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USE OF VP60 RT-PCR TO OVERCOME THE LIMITATION OF HAEMAGGLUTINATION INHIBITION DIAGNOSIS OF RABBIT VIRAL HAEMORRHAGIC DISEASE

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Abstract: Rabbit viral haemorrhagic disease (RVHD) is a highly contagious, highly fatal, peracute and acute viral disease of both wild and domestic rabbits caused by rabbit haemorrhagic disease virus (RHDV). Testing for haemagglutination activity (HA) in processed liver samples is one of the cornerstones for rapid diagnosis of RHDV outbreaks in national rabbitries. However, RHDV isolates exhibiting no HA activity are increasingly being reported. The extent of deviation from classical HA activity patterns for RHDV strains in Egypt has not been investigated. This study compared the HA activity patterns of samples collected from 61 RHDV outbreaks that occurred between 1999 and 2005 to determine whether dependence on HA test (HAT) for diagnosis of RHDV outbreaks needs to be reviewed. All samples were confirmed RHDV positive using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Using slide HAT, only 36.1% of samples were positive (22 samples). Plate HAT conducted at 4ºC detected an additional 16 positive samples bringing the total HA-positive samples to 38 (62.3%). Plate HAT conducted at 22ºC failed to detect additional positive samples. The majority of samples detected after plate HA testing (62.5%) had HA titres comparable to those obtained from slide-HA-positive samples, indicating that the difference in HA activity is dependent on the nature of the HA antigen rather than its presence. Direct detection of HA activity failed in 37.7% of samples despite the presence of classical signs, pathology, and being reverse transcription polymerase chain reaction (RT-PCR) positive for three different VP60 regions. Experimental infection of seronegative rabbits with 9 HA negative RHDV samples showed that 5 isolates were in-fact HA positive, while only 4 isolates remained HA negative. The increased detection of viruses lacking HA activity and the low HAT sensitivity mandates the use of molecular techniques for rapid confirmation of RHDV diagnosis in the Egyptian environment.

Key Words: rabbit haemorrhagic disease virus, slide haemagglutination, plate haemagglutination, non-haemagglutinating, experimental infection.

INTRODUCTION

Rabbit viral haemorrhagic disease (RVHD) is a highly contagious, highly fatal, peracute and acute viral disease of both wild and domestic rabbits. The disease is characterised by a short course (2-3 d), high levels of morbidity (100%) and mortality (80-90%), severe signs (dyspnoea, anorexia, depression, convulsions and epistaxis) and by severe necrotising hepatitis (Peeters *et al*., 1990). The disease is caused by rabbit

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haemorrhagic disease virus (RHDV). Its members belong to lagovirus of the *Caliciviridae* (Ohlinger *et al*., 1990; Parra and Prieto, 1990).

In addition to RHDV, *Caliciviridae* comprise important human and animal pathogens including noroviruses or Norwalk-like viruses (which cause severe gastroenteritis in humans), and vesiviruses (e.g. the vesicular exanthema of swine virus). A similar RVHD virus, the European Brown Hare Syndrome Virus (EBHSV), afflicts European hares of the Lepus genus (Wirblich *et al*., 1994). The nearest relation to RHDV, however, is a non-pathogenic calicivirus (Capucci *et al*., 1996b; Bergin *et al*., 2009; Strive *et al*., 2009). The RHDV strains and non-pathogenic calicivirus form 2 distinct groups, 2 groups that are clustered together and apart from all the other virus strain within the family (Abrantes and Esteves, 2010). The evolutionary analyses of the capsid VP60 of RVHD showed the occurrence of recombination (Abrantes *et al*., 2008; Forrester *et al*., 2008) and allowed the detection of positive selection (Esteves *et al*., 2008).

