Preliminary diagnosis of non haemagglutinating strain of Rabbit Haemorrhagic disease virus

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SUMMARY

During investigation of some rabbit hemorrhagic disease (RHD) outbreaks in different governorates in Egypt we recorded 7 out of 22 outbreaks caused by variant strain of RHDV which cause high morbidity and mortality with similar clinical symptom, PM and pathogenicity to that of the other RHD outbreaks. In contrast to other RHD isolates the isolated virus lacked haemagglutinating (HA) activity while it gave positive results in counter immuno-electrophoresis (CIE) and agar gel diffusion test (AGDT). The isolated virus gave negative results in HA after its passage in experimentally infected rabbits. The virus confirmed to be rabbit hemorrhagic disease by Western immunoblot and reverse transcription polymerase chain reaction (RT-PCR). By Western immunoblot the virus exhibited viral protein of 60kDa (VP60) and gave predicted band at 320bp by RT-PCR. This result indicated the limitation of HA test alone in diagnosis of the virus. Western immunoblot and RT-PCR consider tests of choice for conformation the diagnosis of RHD.

INTRODUCTION

Rabbit hemorrhagic disease (RHD) is an acute, fetal, viral disease of rabbits characterized by short incubation period, rapid course and high mortality rate (Patrich et al., 1994). The disease caused by RHD virus a member calciviridae family (Angeles et al., 2001). The virus is non enveloped with a diameter of about 35 to 39nm. The viron is composed of a single major capsid protein with a
molecular mass of 60 to 71 kDa. Its genome is linear, polyadenylated, positive-sense single-stranded RNA of about 7.3 to 8.3 Kb with either two or three open reading frames (ORFs), Tomoichiro, et al. 2007).

RHD was recorded for the first time in China (Liu, et al. 1984), two years later the disease has been reported subsequently in several countries of the world (An et al. 1988; Marcato et al. 1988; and Ohlinger et al. 1990). In Egypt, RHD virus was first reported during spring of 1991 in Sharkia province (Ghanem and Ismail 1992); Kaluobia province (Sharawi, 1992) in Minia and Sohag (El-Zanaty 1994) and during winter of 1992 in Assuit province (Salem and El-Ballal 1992).

Outbreaks of disease have occurred mainly in rabbits older than two months, female particularly those pregnant or lactated were more susceptible to infection. Transmission of virus is horizontal by direct contact with secretion and excretion of infected rabbits and the transmission is facilitated by high stability of the virus in the environment (Du, 1990). Clinically, rabbits exhibits fever and soon die within 72 hrs sometimes with epistaxis (Patrich et al., 1994 and OIE, 2007). Diagnosis of RHD based on flock history, clinical symptom and postmortem. Hemagglutination test (HA) was the first test applied for routine laboratory diagnosis of RHD virus (Biermann et al., 1992 and Song, 1999). However, other tests (western blot, polymerase chain reaction (PCR), agar gel diffusion test (AGDT) and counter immunoelectrophoresis (CIE) can use for detection of the virus in liver suspension (Dai et al., 1987 and Tian et al., 2007).

In our previous study (El-Sissi, 2002) we detected 4 out of 66 liver samples represented two different outbreaks look like that of RHD were consistently HA negative.

So, this work was planned to investigate some RHD outbreaks with detection of the virus in liver suspension by HA, AGDT and CIE. Also confirm the detection of non hemagglutinating isolates by western blot and RT-PCR and the isolates inoculated in and reisolated from rabbits.

MATERIAL AND METHODS

Samples: Seventy eight liver samples were obtained from rabbits that had dead from 22 natural RHD outbreaks. The samples were collected from different governorates during different seasons of years 2007 and 2008; the dilated data are shown in (Table, 1).

Sample preparation:
For each sample, 10% w/v
liver suspension in PBS (7.2) was prepared then clarified by centrifugation at 3000 rpm at 4ºC, followed by 8000 rpm for 60 min. at 4ºC the supernatant were used for viral detection using HA, AGDT and CIE.

