Molecular and serological studies of Egyptian strains of rabbit haemorrhagic disease virus and their comparison with vaccine strains

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Summary

Vaccination is the major control measure for rabbit haemorrhagic disease virus (RHDV). The co-circulation of different RHDV genotypes in Egypt has led to the need to determine the most effective vaccine strain and the cross-protection between these genotypes. Rabbits seronegative for RHDV were vaccinated with the commercial GI.1a (RHDVa) vaccine strain Giza2006 and the GI.1d (G5) vaccine strain Giza97. The rabbits were challenged three weeks post vaccination with GI.1a (RHDVa) strains Giza2010 and Kal2012 and GI.1d (G5) RHDV Giza97 and RHDV2014 to determine the degree of cross-protection and evaluate immunity and cross-reactivity by haemagglutination inhibition (HI) and indirect enzyme-linked immunosorbent assay (iELISA). Both vaccines were fully protective three weeks post vaccination, with 95% protection percentage for the GI.1a vaccine and 94.7% for the GI.1d vaccine, with no direct

relationship between mortality rates and the genotype of the challenge strain. The antibody titres obtained using the HI test were one log higher for the GI.1a compared with the GI.1d vaccines, but post-challenge titres showed increased responses, expressed as 1−3 $\log_2$ higher titres, for the GI.1d vaccine. Sequence and phylogenetic analysis of the Egyptian strain RHDV2014 revealed its relatedness to the GI.1d genotype and showed no evidence of the presence of GI.2 in Egypt until 2014. In conclusion, both GI.1d (G5) and GI.1a (RHDVa) based vaccines are protective against both RHDV genotypes present in Egypt but continuous monitoring of circulating strains is essential because the arrival of GI.2 in Egypt will require new vaccination strategies.

Keywords

Cross-protection – Phylogeny − Rabbit haemorrhagic disease virus − RHDV − Sequence analysis − Vaccination.

Introduction

Rabbit haemorrhagic disease virus (RHDV) is still a highly contagious and fatal disease in Egypt and worldwide despite the available vaccination strategies (1, 2). Rabbit haemorrhagic disease (RHD) has a high mortality rate and is responsible for large economic losses in the rabbit industry worldwide.

Rabbit haemorrhagic disease virus is an icosahedral, non-enveloped virus in the genus *Lagovirus*, family Caliciviridae (3, 4). The viral genome is a positive sense single-stranded ribonucleic acid (RNA) molecule, 7.4 kilobases (kb) long. The genomic RNA is arranged into two open reading frames (ORFs); ORF1 encodes a 257 kiloDalton (kD) polyprotein that, upon processing, originates several non-structural proteins and the capsid protein VP60 (5). The VP60 protein contains antigenic determinants recognised by the rabbit immune system and, thus, plays a crucial role in evoking a sufficient immune response against RHDV infection (6).

Until 2010, RHDV strains could be assigned to four genotypes, GI.1a, GI.1b, GI.1c and GI.1d (7), corresponding to the former G6, G1, G2 and G3–G5, respectively, with GI.1a representing the antigenic variant referred to previously as RHDVa) (8).

In 2010, a new RHDV-related virus, originally designated as RHDV2, or RHDVb, but now named GI.2, was reported in France (9, 10). The GI.2 virus rapidly spread to several countries (9, 11, 12, 13, 14). The unique phylogenetic and antigenic profile of RHDV2 − different from that of GI.1a – led to a consideration that RHDV2, or 'RHDVb', is a newly emerged virus and not just another GI.1a (9).

In Egypt, RHDV was first reported in Sharkia province in 1991 (15). A retrospective study tracking the evolution from 1997 to 2012 of locally isolated RHDV strains showed that both GI.1d and GI.1a RHDV co-circulated in Egyptian rabbit farms and backyard rabbit units until a more recent period when GI.1a became more prevalent (16).

Although GI.1a and GI.1d belong to the same serotype, comparison of Egyptian strains showed significant differences in amino acid composition, with most substitutions clustered within the hypervariable region E of VP60, which resulted in changes in the antigenic index and Protean analysis (16). The Giza2006 GI.1a vaccine used in Egypt showed major differences in the antigenic index from GI.1d strains and minor differences from the other local GI.1a strains (16).

The introduction of a prototype RHDV vaccine in the 1990s (17) provided good coverage for the strains circulating at that time (18), but the continued occurrence of mortalities on rabbit farms and the emergence of GI.2 in many countries, with outbreaks in vaccinated rabbits, may alter the epidemiology of the disease (19, 20). Therefore, the aim of this study was to perform cross-protection studies between locally produced vaccines and both GI.1d and GI.1a Egyptian strains, and vice versa, with determination of the antigenic relatedness between them. In addition, molecular identification of recently isolated RHDV strains was carried out in order to investigate the

presence of GI.2 in Egypt, allowing a better evaluation of the current vaccination strategies.