RHDV, like other caliciviruses, forms 28-32 nm diameter, non-enveloped, icosahedral virus particles that harbour a 7.4 kb positive sense oriented single-stranded RNA genome that encodes a 257 kDa polyprotein (Wirblich *et al*., 1996). The strains in this genus form a distinct phylogenetic clad within the family, and differ from known caliciviruses in 2 main aspects: haemagglutinating activity and failure to propagate *in vitro* (Studdert, 1978; Cubitt, 1987; Forrester *et al*., 2008).

The genome is organised into 2 major open reading frames (ORFs). ORF1 encodes the non-structural polyprotein, and the major structural CP gene (VP60) in frame with the non-structural polyprotein coding sequence. ORF2 overlaps the ORF1 by 17 nucleotides in the RHDV genome and 5 nucleotides in the EBHSV genome. The ORF2 encodes a small protein (VP10) of unknown function that has been identified as a minor structural component in the RHDV virion (Forrester *et al*., 2008).

Only a single serotype of RHDV is known to exist (Capucci *et al*., 1995). Of particular interest however, has been the emergence of an antigenic variant strain or subtype of RHDV known as RHDVa. RHDVa is replacing original strains of RHDV in Italy. Two recent French isolates belonging to the RHDVa antigenic subtype have been identified (Schirrmeier *et al*., 1999). Phylogenetic analysis of partial VP60 sequences from isolates dating back to 1988 also revealed the emergence of the RHDVa strain in a 2003 outbreak of RHD in Hungary. The World Organisation for Animal Health (OIE) has reported that the RHDVa subtype was responsible for the first ever recorded outbreak of RVHD in Uruguay near the end of November of 2004. At the same time, a large outbreak of RVHD attributed to RHDVa occurred in Cuba. These combined observations confirm that the spread of RHDVa is a pandemic and suggest a selective advantage for infectivity or replication of RHDVa over the original serotype of RHDV (McIntosh *et al*., 2007). In Egypt, RHDV was reported for the first time in 1991 in Sharkia (Ghanem and Ismail, 1992). Outbreaks continue to appear in the Egyptian environment (Salman *et al*., 1999).

Haemagglutination test (HAT) using human red blood cells (RBC) type "O" is the primary test for routine field and laboratory diagnosis of RHDV in Egypt, in addition to ELISA (Capucci *et al*., 1991). Other diagnostic methods (Western blot, RT-PCR) have been employed successfully for investigative studies (Liu *et al*., 1984). Most RVHD isolates, so far described in the literature, haemagglutinate human RBC. However, new types of virus particles with different haemagglutinating properties have been reported. In 1991, a second type of virus particle was identified as the predominant virus population in approximately 5% of the RVHD-positive specimens (Capucci *et al*., 1991), and a variant strain designated "Rainham" was isolated from a small localised outbreak of the disease in southern England (Capucci *et al*., 1996a). Another non haemagglutinating strain was isolated from an RHDV outbreak that occurred in March 1996 in Asturias (North-western Spain) (Prieto *et al*., 2000). The situation regarding the presence of "non-classical" RHDV types in Egypt is largely unknown. Knowledge regarding the existence of RHDV field

strains with atypical HA activity will determine whether dependence on HAT for diagnosis of RHDV outbreaks needs to be reviewed.

Therefore, the aim of the work was to examine the possible existence and distribution of non-haemagglutinating RHDV isolates in Egypt, and the evaluation of some molecular techniques for the detection of these non-classical RHDV isolates.

MATERIALS AND METHODS

Samples

Livers from freshly dead rabbits were collected from 61 suspected RHDV outbreaks in different localities in Egypt during the period from 1999 to 2005. Liver specimens were mechanically homogenised in phosphate buffered saline solution (PBS) pH 7.2 (10% w/v), filtered through cheese cloth, and clarified by centrifugation at 5000 *g* for 15 min. A mixture of 100 IU penicillin-G sodium, 100 µg streptomycin sulphate, and 100 µg clotrimazole per mL was added to the clarified liver homogenate. The supernatant was filtered through a 0.22 µm filter, and examined for bacterial and fungal contaminants. The prepared samples were designated R2-R62, and stored at −20ºC until use. A reference RHDV isolate designated R1 was prepared from rabbit livers infected with a previously characterised RHDV isolate with classical HA, and pathogenicity patterns (Salman, 1999).

