Molecular diagnosis of rabbit hemorrhagic disease virus (RHDV)

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SUMMARY

Rabbit hemorrhagic disease (RHD) is a highly contagious and acute fatal disease of the rabbits caused by a calicivirus. RHD is characterized by high morbidity and mortality rates and spreads very rapidly by direct and indirect transmission. As there are no satisfactory growth conditions, invitro isolation of RHD virus cannot be included among the diagnostic methods. Direct detection using molecular methods like Western blotting analysis, Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR are applied for accurate and rapid diagnosis.

In this study, liver samples were submitted for RHDV diagnosis from three provinces (Giza, Kalubia and Kafr-El-Sheikh). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of extracted viral proteins revealed the presence of high molecular weight polypeptide proteins of nearly 120, 80 and 64 KDa. The low mol. wt. was nearly 40, 36, 26 and 17 KDa. The immuno-blotted picture on nitrocellulose membrane to positive antiserum was visualized similar molecular weight of major bands about 120, 80 and 63.9 KDa with several minor smaller distinct protein bands of about 40.7, 36.7, 27.6 and 16.6 KDa. RT-PCR was applied using specific primers for genomic region encoding the capsid protein (VP60). The amplified cDNA gives size of approximately 538 bp. The samples were also confirmed by 2 steps real-time RT-PCR using SYBR green PCR mix with melting temperature (Tm) at 86°C. In this study, it was proved that SDS-PAGE followed by Western blotting is specific and sensitive test while PCR and real-time RT-PCR proved to be confirmative for the diagnosis of the RHDV.

Key words: rabbit hemorrhagic disease virus (RHDV), polyacrylamide gel electrophoresis SDS – PAGE, PCR, Real time PCR.

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**INTRODUCTION**

Rabbit hemorrhagic disease (RHD) is an acute highly contagious viral disease affecting wild and domestic rabbits of all ages. RHD is caused by Rabbit hemorrhagic disease virus (RHDV), a member of the *Caliciviridae* family (*Thiel and Konig, 1999*). It is a small, positive single-stranded RNA virus with a genome of 7.5 kb and contains two or three functional open reading frames (ORFs) (*Gromadzka et al., 2006*). It is considered as a major pathogen of rabbits in many countries (*Grazioli et al., 2000; Prieto et al., 2000 and McIntosh, et al., 2007*). The disease characterized by high morbidity and high mortality (40-90%) of all ages but clinical disease is observed only in adults and young rabbits older than 40-50 days and spreads very rapidly by direct and indirect transmission. Infection can occur by nasal, conjunctiva or oral routes (*OIE, 2008*).

All known isolates of RHDV appear to belong to one serotype but like other RNA viruses, Calicivirus has a high genetic mutation rate (*Gould et al., 1997*).

Recent work suggests that RHDV in rabbit carcasses can survive for at least 3 months in the field, while virus exposed directly to environmental conditions is viable for a period of less than 1 month (*Henning et al., 2005*).

A consistent genetic and antigenic RHDV variant (RHDVa) has been identified in Italy (*Capucci et al., 1998*) and Germany (*Schirrmaier et al., 1999*). RHDVa presents amino acid changes in the surface exposed E region (AA 344-434) that contains the main antigenic epitopes of calicivirus, three times higher than in all previously sequenced RHDV isolates. However, rabbits experimentally vaccinated with RHDV vaccine were protected against challenge with RHDVa, even if with a lower efficiency (*Capucci et al., 1998*).

In Egypt, RHD has been reported for the first time during the spring of 1991 in Sharkia province with heavy losses 90% and during winter and spring of 1993 in Upper Egypt (Minia, Assiut and Sohag provinces) with mortality rate of 26.7 up to 100% in 14-16 old rabbits (*El-Zanaty, 1994*). **EL-Mongy (1998)** investigated 25 outbreaks of RHDV during 27 months from September 1994 to November 1996 in many governorates in Egypt (Giza, Cairo, Marsa-Matroh, Kalubia, Kafr-El-Sheikh, Dakahlia and Gharbia). **Ibrahim et al., (1999)** isolated RHDV and Pasteurella multocida from examined cases in three rabbits in
Egypt. The isolated virus was identified by HA, ELISA and SDS-PAGE. Daoud et al. (1998) prepared an inactivated RHDV vaccine from the local isolate of RHDV (Egypt 96) by 0.4% formalin at 37°C/48 hours adjuvant with aluminum hydroxide. Recently, Metwally and Madboully (2005) investigated 15 outbreaks with clinical picture and post-mortem lesions similar to that of RHDV in vaccinated flocks at Kafr –El-Sheikh governorate.