**Passage in rabbits:**
Two viruses supernatant that lack hemagglutination activity but gave positive results in AGDT and CIE were isolated in seronegative susceptible rabbits (HI titre less than 8). Each rabbit was inoculated (i.m) by 1ml of liver suspension. The rabbits were observed for one week, the liver of dead rabbit were examined by, HA, AGDT and CIE and compared with a hemagglutinated isolate by western blot and RT-PCR.

**Preparation of hyperimmune serum:**
One rabbit surviving from four rabbits inoculated with local RHD virus (isolated in our previous study, EL-Sissi, 2002), was reinoculated i.m. with 1ml of the same RHD virus followed by two-booster inoculation at 3-week interval. Then serum was collected at weekly interval.

**Hemagglutination (HA) test:**
HA test was used for detection and titration of RHD virus in liver suspension according to Chasey, et al. (1995) in which two fold dilution of 10% liver suspension in PBS 7.2 were incubated with equal volume of 1% washed human type (O) red blood cell in U-bottom microtitre plate at 4ºC for one hour. Agglutination at titers greater than $2^3$ was considered positive and confirmed as virus-mediated by blocking with known RHDV hyperimmune serum (HI).

**Agar gel diffusion test (AGDT):**
The test was carried out according to the method described by Dai, et al. (1987) in which the liver suspensions were examined against RHDV antisera in agarose gel (1% in phosphate buffer saline pH 7.2 with 0.25 gm sodium azide) on Petri dishes and incubated at 37ºC in humid chamber for 5days, candled daily in dark room for precipitation line.

**Counter immunoelectrophoresis (CIE):**
This test was conducted for quick detection of the virus according to Tian, et al. (2007) in which the tested liver samples examined against RHDV antisera on agarose gel (1% in acetate barbitaline buffer pH 8.2 with 0.25gm sodium azide) using Helena electrophoresis unit, at 120 V for 45 minutes then the gel washed, pressed, dried and immersed in Coomassie blue stain for 3-5 minutes destained by three successive
washing using destaining solution.

**Western immunoblot:**

The test was performed according to (Christoph, et al. 1994). Briefly, liver extracts were denatured for 2 min. at 100 ºC in sample buffer (5% b-mercaptoethanol, 2%SDS, 62.5 mMTris-HCl pH 6.8) and electrophoresed on SDS-10% polyacrylamide gels. Transfer to nitrocellulose membrane was performed in a Hoefer Semiphor apparatus for 1 h at 100 mA as described by Towbin et al. (1979). The nitrocellulose membrane incubated over night at 4C in PBS containing 2.5% low-fat milk powder to block nonspecific binding. RHDV hyperimmune serum added and allowed to bind for 2 h. The membrane was washed three times with PBS containing 0.1% tween 20 (PBST), and peroxidase-conjugated anti-rabbit antibodies (Bio Rad) were added at 1:1000 dilution. After 1h of incubation, the membrane was washed three times with (PBST). The blots were developed with AEC (3-Amino-9 Ethyle Carbazde) (Sigma) as substrate. The marker used was Bio Basic Inc. prestained m.w. Marker-D.

**Polymerase chain reaction:**

RHDV RNA was extracted from liver samples by guanidine thyocyanate, 2-meccaptoethanol and phenol extraction using Instapure System (Eurogenetec, Se-raing, Belgium). After ethanol precipitation and a 70% ethanol wash, the pellet was dissolved in 50ul RNAsc free water.

RT-PCR amplification was performed using oligonucleotide primers were as published (Gen Bank) Accession NO. Z49271. Cap14 (5 GCACCTGCAAGTCC-CAATCCG; position 7051-7071) and Cap 17 (5 ACCCACTACGGCACAGGCTC; position 6751-6770) located within the conserved portion of the RHDV capsid region (Meta Bion Company, Germany). The RT-PCR amplification was performed according to (Guittre et al., 1996) A coupled one-step reverse transcription PCR procedure was performed to amplify target sequences (Aatsinki et al., 1994). Initially, 100 pmol of each primer, 40 URNasin and 0.5-1 µg of total RNA were mixed, denatured at 65ºC for 15 min and cooled to 4ºC. Then, a mixture containing 10 µl of 10 X Taq buffer (Pharmacia), 5 µl of MgCl2 (Pharmacia), 200 µM of deoxynucleotide triphosphate (Pharmacia), 200u of M-MLV reverse transcriptase (Gibco, Eragny, France), 2.5 units of Taq polymerase (Pharmacia) and water to a final volume of 50µl was added and overlayed with mineral oil. The RT-PCR reaction was performed in a programmable DNA thermal cycler according to the following conditions: reverse transcription at 42ºC for 45 min fol-
followed by amplification for 30 cycles (95°C for 30s, 55°C for 30s, 72°C for 1.5 min).