Slide haemagglutination test (sHAT)

The liver extracts from all 61 RHDV outbreak samples were tested for HA activity on slides according the technique described elsewhere (Du, 1990). Briefly, one drop of washed 10% human RBCs type “O” was mixed with one drop of the liver extract on a glass slide. The mixed drops were several times. The presence and degree of agglutination was recorded within 1min.

Quantitative plate haemagglutination test (pHAT)

The liver extracts from all 61 RHDV outbreak samples were tested for HA activity and titre in micro HA plates according to the method described elsewhere (Capucci *et al*., 1996a; OIE, 2008). Briefly, duplicate sealed round-bottom micro-titre plates containing two-fold dilutions of each liver extract were incubated with an equal volume (50 mL) of washed 0.75% human RBCs type "O" at two different temperatures, 22°C and 4°C. Lattice and button shapes were recorded for each sample at each incubation temperature after 1-2 h. The reciprocal of the end dilution (last well giving complete HA) was considered the end titre. Results were considered positive when having an agglutination end-point dilution of > 1/16 (2^4 HAU/50 µL) (OIE, 2008).

SDS-PAGE and Western blotting

The presence of RHDV antigens in liver extracts from the investigated RHDV outbreaks was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using positive control reference RHDV antiserum supplied in an enzyme immunoassay kit for detection of antibodies to RHDV (Kalon Biological Ltd. UK). SDS-PAGE followed by Western blotting was carried out according to the method described elsewhere (Sambrook *et al*., 1989; OIE, 2008).

Propagation of RHDV from HA-negative liver extracts in susceptible rabbits

Experimental infection of susceptible seronegative rabbits using the prepared samples was done according to the method described elsewhere (Capucci *et al*., 1990). Briefly, liver extracts from 9 RHDV-positive samples with no HA activity using either sHAT or pHAT were each inoculated into 5 susceptible rabbits.

The different RHDV outbreak samples selected were R2, R4, R9, R10, R14, R26, R30, R47, and R48. Rl, the local characterised reference strain of RHDV (Salman, 1999), was also inoculated in 5 seronegative susceptible rabbits for comparison. A group of 5 rabbits was mock infected and observed as a negative control. Rabbits were observed for 15 d post inoculation. Mortalities were recorded. Liver samples from dead animals were collected and tested using pHAT.

Reverse transcriptase polymerase chain reaction (RT-PCR)

HA-negative liver extracts were tested for the presence of RHDV nucleic acid using RT-PCR. The RHDV-specific primers and RT-PCR reactions were conducted according to methods described elsewhere (Le Gall *et al*., 1998). Twenty-six RHDV isolates previously identified by SDS-PAGE and Western blotting were tested. Total RNA extraction of RHDV from liver tissue was done using SV Total RNA isolation system (Promega, USA), according to the manufacturer's instructions. Purified RNA extracts from the livers of infected rabbits were used for cDNA synthesis by AMV RT with the regions A, B and C specific downstream primers. Regions A and B are within the viral VP60 while region C is in a gene coding a non-structural protein located between the viral helicase and VPg. For the RT reaction, 2 µg of total RNA were heated at 65ºC for 5 min with 100 pmol of each of the downstream specific primers independently. Each mixture (RNA and primers) was cooled to room temperature for 5 min and the following were added: 20 U of AMV reverse transcriptase enzyme (Pharmacia, USA), 500 µM dNTPs, 40 U of RNAsIn Ribonuclease Inhibitor (Promega, USA). The reaction volume was completed to 25 µL with RNAse free double distilled water. The reverse transcription reaction was conducted at 42ºC for 60 min, followed by enzyme inactivation at 95ºC for 5 min. For amplification of A, B and C region sequences, RHDV upstream and downstream specific primers were used (Le Gall *et al*., 1998). The PCR mixture contained 1.5 mM $MgCl_2$, 200 mM of each of the dNTPs, 100 pmol of each primer and, 2.5 U of the Taq polymerase. The amplification reactions were performed using Perkin Elmer Gene Amp 9700 thermal cycler. Cycling was biphasic. The first phase was 5 cycles at 94ºC for 1 min, 50ºC for 1 min, 72ºC for 2 min. The second phase was 25 cycles using the following conditions: 94ºC for 1 min, 65ºC for 1 min, 72ºC for 2 min. The reaction was terminated after a final extension step at 72ºC for 1 min. The amplified product was analysed by electrophoresis in 1.5% ethidium bromide agarose gels.