The incubation period varies from 1 to 3 days, and death usually occurs 12-36 hours after the onset of fever. Nervous and respiratory signs have been described in acute infection. The most severe lesions are in the liver, trachea and lungs. Petechial hemorrhages are evident in almost all organs and are accompanied by poor blood coagulation (OIE, 2008). The transmission occurs by contact with secretions or excretions of infected animals and usually oral transmission. Wild animals (Cooke, 2002), flies and other insects (OIE, 2008) are very efficient mechanical vectors. Gall and Schirrmeier (2006) described for the first time the persistence of RHDV genome for at least 15 weeks in rabbits immunized with an inactivated vaccine as well as a subunit vaccine and subsequently challenged with virulent RHDV. The results presented an evidence for the existence of carrier animals as an important factor in the epidemiology of RHD.

The capsid is composed of four structural polypeptides: VP1: 60-61 KDa, VP2: 54.7 KDa, VP3: 52 KDa and VP4: 26-28 KDa. VP1 is a major structural polypeptide which constitutes approximately 54.7% of the total viral proteins (Du, 1991). The application of RT-PCR to the detection of RHDV-specific nucleic acid has been described by several authors (Vende et al., 1995, Moss et al., 2002 and White et al., 2004).

Haemagglutination (HA) is the first routine laboratory diagnosis used for RHDV using human type “O” RBCS (OIE, 2008). So owing to arising of new non-Haemagglutinating strains of RHDV, HA test became an undependable test for diagnosis.

Therefore the aim of this study was to confirm the preliminary diagnosis RHD by clinical signs and identification of RHDV using recent diagnostic techniques as RT-PCR, real time RT-PCR and sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting as rapid and accurate methods for RHDV diagnosis.

MATERIAL AND METHODS
Sample preparation and virus purification: Nineteen Liver sam-
samples from rabbits suffering from nervous and respiratory signs were submitted for identification from three provinces (Giza, Kalubia and Kafr-El-Sheikh). They were homogenized in 10% (W/V) phosphate buffered saline solution (PBS), pH 7.2, filtered by cheese cloth and clarified by centrifugation at 5000 g for 5 min. Second centrifugation for the supernatant at 12000 g for 15 m (OIE, 2008).

Positive control and hyper immune serum against RHDV: They were kindly supplied from Vet. Serum and Vacc. Res. Inst. Abbassia.

SDS- PAGE: The proteins were denaturized for 2 minutes at 100°C in the presence of Tris, pH 6.8, 2% SDS, 2% betamercaptoethanol, and 5% glycerol. Then separated on 10% SDS- PAGE according to OIE, 2008 in vertical gel apparatus (Biometra) with running buffer and Page Ruler pre-stained protein Ladder (Fermentas, Lot. 00021165) at 90 volt for about 2 hours until the bromophenol blue dye almost reaches the lower edges of the glass plates (0.5 cm).

Western blot: The samples were prepared as described previously and concentrate viral particles by ultracentrifugation. After separation of protein by PAGE, transferred by electro blotting to nitrocellulose membrane in 25mM Tris, 192 mM glycine pH 8.3 and 20% (V/V) methanol (OIE, 2008). After transfer, the membrane is saturated for 1h with 2% bovine serum albumin (BSA), dissolved in phosphate buffer pH 7.4 and incubated for 2 hours at room temperature with RHDV antiserum diluted in PBS, pH 7.4 and 1% BSA. The membrane is washed thoroughly with PBS and incubated for 1h at room temperature with anti-species conjugate. Finally the membrane is again washed and the chromogenic substrate is added. Positive and negative controls were considered.