Materials and methods

Virus

Frozen liver tissue from field cases (rabbits on meat production farms and backyard meat rabbits) with clinical signs of RHD (fever, anorexia and paralysis) in Kalubia district in 2014 was investigated in the authors' laboratory. Genotyping was performed on each isolate in accordance with the authors' previous work, as well as on six RHDV isolates that had been isolated and genotyped as previously described (21). Two viruses were used for vaccine preparation: Giza2006, a GI.1a vaccine, and Giza97, a GI.1d vaccine. Four isolates were used as challenge viruses; two of these (Giza2010 and Kal2012) belong to GI.1a RHDV, while Giza97 and RHDV2014 represent GI.1d RHDV.

Virus isolation and propagation

All procedures were carried out in accordance with the guidelines and requirements of ethical approaches in dealing with experimental animals in research and teaching, by following the World Organisation for Animal Health (OIE) institutional standards (22).

Twenty cross-bred three-month-old susceptible rabbits (1.5–2 kg) were obtained for virus isolation and propagation. The rabbits used in all experiments were confirmed to be seronegative for RHDV by the haemagglutination inhibition (HI) test (Table I). All strains were passaged and propagated in susceptible rabbits as reported previously (21). Each rabbit was inoculated by the intramuscular (IM) route with 1−2 ml of 10% weight/volume liver homogenate in saline solution, pH 7.2, clarified by centrifugation at 5000 ×*g* for 15 min (23). The rabbits were observed for one week and the mortality pattern and post-mortem lesions were recorded. Liver samples were collected from infected rabbits under aseptic conditions.

Table I

Mean haemagglutination inhibition titres (log_2/ml) of rabbits vaccinated with classical and variant rabbit haemorrhagic disease virus vaccines before and after challenge, when reacted against their homologous and heterologous antigens

Vaccine group	GI.1a vaccine (Giza2006)				GI.1d vaccine (Giza97)			
	Pre-vaccination		3rd week		Pre-vaccination		3rd week	
Ag97 (a)	0		7		0		6	
Ag2006 (b)	0		7		0		6	
Challenge virus	Giza 97 (c)	RHDV 2014 (c)	Giza 2010 (d)	Kal 2012 (d)	Giza 97 (c)	Kal 2014 (c)	Giza 2010 (d)	Kal 2012 (d)
Ag97	9	8	8	7	10	10	10	10
Ag2006	6	5	8	7	8	6	6	6

(a) refers to antigen prepared from Giza97 strain
(b) refers to antigen prepared from Giza2006 strain
(c) GI.1d virus type
(d) GI.1a virus type

Reverse-transcription polymerase chain reaction test for detection of rabbit haemorrhagic disease virus

For detection of viral RNA in suspected field cases and vaccinated rabbits, either dead or surviving, specific primers flanking 600 bp of the highly variable region of the VP60 gene (regions C−E) located in the most exposed part of the capsid (the P2 sub-domain) were used, as reported previously (21).

The upstream primer (nucleotide positions 6,106−6,125) 5′ CCT GGA GGG TTT TCT ACG TG 3′ and the downstream primer (nucleotide positions 6,688−6,706) 5′ AGA CGA CAG ACG CGA ACA T 3′ were synthesised by Bio Basic Inc. (Markham, ON, Canada). Total RNA extraction from fresh liver tissues was performed with the SV Total RNA isolation system (Promega, Madison, WI, United States of America [USA], Catalogue No. Z3100), according to the manufacturer's procedure.

The reverse transcription (RT) reaction for synthesis of complementary deoxyribonucleic acid (cDNA) involved incubation of 2 µg of viral RNA and 100 pmol of the downstream primer with nuclease-free water at 65°C for 5 min. The mixture was cooled on ice for 5 min, and the reaction proceeded by adding 5 µl of 5× reaction buffer, 1.25 µl (500 µmol) of dinucleotide triphosphate (dNTP) Mix (Promega, Madison, WI, USA), 10 mM, 1 µl (40 U) of recombinant RNAseine (rRNasine) ribo-nuclease inhibitor (Promega, Madison, WI, USA), 1 µl (200 U) of RevertAid M-MuLV (Fermentas, Waltham, MA, USA) and nuclease-free water up to 25 µl. The thermal cycler programme used was 42°C for 60 min followed by 95°C for 5 min.

Polymerase chain reaction

The polymerase chain reaction (PCR) was performed using 25 µl of 2× Dream TaqGreen PCR Master Mix (Fermentas, Waltham, MA, USA), 100 pmol of upstream primer, 100 pmol of downstream primer, 8 µl of cDNA and nuclease-free water up to 50 µl. The amplification reactions were performed using a Perkin Elmer Gene Amp PCR system 9700 thermal cycler (Waltham, MA, USA). The thermal cycler was adjusted to one cycle at 94°C for 1 min, then 25 cycles at 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, followed by one cycle at 72°C for 10 min.

The amplified product was analysed by electrophoresis using a 1% agarose gel and the product size was measured using a 1 kb DNA Ladder (ThermoFisher Scientific, Waltham, MA, USA).