RESULTS AND DISCUSSION

HAT remains the primary test in routine laboratory detection of the haemagglutinating activity of RHDV in many countries, and especially in Egypt (Liu *et al*., 1984; Peeters *et al*., 1990; Erber *et al*., 1991; Fitzner *et al*., 1992; Salman, 1999). Slide HAT is especially preferred by many field diagnosticians because of its simplicity and limited cost. The liver of RHDV-infected rabbits is considered the best organ to examine for the presence of the virus (Chasey *et al*., 1995). Livers proved to contain the highest concentration of the virus (Zhao *et al*., 1988; Capucci *et al*., 1990; Abd El-Mottelib, 1993). Nowotny *et al*. (1993) detected large quantities of RHDV in the bile.

Out of the 61 liver samples collected by a trained professional from RHDV outbreaks, only 22 liver extracts (36.1%) showed strong positive slide HA activity (Table 1). The titres of sHAT positive extracts ranged from 2^7 to 2^{14} HAU/50 ml using pHAT conducted at 22ºC. The same samples gave 2^8 to 2^{14} HAU/50 mL when the test was carried out at 4ºC (Table 2).

Quantitative pHAT was used to test all sHAT negative samples since the low sensitivity of sHAT may have contributed to the failure to detect HA activity in the majority of samples tested. Sixteen additional liver extracts, out of 39 negative in sHAT, were positive using pHAT carried out at 4ºC (Table 3). Plate HAT conducted at 22ºC failed to detect additional positive samples (Table 3). The effects of temperature on the result of HA tests for RHDV have been reported by others. A new strain, designated RHDV Rainham,

Table 1: Slide haemagglutination test (sHAT) results of 61 rabbit haemorrhagic disease virus (RHDV) outbreak liver extracts.

	sHAT-positive	sHAT-negative
Outbreak extracts	R3, R5, R15, R23, R34, R35, R36, R37, R39, R40, R42, R43, R45, R46, R51, R53, R55, R57, R58, R59, R61, R62	R2, R4, R6, R7, R8, R9, R10, R11, R12, R13, R14, R16, R17, R18, R19, R20, R21, R22, R24, R25, R26, R27, R28, R29, R30, R31, R32, R33, R38, R41, R44, R47, R48, R49, R50, R52, R54, R56, R60
Total No.	22	39
Percentage of total	36.1	63.9

originally failed to cause HA in standard conditions at 22 ºC while HA was clear at 4ºC (Capucci *et al*., 1996a). Nardelli *et al*., (1996) also found that some rabbit liver homogenates identified as RHDV-positive by ELISA do not haemagglutinate human RBCs at room temperature.

Table 2: Haemagglutination activity (HA) titres of slide haemagglutination test-positive liver extracts[1].

	HA titres expressed as $\log_2$ HAU[2]	
Liver Extract	22ºC	4ºC
R3	12	13
R5	12	13
R15	10	12
R23	11	12
R34	12	14
R35	11	13
R36	11	13
R37	9	11
R39	10	13
R40	9	11
R42	10	13
R43	11	13
R45	10	13
R46	13	14
R51	14	14
R53	11	13
R55	10	12
R57	11	13
R58	13	14
R59	12	14
R61	13	14
R62	7	8

[1] Titration was done using quantitative plate hemagglutination test conducted at 22ºC and 4ºC.
[2] HAU: Haemagglutinating units/50 μL.