RT-PCR: viral RNAs were extracted from the samples of liver suspension with RNeasy (QIAGEN, Germany) and amplified using a One-Step RT-PCR kit (Cat. No.210212, QIAGEN, Germany). The reactions were carried out in 25 ml volume reaction using 5 µl of 5X-RT-PCR buffer, 1 µl of RT-enzyme provided with the kit, 1.25 µl of MgCl₂, 1 µl of each primer, 1µl of dNTPs and 10 µl of PCR-water, then 5 µl of RNAs for each sample. The RT-PCR was carried out using oligonucleotide primers according to Vende et al., (1995). The primer sets and their position, numbered according to the RHDV sequence were illustrated in Table (1). It was designed in Metabion Company, Germany.
Table 1: Oligonucleotide primers according to the RHDV sequence.

<table>
<thead>
<tr>
<th>Primer design</th>
<th>Sequence (5’ to 3’)</th>
<th>No.</th>
<th>Gene</th>
<th>Location</th>
<th>Size of amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHDV- P33(F)</td>
<td>CCACCACCAACACTTCAGGT</td>
<td>20</td>
<td>VP60</td>
<td>6473–6492</td>
<td>538 bp</td>
</tr>
<tr>
<td>RHDV- P34(R)</td>
<td>CAGGTTGAAACACGAGTGTGC</td>
<td>20</td>
<td></td>
<td>6992–7011</td>
<td></td>
</tr>
</tbody>
</table>


The amplification reaction was performed using the following temperature profile: an RT step at 50 °C for 30 minutes followed by initial denaturation at 95°C for 15 min, then 40 cycles of 95°C for 1 min, then 56°C for 1 min, and 72°C for 2 min, then a final extension step at 72°C for 10 minutes on thermocycler (Biometra, Germany). To reveal the PCR products, the amplified DNA reaction mixture is loaded into 1.5% agarose gel (molecular biology grade) electrophoresis on, visualized by ultraviolet transillumination with ethidium bromide stain (0.5 μg/ml).

Real-time PCR: RNAs were used for cDNA synthesis in 2 steps real-time PCR. cDNA synthesis was performed with RT- enzyme ACCESS Quick RT-PCR SYSTEM - RT-PCR kit (Cat No. #A1702, Promega) in a 25 ul reaction mixture containing 12.5 ul of kit-supplied mix and 20 pmol of specific RHDV primers, 0.1 ul from Access quick RT- Enzyme, 4.5 ul DEPC water and 5 ul of each RNA and control RNAs were amplified using thermal cycler ABI2720. The RT-PCR program consisted of 30 min at 50 °C and 15 min at 95 °C. The cDNA was amplified by two step real-time PCR using SYBR green PCR mix Maxima™ SYBR Green/ROX qPCR Master Mix (2X), cat#K0221 (Fermentus). The PCR reaction was carried out in a 25 ml volume. The reactions were carried out on Stratagene real time PCR machine. PCR amplification was performed using a program of 10 min at 95°C followed by 40 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C.

RESULT

The results of SDS- PAGE: presented in this study are an evidence for the existence of major structural protein and several minor distinct proteins bands. The molecular weights are approximately 64.4 and 60.4 KDa for the major (prominent) and approximately 120.4, 80.2, 40.8, 36.5,
27.2 and 16.9 KDa for the minor protein components (Photo 1 and Table 2), comparing with molecular weight marker (Fig. 1 and Fig. 2).

Photo (1): SDS-PAGE of RHDV in liver extracts of rabbits visualized by Coomassie blue staining and showing major and minor components. M: High molecular weight marker (Page Rule prestained protein Ladder) is a mixture of 10 recombinant highly purified coloured proteins with the apparent molecular weights from 170 to 11 KDa. Proteins are covalently coupled with a blue chromophore except for one reference 72 KDa coupled with an orange dye.

Lane (1): Infected liver with RHDV.
Lane (2): Positive RHDV.
Lane (3): Negative control from none infected liver.
Table (2): The molecular weights of extracted protein of RHDV comparing with molecular weight marker using SDS-PAGE.