Sequence analysis of the purified reverse-transcription polymerase chain reaction (RT-PCR) product of the RHDV2014 strain, representing 600 bp of the hypervariable C−E region of the VP60 gene, was performed using the PCR primers by Macrogen Inc. (Seoul, Republic of Korea).

Data were analysed using Geneious 3.7 (24). The MEGA5 programme (25) and MegAlign software (26) were used for phylogenetic tree construction with the neighbour-joining method for the nucleotide

sequences and bootstrap values calculated from 1,000 replicates. The analysis was performed with other published RHDV strains from GenBank that were selected to represent the various GI.1 genotypes with different geographical and temporal origins. The accession numbers of the GI.1a strains used were: Giza2006 (JQ995154), KS2000 (JX409902), Kal2000 (JX429925), Kal2005 (JX436484), Giza2010 (KC920592), Kal2012 (KC788211), IN 05 USA (EU003578), NY 01 USA (EU003581), UT 01 USA (EU003582), Var Iowa 2000 USA (AF258618), Vt 97 Italy (EU250331), JX CHA 97 (DQ205345), WHNRH China (DQ280493), 99−05 France (AJ302016), Triptis (Y15442), 00−Reu (AJ303106), Whn−1 (DQ069280) and Pv 97 (EU250330). The GI.1b–d strains were represented by Giza97 (JX391954), Kal2011 (KF646793), Spain AST 89 (Z24757), Rainham UK (AJ006019), Bs89 Italy (X87607), FRG 91 (M67473), KSA-96 (DQ189078), Bahrain 2006 (DO189077), Hagenow (Y15441), Frankfurt (Y15424), Czech V351 (U54983), WX China 84 (AF402614), 89-Eis (Y15440), 89-SD (Z29514) and 96-Wri (Y15427). The accession numbers of the GI.2 strains used are Portu 6628 (KP862925), Portu 13647 (KP862922) and N11 Spain (JX133161); rabbit calicivirus (RCV) Italy (X96868) was used as the outgroup.

Vaccine preparation

The SERVAC RHDV® vaccine is produced as a commercial product of the Veterinary Serum and Vaccine Research Institute (VSVRI), Cairo, Egypt. It is prepared with the Giza2006 GI.1a strain using aluminium gel adjuvant. Another pilot batch was prepared with the Giza97 GI.1d strain using aluminium gel adjuvant and two other batches with Giza2006 and Giza97 using Montanide ISA206 (Seppic, Colombes, France) for preparation of hyperimmune serum (23).

Animal immunisation

In order to investigate the cross-protection between GI.1d and GI.1a RHDVa vaccines, 60 commercial crossbred susceptible rabbits, three months old and 1.5−2 kg, were obtained for immunisation. Blood samples were collected from their ear veins before vaccination, to

select seronegative rabbits. The rabbits were divided into three groups of 20 rabbits. The first group was immunised with 0.5 ml subcutaneous (S/C) SERVAC RHDV® (corresponding to strain Giza2006) while the second group was immunised with 0.5 ml Giza97 aluminium gel vaccine. The third group was kept as a control group. The rabbits were kept under field conditions (with the hygienic measures of commercial rabbit farms, but without vaccination against pasteurellosis). Food and water were provided ad libitum. Serum samples were collected weekly for three weeks.

Virus challenge

Three weeks post vaccination, each vaccine group was divided into four subgroups, separated from each other, to perform the challenge test. Challenge viruses were selected according to a previous study (16) in which the GI.1a strain Giza2010 showed 100% homology with the Giza2006 vaccine strain, while Kal2012 was the most divergent GI.1a strain, with 97.2% homology. The GI.1d Giza97 vaccine strain showed greater similarity to Kal2011 (94.7%), and lower homology with RHDV2014 (93.4%).

Each vaccine group was challenged with two homologous viruses and two heterologous viruses. The control group was also divided into four subgroups of five rabbits each, and all groups were inoculated with 1 ml of a 10% liver suspension of virulent RHDV (2^{13} haemagglutinating units) by the IM route. The GI.1d strains Giza97 and RHDV2014 and the GI.1a strains Giza2010 and Kal2012 were the virulent strains used during the challenge test.

Mortalities and post-mortem lesions were recorded, and the livers were collected immediately from dead animals. The surviving rabbits were observed for clinical signs, and serum samples were collected from all rabbits that survived three weeks post challenge; after this, all remaining rabbits were euthanised for liver collection.

Safety test

The safety of the administration of an overdose (three doses of the inactivated vaccine) was tested by inoculation of ten seronegative rabbits with 1.5 ml vaccine subcutaneously (23). The rabbits were observed for any clinical signs or mortalities for three weeks. The livers of the rabbits were tested for RHD viral RNA to check the safety and efficacy of virus inactivation during the vaccine preparation process.

Serological analysis

Titration of RHDV-specific antibodies and cross-reactivity between GI.1a and GI.1d antigens and antibodies, in either homologous or heterologous reactions, were checked by HI and confirmed with indirect enzyme-linked immunosorbent assay (iELISA).