For the additional 16 samples identified by pHAT, HA titres ranged from 2^4 to 2^{11} HAU/50 mL (Table 3). The majority (n=10, 62.5%) of these 16 samples produced titres (2^7 to 2^{11} HAU/50 mL) comparable to those obtained from titration of sHAT-positive extracts (Table 3). Since detection of HA activity using sHAT was possible for samples containing 2^7 HAU/50 mL extract (Tables 2 and 3), then viral antigen titre in extracts was probably not the cause of the false negative results obtained by sHAT. The 60 KDa VP60 major antigen of RHDV was identified in all sHAT negative samples in Western blot analysis (data not shown).

It is plausible that the observed deviation from classical slide HA activity in high titre extracts may have been caused by either the nature (conformational changes, proteolytic digestion) of the viral HA antigen or the purity of the preparations, although the latter cause is highly unlikely due to the uniformity of the methodology and the expertise of the specialist conducting the experiment.

This loss of HA activity of RHDV in liver extracts from the different outbreak samples may also be explained by the stage of the disease at which the samples were collected (peracute, acute, subacute or chronic). The loss of haemagglutinating activity of RHDV has been reported to be associated with the chronic stage of RVHD (Granzow *et al*., 1996). However, the combined HAT results detected RHDV HA activity in only 62.3% of the

Table 3: Haemagglutination activity (HA) titres of slide haemagglutination test negative liver extracts[1].

	HA titres expressed as $\log_2$ HAU[2]				HA titres expressed as $\log_2$ HAU[2]		
Liver Extract	22ºC	4ºC	Interpretation	Liver Extract	22ºC	4ºC	Interpretation
R2	0	0	−	R26	0	0	−
R4	0	3	−	R27	0	3	−
R6	0	4	+	R28	0	6	+
R7	0	11	+	R29	0	9	+
R8	0	11	+	R30	0	0	−
R9	0	0	−	R31	0	11	+
R10	0	3	−	R32	0	0	−
R11	0	10	+	R33	0	11	+
R12	0	0	−	R38	0	5	+
R13	0	3	−	R41	0	0	−
R14	0	0	−	R44	0	0	−
R16	0	0	−	R47	0	0	−
R17	0	4	+	R48	0	0	−
R18	0	3	−	R49	0	0	−
R19	0	0	−	R50	0	0	−
R20	0	11	+	R52	0	9	+
R21	0	7	+	R54	0	0	−
R22	0	7	+	R56	0	0	−
R24	0	11	+	R60	0	5	+
R25	0	0	−				

[1] Titration was done using quantitative plate hemagglutination test conducted at 22ºC and 4ºC.
[2] HAU: Haemagglutinating units/50 µL.

61 outbreaks. This is far lower than previously reported. The phenomenon that about 5% of the RHDV-positive samples sometimes produced negative HA results has been reported by others (Capucci *et al*., 1991; Biermann *et al*., 1992). After those early reports, research groups have also identified non-haemagglutinating viruses (Chasey *et al*., 1995; Kesy *et al*., 1996; Prieto *et al*., 2000).

To investigate whether the failure of HA activity of some outbreak viruses is a true viral character, 9 HAT-negative samples were selected for virus propagation and retesting. As no satisfactory growth conditions and/or sensitive cell substrates have been established, *in vitro* isolation of RHDV cannot be included among the virological methods for diagnosis of the virus. Rabbit inoculation therefore remains the only way of isolating, propagating and titrating virus infectivity (OIE, 2008).

RHDV was successfully propagated from the 9 outbreak samples selected (Table 4). The characteristic clinical signs and PM lesions for RHDV were replicated in the inoculated rabbits. The rabbits inoculated with the local strain of RHDV R1 developed the specific and characteristic clinical signs and PM lesions of RHDV (data not shown). Mock-infected negative control rabbits remained normal. Viruses from the outbreaks caused between 20% and 60% mortalities. The local reference R1 isolate resulted in the death of 80% of the inoculated group (Table 4). These findings agree with the epidemiological picture of the outbreak from which these samples were collected.