The analysis of PCR product was carried out according to (Sambrook, et al. 1989) The product were analyzed for the presence of specific fragment of expected length in agar gel electrophoresis 2% stained with ethedium bromide. PCR marker (DNA ladder) 100 bp was also included. PCR products were stored at -20°C until used.

RESULTS

Investigation of some outbreak of RHD revealed that RHD showed high rate of mortality within short period, no variation in susceptibility of affected breads, rabbits less than two months are resistant to the infections while females particularly those pregnant or lactated were more susceptible to infection. The outbreak distributed allover the different seasons. In addition, there were three outbreaks of rabbits previously vaccinated with locally prepared vaccine. Clinically some rabbit showed fever, squeals followed by death, while others developed dullness anorexia, pyrexia, sometimes, neurological and respiratory signs.. The common PM findings were congestion all over the body with bloody tinged fluid in tracheal lumen. Pregnant doe showed dead fetuses in inflamed uterus.

The results of detection of RHD antigen in 78 liver suspension by HA, AGDT and CIE are summarized in (Table, 2) the three test are +ve on (41) samples also (12) samples which gave HA +ve with titer less than 2^5 could not be detected either by AGDT nor CIE. On the other hand (23) liver samples (represented almost sample from 7 out of 22 outbreaks), gave +ve results by AGDT and/or CIE and failed to hemagglutinate human RBCs, type O.

Comparing between AGDT and CIE diverge results were obtain in 5 sample (HA-ve) three of them were +ve by CIE and –ve by AGDT and two give +ve in AGDT but –ve in CIE. Also two samples (HA +ve) gave +Ve results in CIE and –ve in AGDT.

When two of this non hemagglutinating isolates inoculated 6 seronegative rabbits, resulted in 66% mortality, liver extracts from dead rabbit again failed to hemagglutinate human RBCs, type O and give +ve in AGDT and CIE. The course of the disease was rapid, 3-4 day incubation period ranged between 1 to 3 days followed by depression, anorexia, pyrexia (>41°C) then rabbit died after showed convulsion and paddling movement The most prominent PM lesions were friable, discolored liver, hemorrhagic thymus, spleenomegaly and consolidation.
in lung and trachea with foamy bloody fluid in their lumen and also seen in abdominal and thoracic cavities.

The results of Western immuno blot of hemagglutinating and non hemagglutinating RHD virus isolates are shown in (Fig., 1) and demonstrated the presence of a major capsid protein of molecular weight 60 kDa, and there were no differences between the two isolates.

When hemagglutinating and non-hemagglutinating RHDV isolates were tested by RT-PCR, electrophoresis of the amplified products of the two isolates showed the presence of specific PCR product at correct expected size (320 bp) (Fig., 2).

Table (1): Epidemiological data of some investigated RHD outbreaks

<table>
<thead>
<tr>
<th>Localities</th>
<th>No. of outbreak</th>
<th>No. of sample</th>
<th>Hemagglutination</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>+ve</td>
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<tr>
<td>Giza 1</td>
<td>3</td>
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<td>10</td>
</tr>
<tr>
<td>Giza 2</td>
<td>3</td>
<td>11</td>
<td>7</td>
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<tr>
<td>Giza 3</td>
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<td>8</td>
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<td>7</td>
</tr>
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<td>4</td>
</tr>
<tr>
<td>Kafer El Sheikh</td>
<td>2</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>Mnofia</td>
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<td>6</td>
</tr>
<tr>
<td>Cairo 1</td>
<td>3</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Cairo 2</td>
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<td>8</td>
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<tr>
<td>Total No.</td>
<td>22</td>
<td>78</td>
<td>55</td>
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Table (2): Detection of RHDV in liver suspension by HA, AGDT and CIE