Antigen and serum preparation

Two different strains were used as the antigen for the HI and enzyme-linked immunosorbent assay (ELISA) tests, the GI.1d Giza97 (Ag97) and GI.1a Giza2006 (Ag2006) strains. Livers from freshly dead infected rabbits were homogenised in 10% phosphate buffered saline (PBS) (wt/vol) pH 6.4, and then subjected to freezing and thawing three times. The homogenates of both virus strains were clarified by low-speed centrifugation at 500 ×g for 20 min, and then at high speed (6000 ×g) for 30 min. The supernatant was drawn carefully to avoid the superficial lipid layer and filtered through a 0.22 µm pore size filter (Cornell®, Ithaca, New York, USA) (23).

The serum samples collected pre-vaccination, weekly afterwards and post challenge were incubated at 56°C for 30 min for heat inactivation to remove non-specific haemagglutination inhibitors, kept at 4°C for 1 h followed by centrifugation at 3000 ×g for 15 min at room temperature and divided into aliquots (27).

Haemagglutination inhibition

The HI procedures were done as previously reported (23) using V-shaped microtitre plates (Nunc, Roskilde, Denmark). Each serum sample was retested once with GI.1d Giza97 antigen and again with GI.1a Giza2006, used as homologous and heterologous antigens. Two-fold serial dilution of serum samples was performed in 50 µl PBS, and an equal volume of virus antigen was added that contained eight haemagglutinating units, before incubation at 37°C for 30 min. Human red blood cells (RBCs) (type O) 0.75% were added (50 µl) and incubated at 4°C for 1 h. The endpoint was the serum dilution showing inhibition of haemagglutination, expressed as mean HI $\log_2$/ml titres. The titre of the negative control serum was in the range of 20–80 (23).

Indirect enzyme-linked immunosorbent assay

All serum samples collected were re-tested using an iELISA test for additional evaluation of the cross-reactivity between GI.1d and GI.1a RHDV subtypes. Two types of hyperimmune serum were prepared, to be used as positive controls, by inoculation of two groups of seronegative rabbits with pilot batches of inactivated vaccines prepared with Montanide ISA206 representing GI.1d and GI.1a RHDV strains (0.5 ml vaccine subcutaneously), as previously reported (28), to provide a better immune response than that obtained by aluminium gel based vaccines (29). Vaccination was repeated twice weekly for three successive weeks and serum samples were collected every week. Maxisorb plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with both GI.1d Giza97 and GI.1a Giza2006 antigens in carbonate buffer (pH 9.6). The plates were washed with PBS–0.1% Tween-20 (PBST) five times and blocked with 5% skimmed milk for 1 h at 37°C. Serum samples were added and incubated for 1 h at 37°C, washed five times with PBST, sheep anti-rabbit immunoglobulin G (IgG)−horseradish peroxidase conjugate was added at a dilution of 1:7000 and the samples were incubated at 37°C for 1 h. After washing the plates five times, the reaction was developed with 0.4 mg/ml ortho-phenylenediamine (OPD) in citrate buffer at pH 5 for 10 min in

the dark. A stop solution of 2.5 M sulphuric acid was added and the optical density (OD) was measured at 492 nm using a Biotek ELISA reader (30).

Results

Reverse-transcription polymerase chain reaction and sequence analysis

The PCR analysis of the RHDV suspected field sample (RHDV2014) showed a specific 600 bp band of the hypervariable region of VP60, indicating that the sample was RHDV positive. The sequence of RHDV2014 was submitted to GenBank with accession number KR057763. The RHDV2014 strain showed the highest homology with GI.1 RHDVs at both the nucleotide and amino acid levels. It showed 93.4% and 94.3% homology with Egyptian GI.1d strains Giza97 and Kal2011, respectively, while it showed 84.7% homology with all Egyptian GI.1a strains at the nucleotide level. The percentage homology of RHDV2014 at the amino acid level was 99.8% with Egyptian GI.1d isolates and 98.3% with Egyptian GI.1a strains. The percentage homology of RHDV2 with RHDV2014 and Egyptian GI.1 strains was 76–77.3%, while with Egyptian GI.1a strains it was 77−77.9%.

Multiple alignment of 434 deduced amino acids in the hypervariable region (C−E) of the VP60 gene of RHDV2014 with seven local strains and other published strains is shown in Figure 1. The three Egyptian GI.1d strains (Giza97, Kal2011 and RHDV2014) showed a unique amino acid substitution (R299S) in comparison with other GI.1 strains. The RHDV2014 strain showed three unique amino acid substitutions. The first substitution was A309S, where RHDV2014 shared the amino acid S (serine) with GI.1a rather than A (alanine) with GI.1 strains. The other two substitutions (L388I and N414S [in RHDV2014 only]) were unique to Egyptian classical strains (Kal2011 and RHDV2014), distinguishing them from all other GI.1and GI.1a strains studied.

