits presented the same morphological properties as those described by other authors [45, 67, 73], the virions were icosahedral, non enveloped 33-35 nm in diameter (size varied between 29-40 nm in our findings) and displayed a clearly structured surface consisting of regularly arranged cup-shaped depressions, being consistent with previous reports [7, 74]. The detected morphology and size are also in agreement with those recorded VHD virus as a member of the Caliciviridae family [75-81].

For diagnosis of RHD virus, it is worthy to mention that although RHDV hemagglutinates human ″O″ type very efficiently, there is a practical difficulty of obtaining and keeping and the risk from working with human ″O″ type, therefore, our results support the value to perform with other mammalian and avian erythrocytes which are able to exhibit quite good levels of sensitivity. Additionally, subsequent detection and identification of the isolated RHD virus can depend on indicators such as cytopathic effect induced by a propagated RK-13 cell line. The "open view" approach of electron microscopy permits rapid and "catch-all" detection of RHD virus and makes it especially useful in emergency situations.

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