Development and Production of a Novel Bivalent Inactivated Rabbit Haemorrhagic Disease Virus (RHDV) Vaccine

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ABSTRACT

Rabbit Haemorrhagic Disease (RHD) is a deadly threat to rabbit populations hurting livability with severe economic losses. During 2018 and 2019 a new variant RHDV2 emerged in some Egyptian governorates, causing a typical outbreaks in commercial rabbitries, with high mortality rates especially in suckling rabbits. Classical RHDV vaccines showed low cross protection against RHDV2, revealing the need for a new vaccination strategy to face the current RHD outbreaks. In this study the development and production of a novel bivalent inactivated RHDV vaccine was applied. three hundreds six weeks old, hybrid rabbits were randomly divided into 3 groups; the first group (100 rabbits) was vaccinated with the prepared bivalent inactivated RHDV vaccine once in a dose of 0.5 ml per rabbit injected S/C (group 1), the second group (100 rabbits) was vaccinated twice with 14 days apart (boaster dose) (group 2), and the third group (100 rabbits) was kept unvaccinated as negative control (group 3). Clinical signs and mortality were monitored after challenge (performed 1 week post-vaccination, 2 WPV, 3WPV and 24 WPV). Blood samples have been collected at day 0 (before vaccination) up to 4 weeks post vaccination (WPV), every 2 weeks up to 10 weeks then every 4 weeks up to 24 WPV. In order to determine the antibody response against RHDV a and RHDV2 by Haemagglutination inhibition (HI) tests. No clinical signs or adverse reactions were observed in the two vaccinated groups (1and 2) suggesting that the prepared bivalent RHDV vaccine was safe, potent and survived the experimental challenge where the mortality rate in the control group was higher. All animals from both vaccinated groups (1 and 2) showed clear seroconversion (Mean titers for RHDV HI antibodies higher than protective HI titer for RHDV a and RHDV2) beginning from seven days post vaccination. No significant differences were observed between HI titers among group 1 and group 2. These results revealing that the administration of single dose of the prepared bivalent RHDV vaccine was adequate and recommended, performing a challenge 7DPV. Furthermore, serological analysis demonstrated that the prepared bivalent vaccine was proved to be sterile, safe and potent protecting rabbits against 2 viruses (RHDV a and RHDV2) in one shot consequently saving time, efforts of labor and avoiding stress of rabbits during vaccination.

Key words: RHDVa, RHDV2, Vaccine, Production, A novel, Bivalent, Rabbit

INTRODUCTION

Rabbit Haemorrhagic Disease (RHD) is an acute febrile highly fatal infectious disease causing heavy losses among rabbits (Cao et al., 1986). The disease was first reported in China in 1984 (Liu et al., 1984). In Egypt, the RHD has been reported for the first time in 1991 in Sharkia governorate (Ghanem and Ismail, 1992). In france during 2010, a new lagovirus genotype related to RHDV was emerged and found to be new RHDV variant desigated RHDV2 which is genetically and antigenically different from classic RHDV (Le Gall-Reculé et al., 2013). In Egypt at mid 2018 and continued 2019 severe mortalities were recorded in vaccinated rabbit flocks. Samples were confrmed to be positive for RHDV from different Governorates. Genotyping of these recent isolates was done by sequencing and phylogenetic analysis of (C to E) region of highly variable region of VP60 capsid gene that revealed clustering of all Egyptian isolates with RHDV2 Genogroup I.2 (GI.2) strains with high homology up to 98.4% and was genetically distinct from classical and variant RHDV with divergence around 23%. This is the first report to identify the arrival of RHDV2 (GI.2) in Egypt (unpublished data). The clinical evolution of the

disease can be per acute, acute, sub-acute or chronic (Marcato et al., 1991). RHD is characterised by high morbidity and high mortality of 70–90% for RHDV/ RHDVα and 5–70% for RHDVβ. In rabbits younger than 4–6 weeks, the RHDV/RHDVα infection course is subclinical, but when the causative agent is RHDV2, clinical signs and mortality are observed even in young animals from 15 to 20 days of age (Puggioni et al., 2013).