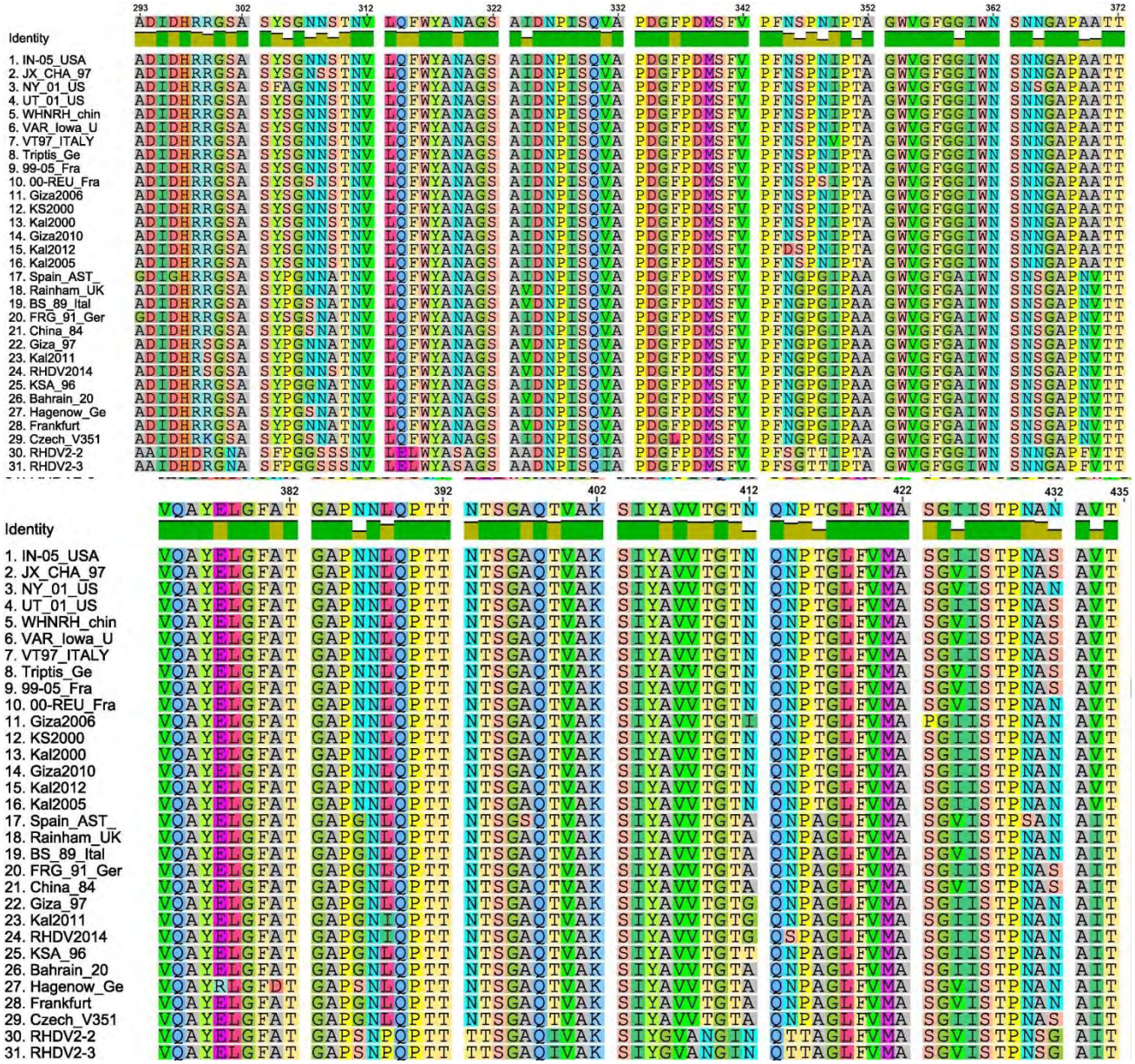

Fig. 1

Multiple alignment of 143 deduced amino acid sequences of the highly variable region (C–E) of the VP60 gene

Phylogenetic analysis

The phylogenetic tree constructed using the neighbour-joining method for the nucleotide sequence of the hypervariable region of the RHDV VP60 gene is shown in Figure 2. The RHDV2014 strain clustered with the GI.1d RHDV clade in a separate subclade with Kal2011 and is

closely related to Giza97. The three Egyptian GI.1d isolates were closely related to 96-Wri, representing the G5 genogroup (GI.1d genotype).