<table>
<thead>
<tr>
<th>Rows</th>
<th>Marker (mol.w.)</th>
<th>Lane 1 (mol.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>r1</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td>r2</td>
<td>130</td>
<td>120.42</td>
</tr>
<tr>
<td>r3</td>
<td>95</td>
<td>80.23</td>
</tr>
<tr>
<td>r4</td>
<td>72</td>
<td>64.393</td>
</tr>
<tr>
<td>r5</td>
<td></td>
<td>60.432</td>
</tr>
<tr>
<td>r6</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>r7</td>
<td>43</td>
<td>40.832</td>
</tr>
<tr>
<td>r8</td>
<td></td>
<td>36.451</td>
</tr>
<tr>
<td>r9</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>r10</td>
<td></td>
<td>27.284</td>
</tr>
<tr>
<td>r11</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>r12</td>
<td>17</td>
<td>16.888</td>
</tr>
</tbody>
</table>

Figure (1): The molecular weights of the marker using SDS-PAGE.

Figure (2): The molecular weights of rabbit hemorrhagic disease virus (RHDV) using SDS-PAGE.
Results of Western blot: RHD viral proteins were transferred by electro blotting to nitrocellulose membrane were reacted with RHDV antiserum using Western blot technique. Positive test samples and the positive control will produce a pattern consistent with reaction of major protein antigen which have similar molecular weight of about 120, 80 and 63.9 KDa with several smaller distinct protein band react positively with molecular weight of about 40.7, 36.7, 27.6 and 16.6 KDa were illustrated in photo (2) and table (3). The control (non infected liver) revealed no observed band comparing with molecular weight marker (Fig. 3 and 4).

Photo (2): Identification of RADV protein by Western blot with polyclonal antibodies. Proteins of RHDV were electrophoresed on SDS-PAGE and blotted onto nitrocellulose membrane. Viral proteins were detected with anti-RHDV polyclonal antiserum. Lane (1), M: High molecular weight marker (Page Rule prestained protein Ladder) is a mixture of 10 recombinant highly purified coloured proteins with the apparent molecular weights from 11 to 170 Kda. Lane (2) and (3): Infected liver with RHDV. Lane (4): Negative control.
Table (3): The molecular weights of extracted protein of RHDV comparing with molecular weight marker by Western blot technique.

<table>
<thead>
<tr>
<th>Lanes:</th>
<th>Marker (mol.w.)</th>
<th>Lane 1 (mol.w.)</th>
<th>Lane 1 (amount)</th>
</tr>
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<tbody>
<tr>
<td>r1</td>
<td>170</td>
<td></td>
<td></td>
</tr>
<tr>
<td>r2</td>
<td>130</td>
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</tr>
<tr>
<td>r3</td>
<td>120</td>
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<td>r4</td>
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<tr>
<td>r5</td>
<td>72</td>
<td>80.05</td>
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<td></td>
<td>63.864</td>
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<td>r7</td>
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<td>r8</td>
<td>43</td>
<td>40.78</td>
<td>5.54</td>
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<td>r9</td>
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<tr>
<td>r12</td>
<td>11</td>
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<td>0.864</td>
</tr>
</tbody>
</table>

Figure (3): The molecular weights of the immunoblotting of the marker by Western blot technique.

Figure (4): The molecular weights of the immunoblotting of RHDV by Western blot technique.
Results of the conventional 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 electrophoreses revealed a size of 538 bp with marker 100bp DNA ladder. No amplification in any product was shown in negative control (photo 3).

Photo (3): The amplification of PCR product form infected rabbit’s liver of RHDV: In three different regions in Egypt yielded PCR products of about 538 bp. PCR products were analyzed by electrophoresis and visualized by ultraviolet rays with ethidium bromide. M: 100bp marker.
Lane 1: Positive control.
Lane 2 to 10: Infected liver with RHDV (positive).
Lane 11: Negative control.

Results of the Real-time RT-PCR: The samples were also confirmed by 2 steps real-time RT-PCR using SYBR green PCR mix (Stratagen machine). The dissociation curves of nine positive RHDV liver samples were approximately at 86°C for positive controls while the dissociation curve for the negative control was at 76°C.
DISCUSSION

Rearing of rabbit for its meat in Egypt may be an important line shared in poultry industry. RHDV will be a destructive agent for industry through economic losses in rabbit production, high morbidity and mortality. On the bases of biotechnological technique, four laboratory techniques were applied for confirming the diagnosis of RHDV, SDS-PAGE, Western blotting, RT-PCR and real time PCR. As no satisfactory growth condition or sensitive cell substrates have been established, in-vitro isolation cannot be employed (OIE, 2008).