Table 4: Effect of virus propagation in rabbits on the haemagglutination activity (HA) of haemagglutination test negative rabbit haemorrhagic disease virus isolates.

	HA before propagation	No. of rabbits inoculated	No. of dead rabbits	Percentage of mortality	HAT-positive extracts[1]	VP60 RT-PCR	Mean HA titres[2]
R1	+	5	4	80	4	+	11.75
R2	−	5	2	40	0	+	0
R4	−	5	1	20	0	+	0
R9	−	5	3	60	2	+	11
R10	−	5	2	40	1	+	9
R14	−	5	1	20	0	+	0
R26	−	5	2	40	1	+	9
R30	−	5	2	40	1	+	9
R47	−	5	1	20	0	+	0
R48	−	5	2	40	1	+	9

[1]HA was determined using slide HAT for liver extracts obtained from the dead rabbits.
[2] Titration was done using quantitative plat HAT conducted at 4ºC for liver extracts obtained from dead rabbits.
Titres are expressed as $\log_2$ Haemagglutinating units (HAU)/50 µL.

Liver extracts of dead rabbits used for propagation of HA-negative RHDV samples, as well as original outbreak extracts lacking HA activity were all positive for the presence of RHDV nucleic acid using VP60 RT-PCR assays (Figure 1). HA activity was detected in only some of the livers of dead infected rabbits used for propagation of outbreak viruses R9, R10, R26, R30 and R48 (Table 4). Liver extracts prepared from freshly dead rabbits showed variable HA titres ranging from 0 to 2^{14} HAU/50 mL.

Four outbreak viruses retained the HA-negative phenotype after propagation in rabbits. This phenomenon was recorded by others (Park *et al*., 1991; Capucci *et al*., 1996a). Five outbreak viruses exhibited HA activity after propagation in susceptible rabbits (Table 4). Interestingly, outbreak viruses exhibiting lower mortality rates (20-40%) were associated with loss of HA activity in 50% of the liver extracts of dead rabbits. R1-inoculated rabbits livers were all HA-positive (Table 4). R9-inoculated rabbits showed a relatively high mortality% and the liver extracts of two of the three rabbits that died due to infection were HA-positive (Table 4). Data collected during experimental infection trials point to the presence of a correlation between the HA activity of liver extracts and virulence (data not shown). Further studies are required to affirm this observation.

Regaining RHDV HA activity after rabbit inoculation may be a result of increased virus replication in the hepatocytes of susceptible rabbits. All HA positive extracted from rabbit livers used for virus propagation contained high HA titres (Table 4). However, it is evident that other immunopathological factors, in addition to virus replication, may be at play. Negative HA viral particles were recovered from the liver of an experimentally infected rabbit showing a protracted course of the disease. Such particles may have evolved from a truncated RHDV genome or defective expression (Granzow *et al*., 1996).

Barbieri *et al*., (1997) considered the recovery of negative HA viral particles from rabbit liver as a marker of the subacute/chronic form of RVHD that usually evolves between 4 and 8 d post-infection. Another explanation offered argues that the determinant of whether the initial infection with RHDV resulted in acute disease or chronic infection may be the viral 'dosage' received (White *et al*., 2002). Following infection, virus replicates at a rapidly accelerating rate. The antibody-mediated immune response occurs 2-3 d post-