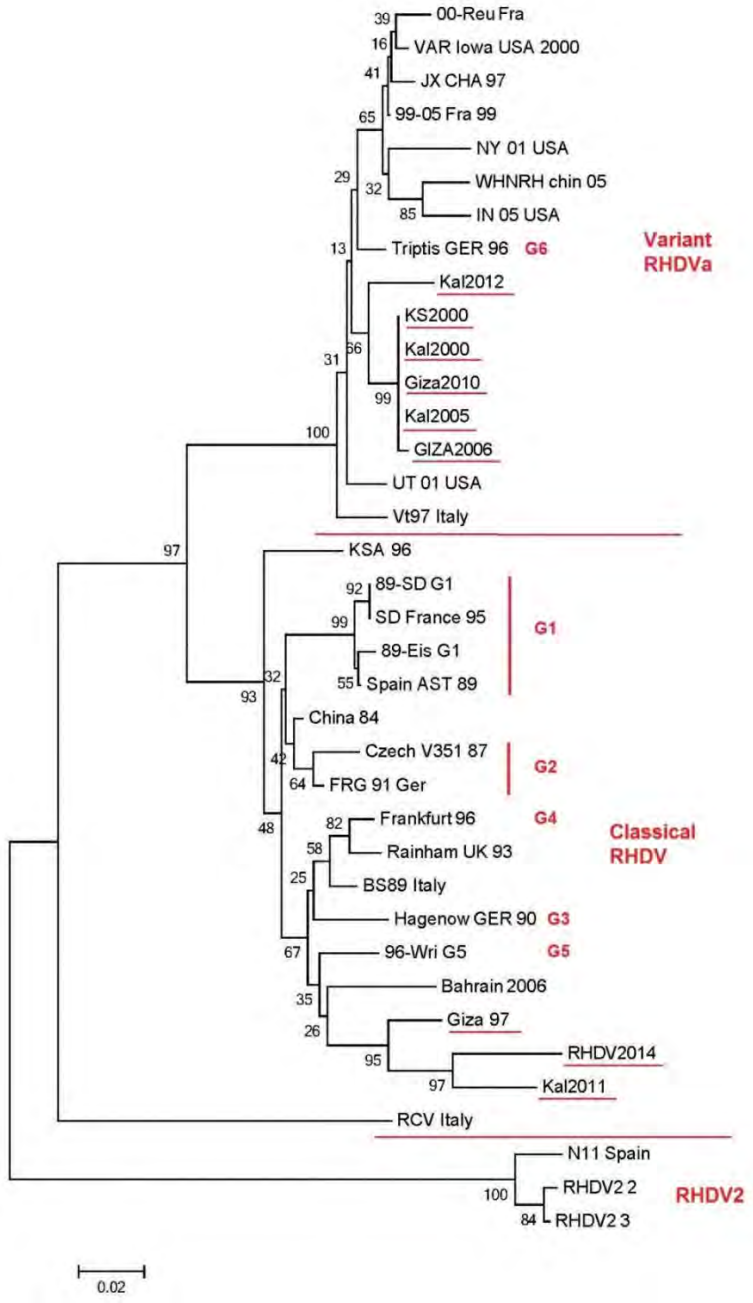

Fig. 2

Phylogenetic analysis of nucleotide sequences using the neighbour-joining method

Rabbit haemorrhagic disease virus (RHDV) genotypes were separated into three clades: GI.1a, GI.1d RHDV and RHDV2. Egyptian strains are underlined in red.

Rabbit challenge test

The rabbits from all control groups showed the typical clinical signs of RHD. The control groups that were inoculated with GI.1d RHDV strains (Giza97 and RHDV2014) showed a case fatality rate of 100% within 3−4 days post infection, while the control groups infected with GI.1a strains (Giza2010 and Kal2012) showed milder clinical signs (typical RHD signs but less severe) but 100% mortality 7−8 days post inoculation. The rabbits vaccinated with either GI.1a or GI.1d vaccines survived 17−21 days post challenge, at which time mild respiratory signs began to appear in some rabbits. This was followed by 20% (1/5) mortality in group 1 (vaccinated with GI.1a Giza2006 vaccine and challenged with homologous GI.1a Giza2010) and 40% (2/5) mortality with homologous GI.1a Kal2012 challenge in group 2. Group 3, vaccinated with GI.1d Giza97, showed 20% (1/5) mortality when challenged with heterologous GI.1a Giza2010 and group 4, vaccinated with the same vaccine but challenged with the homologous Giza97 strain, showed 50% mortality (2/4; one of the rabbits died of unrelated causes before vaccination). Groups 5 and 6, vaccinated with GI.1a Giza2006 and challenged with heterologous GI.1d Giza97 and RHDV2014, as well as groups 7 and 8, vaccinated with GI.1d Giza97 and challenged with homologous GI.1d RHDV2014 and heterologous GI.1a Kal2012, showed no mortalities. The challenge test results are summarised in Table II.