The liver 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. A severe disseminated intravascular coagulation (DIC) is evident in all organs and tissues (El-Mongy, 1998 and OIE, 2008).

In this study, the identification of RHDV protein SDS-PAGE was carried out on extract of liver samples from infected rabbits from different provinces (Giza, Kalubia and Kafr-El-Sheikh). Fig.(1) and Table (2) revealed mostly major protein of approximately 64.4 and 60.4 KDa and approximately 120.4, 80.2, 36.5, 27.2 and 16.9 KDa for the minor protein components (photo 1 and table 2), comparing with molecular weight.
marker (Fig. 1 and Fig. 2).

Our results were nearly similar to the results of EL-Mongy, (1998) who revealed four molecular weight distance (43-49, 66-78, 84-85, and 100-115 KDa) in homogenated liver from selected six cases by SDS – PAGE. Fragments of VP 60 having different molecular weight (41-30 KDa) during transition from RHDV to S-RHDV have been observed by Barbieri et al. (1997) and Gromadzka et al. (2006). Only one polypeptide of 63 to 67 KDa was found in highly purified RHDV preparations using PAGE. The main polypeptide of 60 KDa is sufficient to classify this virus as a member of Caliciviridae (Clouet et al., 1995). Wu et al. (1990) separated four major protein components with molecular weight of 52, 17, 16 and 14.5 KDa. RHDV contains viral proteins with molecular weight 0f (58-60 KDa) VP1, (54.7) VP2, (15-53) VP3 and (26 KDa) VP4 (Du, 1991) while Rodak et al. (1991) added three major proteins with values of 61, 52 and 38 KDa.

In this study, the major polypeptide bands is considered the main structural protein in local RHDV while the other minor protein bands may be resulting from proteolytic degradation of major protein which can range in size from 50 to 28 KDa or smaller polypeptide as recorded by OIE, (2008).

Using Western blotting analysis, the reacted RHDV proteins with positive antiserum were visualized with the presence of high molecular weight antigen of approximately 120, 80 and 63.9 KDa and minor proteins of molecular weight 40.7, 36.7, 27.6 and 16.6 KDa in comparison with positive and negative controls (photo 2 and Table 3), and comparing with molecular weight marker (Fig. 3 and 4).

Three specific reactive bands of RHDV by Western immunoblot, one at the site of 63-65 KDa and two at the sites 43-49 and 84-100 KDa (EL Mongy, 1998). Also, Salman (2008) performed Western blotting and demonstrated the presence of major antigen of molecular weight of 50-55 KDa approximately and several minor distinct proteins bands with lower intensities which had similar molecular weight of 124, 80, 64, 59, 45, 40, 35, 30, 20 and 15 KDa approximately. There were several minor bands visualized (photo 2). However, the percentage of samples showing viral degradation is higher and therefore several fragments of lower molecular weight, originating from the VP60 structural protein are often observed (OIE, 2008).

In the study, the results of RT-PCR were matched with results of Western blot analysis. The ampli-
fied PCR product was visualized by UV in agarose gel electrophoreses revealed a size of 538 bp (photo 3).

Asgari *et al.* (1999) and Van de Bildt *et al.* (2006) examined liver samples using RT-PCR for the capsid protein (VP60) gene with different approaches. Diagnosis by Western blotting and RT-PCR agree with Capucci *et al.* (1998) that identified two RHDV isolated from geographically distant Italian regions in 1997 by using HA, ELISA, Western blotting and RT-PCR.

In this study, use of real-time PCR for RHDV confirmation was very successful, the dissociation curves of nine positive RHDV liver samples were approximately at 86°C (photo 4). A real-time RT-PCR using SYBER Green as a diagnostic tool for the detection of RHDV revealed a specific and sensitive results. PCR and Western blot have shown a higher level of specificity and sensitivity (OIE, 2008). The viral RNA loads were determined by real-time reverse transcription-polymerase chain reaction (Gall and Schirrmeier, 2006).