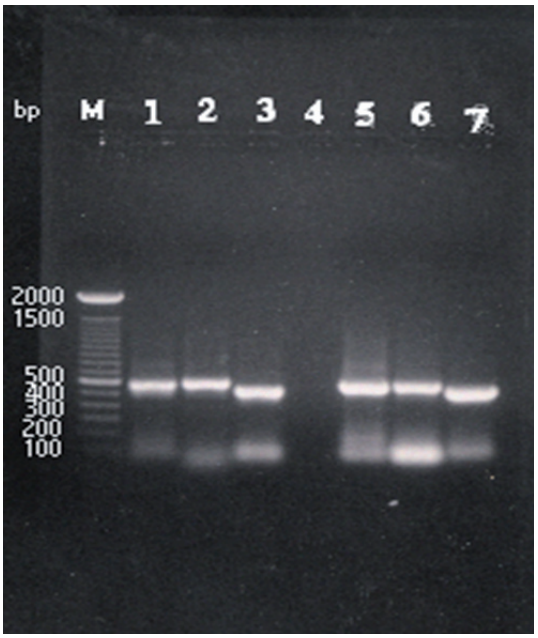

Figure 1: PCR for 2 rabbit haemorrhagic disease virus (RHDV) local strains showing the amplification of 3 genomic fragments A, B and C. Lane M: Marker 100 bp DNA ladder. Lanes 1, 2 and 3: Amplification products A, B and C, respectively, for RHDV (R1). Lane 4: Negative control. Lanes 5, 6 and 7: Amplification products A, B and C for RHDV (R2), respectively.

infection and if this immune response succeeds in arresting viral amplification before a fatal amount of liver damage occurs, then the rabbit survives and develops chronic infection, otherwise death results (White *et al*., 2002). The time taken for fatal damage to occur will depend upon the size of the initial viral inoculum, with acute infection resulting from a 'large' inoculum (received from an acutely infected rabbit) enabling infection to progress rapidly, usually overwhelming the host before antibodies have been produced in sufficient quantity to control the infection. A smaller inoculum from a chronically infected rabbit would allow more time for the host immune system to respond before fatal liver damage occurs.

During the trial for isolation of RHDV, it was noticed that the majority of deaths occurred within 1 to 3 d from onset of experimental infection, and the liver extracts of those samples were HA-positive. Later deaths lacked HA activity (data not shown). Results were comparable to those of previous experimental infection experiments (Capucci *et al*., 1990; Park *et al*., 1991; Ghanem and Ismail, 1992; Sharawi, 1992; Salman, 1999). Overall, the results of this work support existing concepts about the limitations of HAT in the diagnosis of RHDV and demonstrate an influence on the outcome of HAT analysis for the sampling time and disease grade.

Many authors have used tests other than HAT for RHDV diagnosis (Capucci *et al*., 1990; Ohlinger *et al*., 1990; Du, 1991; Lieberman *et al*., 1992; Motha and Kittelberger, 1998). Five laboratory techniques are commonly used for confirmation of RHDV diagnosis: SDS-PAGE, Western blotting, immuno-electron microscopy (IEM), histopathological examination, and RT/PCR assay. RT-PCR analysis was the method of choice in our analysis because of sensitivity and specificity of the reaction. It was decided to use the technique adopted by others (Le Gall *et al*., 1998) to avoid having to establish sensitivity and specificity required for new primer development.

Specific PCR products of the expected size were obtained for the three regions tested. PCR products were approximately 500 bp for A and B regions, and over 400 bp for C region for both HA-positive (R1) and HA-negative (R2) viruses (Figure 1). Both strains were sequenced to confirm the analysis (data not shown). The negative controls showed no amplification. This was recorded by the original designer of the primers (Le Gall *et al*., 1998) and others who have designed primers for the VP60 (Prieto *et al*., 2000; Moss *et al*., 2002).

With the obvious increase in deviation from classical HA activity of recent RHDV isolates in Egypt, and considering the need for rapid confirmatory diagnosis of classical and emerging RHDV in rabbit populations, our data demonstrate the inevitability of the shift towards use of molecular diagnostic techniques. VP60 RT-PCR will provide a sensitive and rapid tool for virus isolation in addition to facilitating the identification of the molecular epidemiology and evolution of the virus, leading to better vaccine and control strategy

development. Without this shift, it will be impossible to curb the future economic impact of RHDV infections on the regional rabbit industry.

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