Table II

Animal challenge results

	Vaccine used	Challenge virus	No. of animals	No. of deaths	No. of RHDV positive PCR	No. of *Pasteurella* positive PCR
Group 1	Giza2006 v [(a)]	Giza2010 v	5	1	0	0
Group 2	Giza2006 v	Kal2012 v	5	2	1	1
Group 3	Giza97 c [(b)]	Giza2010 v	5	1	0	0
Group 4	Giza97 c	Giza97 c	4	2	1	0
Group 5	Giza2006 v	Giza97 c	5	0	0	0
Group 6	Giza2006 v	RHDV2014 c	5	0	0	0
Group 7	Giza97 c	RHDV2014 c	5	0	0	0
Group 8	Giza97 c	Kal2012 v	5	0	0	0

[(a)] v: GI.1a virus type
[(b)] c: GI.1d virus type
PCR: polymerase chain reaction
RHDV: rabbit haemorrhagic disease virus

The dead rabbits were examined for typical RHDV post-mortem lesions. The lesions detected were mild congestion in the liver and kidneys, and various respiratory lesions in some rabbits, with a large amount of pus in the thoracic cavity of one rabbit from group 4. Liver samples were taken from the unvaccinated rabbits post-mortem, and from surviving vaccinated rabbits at the end of the study, as well as from the six vaccinated rabbits that died 17−21 days post RHDV challenge. The samples were tested by RT-PCR to check for the presence of RHDV or *Pasteurella* infection that may have been the cause of the deaths. The RT-PCR of the hypervariable region of VP60 was positive for RHDV on liver samples collected from all unvaccinated rabbits and surviving rabbits post challenge. Only two liver samples from dead vaccinated and challenged rabbits were positive for RHDV. The first was from group 4 (vaccinated with GI.1d Giza97 vaccine and challenged with the same virus), while the second was from group 2 (vaccinated with GI.1a Giza2006 vaccine and challenged with homologous GI.1a Kal2012) (Table II).

This liver sample from group 2 was also confirmed to be positive for *Pasteurella multocida* type D by multiplex PCR (31) (data not shown).

The challenge results revealed that the GI.1a-based vaccine (Giza2006) conferred 95% protection while the GI.1d-based vaccine provided 94.7% protection.

Safety test

The rabbits inoculated with three doses of inactivated vaccine showed no systemic signs of RHDV and no mortalities in the three weeks post inoculation. For both tested vaccines, no residual RHDV RNA could be detected by RT-PCR for the hypervariable region of VP60 in any of the liver samples.

Haemagglutination inhibition test and indirect enzyme-linked immunosorbent assay

Serum samples were collected from each group three weeks after vaccination and then collected from surviving rabbits three weeks post challenge. All vaccinated animals demonstrated protective serum antibody responses with measurable HI titres before challenge, whereas none of the unvaccinated rabbits showed any detectable rise in RHDV antibody (Table I). All unvaccinated rabbits died from RHD within 72 h of challenge. All vaccinated animals survived and showed no signs of disease before virus challenge. The HI titres of rabbits immunised with GI.1a vaccine (7 $\log_2$) were one log unit higher than in those vaccinated with GI.1d vaccine (6 $\log_2$) three weeks post vaccination, when tested using both homologous and heterologous antigens. Post challenge, rabbits given the GI.1d vaccine showed much higher titres (10 $\log_2$) than those given the GI.1a vaccine (range 7−9 $\log_2$) when reacted with the GI.1d antigen. The antibody response post challenge of animals given either vaccine showed higher titres against the GI.1d antigen than the GI.1a. The challenge of GI.1a vaccinated rabbits with homologous strains gave the same HI titres when reacted with either the GI.1a or the GI.1d antigen, but sera of

rabbits challenged with heterologous strains showed higher titres with GI.1d antigen (8−9 $\log_2$) than with GI.1a antigen (5–6 $\log_2$).

Sera collected after homologous and heterologous challenge of rabbits vaccinated with GI.1d vaccine presented much higher titres (10 $\log_2$) when reacted with the homologous antigen rather than with GI.1a antigen (6–8 $\log_2$).

The iELISA results (data not shown) could not distinguish between the antibody responses elicited by GI.1d and GI.1a based vaccines and did not show any significant differences.

Discussion

Sequence and phylogenetic analyses of the main antigenic determinant regions (C and E) of the VP60 gene has been used for genotyping of RHDV (16, 32, 33, 34, 35). Sequence and phylogenetic analyses of 600 bp of the hypervariable region of the VP60 gene of the RHDV2014 strain showed its relatedness to Egyptian GI.1d strains (Giza97 and Kal2011), and the three Egyptian GI.1d strains were closely related to classical GI.1. The results confirmed that none of the Egyptian strains from 1997 to 2014 was related to GI.2. A unique amino acid mutation was shared among Egyptian GI.1d strains (R299S) that was different from other GI.1 RHDV studied when compared using multiple alignment (Fig. 1). Notably, RHDV2014 showed an amino acid substitution (A309S) which is shared with GI.1a strains. Both amino acids 299 and 309 are located at L1 of the P2 subdomain, which is the most exposed loop and lies juxtaposed to three putative binding pockets of histo-blood group antigens (HBGA) (10). The accumulation of amino acid mutations may suggest possible antigenic drift in the main antigenic determinants, as some researchers have reported a recombination event between GI.1b and GI.1a strains with two breakpoints, located at nucleotide positions 393 and 1,079 of the VP60 sequence (36). Therefore, future studies should aim to obtain full-length VP60 sequences for the different Egyptian strains to provide a clear picture of their genetic relationships and check for recombination events.