<table>
<thead>
<tr>
<th>No. of sample</th>
<th>HA +ve</th>
<th>HA -ve</th>
<th>Titer of +ve HA*</th>
<th>AGDT +ve</th>
<th>AGDT -ve</th>
<th>CIE +ve</th>
<th>CIE -ve</th>
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<tr>
<td>41</td>
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</tr>
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<td>&lt; 5</td>
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<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Total 78</td>
<td>55</td>
<td>23</td>
<td>-</td>
<td>61</td>
<td>17</td>
<td>64</td>
<td>14</td>
</tr>
<tr>
<td>%</td>
<td>70.5%</td>
<td>29.5%</td>
<td>-</td>
<td>78.2%</td>
<td>21.8%</td>
<td>82%</td>
<td>18%</td>
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</table>

* = Titer expressed as log 2

Fig (1): Immunoblot of capsid protein from non-hemagglutinating RHDV strain lane 1 & 2 and hemagglutinating strain lane 3, showed identity of the VP60 capsid protein.
DISCUSSION

Rabbit hemorrhagic disease is one of the greatest problems facing rabbit during the last few years causing high economic losses in rabbit older than two month (OIE, 2004).

The present investigation declared that RHDV outbreaks were similar to that previously described by several studies except that, we see some outbreak in vaccinated flock. These findings were in agreement with Metwally and Madbouly, (2005) and Ewees, (2007) as they recorded RHDV outbreaks in vaccinated rabbit flock. Although the causative virus was HA positive but it may be of different strain as Matiz, et al. (2006) assigned different strains of RHD in Hungary into three different genogroup RHD variant.
(RHDV<sub>a</sub>). Moreover Ewees (2007) detected more than Egyptian rabbit hemorrhagic disease virus strains.

Liver contain the highest viral titre and the most suitable organ for viral identification (Alexandrov et al. 1992; Carrosco, et al. 1991) so we used liver suspension for detection of the virus.

Detection of RHDV in liver suspension by HA, AGDT, CIE revealed that 23 out of 78 were +ve by AGDT and CIE, and –ve by HA, and the virus after experimental passage through rabbits failed to hemagglutinate. HA-negative RHDV isolate were proved to be RHDV by immunoblot and RT-PCR. The virus appear to resemble all known RHDV isolate worldwide in other respects including its pathogenicity and no significant antigenic differences could be detected when the virus react with antibody of HA +ve isolate in AGDT, CIE and immunoblot. HA-negative RHDV isolate has been detected on different countries by (Capucci et al. 1996; Granzow et al. 1996; Kesey et al 1996; Toru 2003; Vandekerchove et al., (2003) and Tain et al. (2007).

There were two hypotheses have been proposed to explain the presence of HA –ve isolate, Granzow et al. (1996) assumed that HA-negative RHDV isolate is a small particle named core like particles arises from a truncated RHDV genome or due to defective expression. Differently Capucci et al. (1991) and Barbieri et al. (1997) mentioned that samples of normal HA- RHDV sometimes fail to hemagglutinate and this 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 and this occurs mainly in animals with subacute or chronic disease. But in present study the lack of HA is not a consequence of the capsid protein degeneration as western blot analysis of HA –ve isolate exhibit viral protein of 60 kDa and also, there was no sign of degradation. More over, the virus isolated from acute RHDV outbreak.

The non –HA isolate could not be differentiated from HA isolate by CIE, AGDT and western immunoblot, as all this tests using a polyclonal antiserum and it is probable that any antigenic difference would need to be demonstrated by appropriate monoclonal antibodies. Also, it may be differ by only a few amino acid changes in its capsid protein sequence Capucci et al. (1996). Also our results are supported by that by
Tain et al. (2007) he said that the non-HA (whn-1) strain is probably an antigenic variant of RHDVα.