Wilhelm and Truyen (2006) describes a real-time reverse transcription polymerase chain reaction (RT-PCR) assay with SYBR® Green targeting the VP2 (ORF 3) of feline caliciviruses. The sensitivity of the SYBR® Green reaction was shown to be equivalent to $5 \times 10^1$ to $5 \times 10^2$ copies/reaction and the overall sensitivity equivalent to a feline calicivirus titer of 100.6 TCID$_{50}$/100 l in Crandell Reese Feline Kidney (CRFK) cells. A multiplex real-time RT-PCR using TaqMan probes and external standards for absolute RNA quantification have been developed recently as a further diagnostic tool for the detection of RHDV. The test revealed a specificity of 100%, and linearity over a range from $10^1$ to $10^{10}$ copies (Gall *et al*., 2007).

In conclusion Western blotting and RT-PCR appear to be an extremely sensitive method for the detection of RHDV. It is not strictly necessary for routine diagnosis, but it is more appropriate for studies on molecular epidemiology, to study the early stages of the infection and the pathogenesis of RHDV and to detect the virions in young animals (OIE, 2008). Once the presence of RHD has been confirmed in a region, strict quarantine restriction and biosafety procedure for all rabbit related commerce under official supervision should be established in all farms in the surrounding control zone.

All the results of SDS-PAGE, Western blotting, RT-PCR and real-time PCR have shown a
higher level of specificity and sensitivity and proved to be confirmative for the diagnosis of the RHDV.

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التشخيص الجزيئي لفيروس مرض النزف الدموي للأرانب

أيمن حامد محمود

قسم بحوث البيوتكنولوجيا المعمل المركزى للرقابة البيطرية على الانتاج
- معهد بحوث صحة الحيوان - الجيزة.

يعتبر مرض النزف الدموي في الأرانب من الأمراض المعدية الخطيرة وهو مرض calicivirus. ويميز هذا المرض بنسبة إصابة ووفيات عالية وكذلك سرعة انتشار كبيرة بطرق مباشرة أو غير مباشرة وقد تكون طريقة العدوى من الإفرازات الأنفية أو مخاطية عينية أو فمية. ولأن زرع هذا الفيروس لا يعطي نتائج مرجعية في التشخيص بيوتكنولوجيا حديثة مثل الطبع المناعي وتفاعل البلمرة المتسلسل المصاحب لإنيزم النسخ المقلوب وتفاعل البلمرة المتسلسل في الوقت الحقيقي.

تم استخراج الحامض النووي من عينات كبد لأرانب تعاني من أعراض المرض من ثلاث محافظات مصرية هي الجيزة والقليوبية وكفر الشيخ.

وباستخدام الفصل الكهروني لبروتينات فيروس مرض النزف الدموى وجد أن الوزن الجزيئي العالي للبروتين كان حوالي كيلو دالتون ووزن الجزيئي كيلو دالتون وكانت صورة الطبع المناعي للبروتين المنقل على ورق نيترولوز نفس البانادات لبروتينات رئيسية حجمها كيلو دالتون وعدد من البروتينات المصاحبة حجمها كيلو دالتون عند تفاعلها مع مضاد سيرم إيجابي للفيروس.

ويتمبطق تفاعل البلمرة المتسلسل المصاحب لإنيزم النسخ المقلوب باستخدام الباديء لتكاثر جين (VP60) كانت نتائج هذا التفاعل في جل الأجاروز المصبغ بالإيثيدين بروميدي إيجابية لوجود الفيروس عند وحدة مزدوجة بالمقارنة بسواها إيجابية وسلبية. وباستخدام نفس الباناد وصبغة سبير الخضراء لتفاعل البلمرة المتسلسل في الوقت الحقيقي كان منحنى التفاعل للصبغة عند درجة حرارة الدواجن 0 م إيجابيا.

وخلصت الدراسة إلى نجاح طرق الكشف الجزيئي لفيروس مرض النزف الدموى عن طريق الطبع المناعي وتفاعل البلمرة المتسلسل وكذلك تفاعل البلمرة المتسلسل في الوقت الحقيقي التي تميزت بدقتها وحساسيتها وسرعتها وكانت مؤكدة لتشخيص الفيروس.

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