Regarding the cross-protection conferred by the vaccines based on the Egyptian RHDV strains, the challenge test results showed protection rates of 94.7% and 95% for the GI.1d and GI.1a based vaccines, respectively. Thus, both were fully protective and the mortality rates following vaccination and challenge were not directly related to the genotype or phylogenetic position of the challenge strain (Table II). These findings demonstrate that, despite the genetic divergence and antigenic differences between GI.1d and GI.1a genotypes of RHDV (16), vaccines developed for GI.1a strains are still found to be protective against classical strains, and vice versa, when administered correctly (8, 32, 37), and confirm that cross-protection is almost complete between classical RHDV and RHDVa because they belong to the same serotype (9, 38).

The antibody titres of vaccinated rabbits obtained using the HI test showed that GI.1a vaccines gave results one log higher than GI.1d vaccines but, interestingly, the post-challenge titres showed increased reactions, expressed by values 1–3 $\log_2$ higher for GI.1d vaccines.

In addition, the cross-reactivity between the homologous and heterologous strains meant that the classical antigen of Giza97 had higher antigenicity with both vaccines. The sera collected from GI.1a vaccinated rabbits after challenge with the GI.1d strain showed equal or slightly higher titres in comparison with those of GI.1a challenged rabbits (Table I).

This may give a preliminary indication that GI.1d strains of RHDV have stronger antigenicity and reactivity and higher antibody response post challenge, although each vaccine strain can be protective against challenge with the other. Previous reports support our results: antibody titres are dependent on the viral antigen type, but differences in HI cross-reactivity do not exceed the range of two dilutions (39). However, significant differences in immunological response reflect antigenic differences in RHDV (40).

The results of the RT-PCR testing of the livers collected from both euthanised rabbits and those dying as a result of the challenge showed that two out of six dead rabbits were positive for RHDV, one for each

vaccine, and both were challenged with their homologous strain. Moreover, one of them − the GI.1a vaccinated rabbit with pyothorax – was confirmed to have *Pasteurella multocida* type D infection by multiplex PCR, while the other four deaths were of unclear causation. Co-infection of RHDV with *Pasteurella multocida* may explain the occurrence of deaths with clinical signs compatible with RHDV infection despite vaccination, especially in the field, where rabbits may be unvaccinated against *Pasteurella* (41).

Both prepared vaccines were found to be safe, because no clinical signs were seen and no RHDV RNA was found in the livers of tested rabbits three weeks post vaccination with three doses, demonstrating that vaccine virus was not detected. In contrast, the livers collected from rabbits surviving three weeks post challenge were all positive for RHD viral RNA despite a lack of clinical signs, indicating the persistence of RNA in the liver. This reveals the ability of the prepared vaccines to evoke sufficient immune response to overcome the highly lethal classical GI.1 or GI.1a RHDV challenge in rabbits. These results are supported by previous findings that RHDV RNA could be detected in visceral organs of convalescent rabbits until 15 weeks after infection (2, 42), while no viral RNA was found in vaccinated animals without RHDV challenge. Vaccination is associated with reduced viral RNA loads, but is unable to induce sterile immunity. Some vaccinated rabbits had very robust immune responses and this might be manifest either by preventing viral replication or increasing the clearance of antigen, as observed in other studies (43), while others were identified as carriers of low amounts of or no viral RNA (false negatives).

The cellular immune response may also be involved with clearance of the virus and preventing the early stages of RHDV infection. Indeed, cellular immune mechanisms, which are known to be transiently elevated following infection, may play an important role (27). Further studies are required to evaluate the cell-mediated immune response induced during the vaccination process.

Unfortunately, the unavailability of commercial competitive enzyme-linked immunosorbent assay (cELISA) kits and the difficulty of obtaining monoclonal antibodies for monitoring of RHDV on rabbit farms are considered obstacles to the evaluation of RHDV status in Egypt.

Natural resistance against GI.1 (G1–G6) strains in young rabbits lasts up to four weeks of age (23, 44) and maternal antibodies may provide protection to the age of approximately eight weeks (17). The history of GI.1d Kal2011 and GI.1a Kal2012 strains shows high mortality rates (up to 100%) only in rabbits less than two months old, as previously reported (21, 45). However, the results of this study showed wide divergence between these and GI.2 strains, with only 76% nucleotide identity, indicating that GI.1 strains may appear at younger ages than previously thought. This leads to a recommendation to begin vaccination at earlier ages (4 weeks), rather than at 2–3 months old as advised by the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (23).

Conclusion

The classical GI.1 and GI.1a based vaccines were cross-protective but a vaccine combining both genotypes should be studied to evaluate whether it may evoke a better humoral and cell-mediated immune response. In addition, continuous monitoring of RHDV in Egypt is advised, considering the rapid worldwide dispersion of GI.2 even among vaccinated rabbits.

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