Detection of RHDV in liver suspension using HA, AGDT and CIE revealed that, the percentage of +ve result of CIE (82% ) is more than that of AGDT (78.2% ) so CIE proved its efficiency as simple rapid diagnostic technique in detection of RHDV but its still not sensitive as two sample give +ve result in AGDT and –ve result in CIE and this may be due to slow in migration of RHDV antigen against antibody in electric field (Johnstone and Thorpe, 1988)

Although hemagglutination test using human group O red blood cells consider very simple test and it was the first test applied for routine laboratory diagnosis of RHDV its used alone are not satisfactory for the diagnosis of the RHD virus because non-hemagglutinating isolates of the virus otherwise indistinguishable from others, (Chasey et al., 1995)

It was known from previous studies that RT-PCR was found to be 104 fold more sensitive than ELISA testing for the detection of the virus and was able to detect as few as 12 copies of template DNA. Moreover RT-PCR test demonstrates about 98.7% homology in N-terminal portion of the capsid protein of three isolates from geographically and tempo-

rally separate outbreaks of viral hemorrhagic disease, indicating that this portion of RHDV capsid protein is highly conserved (Guittre et al., 1995) .So RT-PCR technique performed in this work was achieved using two primers chosen from conserved portion of the RHD virus capsid region to help us in detection of different strains of RHD virus and permits efficient amplification of the RHDV cDNA. The two isolates gave predicted band at 320bp. This primer used successfully in screening of the presence of RHDV from flies located within areas Known to be reported by RHD in rabbits (Sassan et al., 1998).

Conclusion

-Isolation of HA negative RHDV in rabbit and detection of the virus by CIE, AGDT, Western immunoblot and RT-PCR.

-Haemagglutination test alone is not satisfactory for diagnosis of RHD due to presence of non- hemagglutinating virus.

-CIE and AGDT can used as rapid diagnostic tests for diagnosis of RHD

-Western immunoblot and RT-PCR have shown a higher level of specificity and sensitivity.

-It was proved that HA-negative RHD strains are circulating in Egypt.
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تشخيص مبكر للفيروس المسبب لمرض الزفيك الفيروسي للأطفال الفجأة على تلقين الدم أشجان فهمي السبسي *و جيهان عبد الله جعفر**
قسم المناعة - معهد بحوث صحة الحيوان بالدقى جيزة
*قسم البيوتكنولوجى - معهد بحوث صحة الحيوان بالدقى جيزة

الملخص العربي

أثناء استبيان بعض الأوقات الخصبة بمرض النزيف الفيروسي للأطفال في محافظات مختلفة في مصر، وجد أن العينة السببية لبعض من أشجان وعشرون وراء غير قادرة على تلقين الدم على عكس العينة المعروفة السببية لمرض النزيف الفيروسي، ولكنها أعطت نتيجة إيجابية من كل من اختيار الأنتشار بالأجار واستخبار الفصل الكهربى المناعي المضاد. وكما أن هذه العينة تسببت في نسبة نفوق عالية ونقص الأعراض المرضية والصفة التشريحية والبيولوجية كتلك المعروفة لمرض النزيف الفيروسي.

و مرة أخرى أعطت نتيجة سلبية في اختيار تلقين الدم بعد تمريره في الأطفال بالعوامل الترجيبية وتم التأكد من التشخيص على أنه الفيروس المسبب لمرض النزيف الفيروسي باختيار الطبع المناعي، وكذلك اختيار النسخ العكسي لتفاعل البلمرة المستقل.

و عند اجراء اختبار الطباع المناعي للعطر على بروتينات الفيروس اظهرت النتائج وجود بروتين واضح حجمه 20 كيلو داتون. واختيار النسخ العكسي لتفاعل البلمرة المستقل أعطى النتيجة المتوقعة عند 20 زوج قاعد.

وقد أظهرت الدراسة محدودة استخدام اختبار تلقين الدم في تشخيص المرض، واعتبر اختيار الطباع المناعي واختبار RT-PCR من الاختبارات المختارة لتأكيد تشخيص مرض النزيف الفيروسي للأطفال.

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