Control policy of RHD mainly depends on vaccination using the appropriate vaccine. Successful control of RHD was easy during the last two decades due to the use of effective vaccine in addition to low antigenic variation of the field virus strains (Lavazza and Capucci, 2012). In Egypt, an inactivated formalized RHDV vaccines have been developed using Egyptian classical strain (Daoud et al. 1998) then using Egyptian variant RHDVa (Salman 2007). However, outbreaks of RHD with dramatic lethality were recorded in rabbit flocks that were vaccinated with commercial available vaccines prepared from classic or variant strains of RHDV (RHDV/ RHDVα). Antigenically different RHDV from the classical was isolated and called RHDV2 (Dalton et al., 2012; Le Gall-Reculé et al., 2013). Cross protection was low between the classical strains (RHDVα) & RHDV2 (Bárcena et al., 2015).

OIE (2018) advised to vaccinate rabbits with vaccine containing both antigenic types (RHDVα & RHDV2) or contain the homologous strain to that identified during the outbreak. Classical RHDV vaccines showed low cross protection against RHDV2 and did not prevent infection and losses of clinical disease (OIE 2019). Therefore the aim of the present work was planned to prepare a bivalent vaccine containing both antigenic types (RHDVa and RHDV2) and assessment its efficacy and safety for controlling RHD outbreaks in Egypt to minimize economic losses, control both viruses in one shot saving time, effort of labor and avoiding stress of rabbits during vaccination.

MATERIALS AND METHODS

Rabbit Haemorrhagic Disease Viruses (RHDV)

RHDVa: Local Egyptian strain of RHDV designated as Giza/2006 (Salman, 2007) with a titer of 10⁶.³ LD₅₀/ml and of haemagglutination (HA) titer equal to 2¹⁴ HA unit was used for vaccine preparation, challenge of vaccinated rabbits and in haemagglutination inhibition (HI) tests, it was obtained from Veterinary Serum and Vaccine Research Institute (VSVRI) Newcastle Disease Department.

RHDV2: Local Egyptian strain of RHDV2 designated as Mahala2019/VSVRI with Accession Number MK736667, titer of 10⁶.⁷ LD₅₀/ml and of HA titer equal to 2¹² HA unit was used for vaccine preparation, challenge of vaccinated rabbits and in HI test, it was obtained from VSVRI Newcastle Disease Department.

Experimental rabbits: Three hundred and thirty six weeks old, hybrid rabbits with an average body weight of 1.25 to 1.5 Kg were purchased from a conventional rabbitry. All rabbits were proved to be sero-negative for RHDV antibodies. The rabbits were required for vaccine preparation and its evaluation.

Serum samples: Blood samples were collected from the experimental rabbits through the ear vein. The collected blood samples were allowed to coagulate and centrifuged (2500 rpm 10 minutes) in order to separate the serum. Sera of individual rabbits were subjected for inactivation process by heating in a water bath at 56°C for 15 minutes then kept in sterile screw capped vials at -20°C till examined serologically using HI test to detect the specific RHDV antibodies.

Positive and negative control sera of RHDVa and RHDV2: These sera were supplied from VSVRI Newcastle Disease Department. It used in HI test.

Erythrocytes suspension: Erythrocytes human type "O" were collected from a healthy volunteer using 3.8% sodium citrate solution as anticoagulant. The packed erythrocytes were suspended in sterile saline in a concentration of 0.75% for micro-technique of HA and HI tests.

Adjuvants: Rehydragel®LV (CHEM TRADE) was used for preparation of the inactivated RHDV vaccine, Aluminium hydroxide (Al OH) low viscosity gel. Stock No. 203120070602. It was supplied by CHEM TRADE - BERKELEY HEIGHTS, NEW JERSEY. It was used according to manufacturer instructions.

Chemicals

Formaldehyde solution (Fluka Riedel-deHaen, Sigma, Germany): Lot No. 52930. 37% by weight stabilized with approximately 10% methanol. It was used for virus inactivation.

Sodium thiomersal (PARK scientific limited Northampton, UK): Lot No. P839F. It was prepared as solution in a concentration of 1/10000 (W/V) and added to the prepared vaccine in a concentration of 1ml/ liter as a preservative.

Haemagglutination (HA) test: A two fold dilution of the RHDV was incubated with an equal volume of 0.75% concentration washed human RBCs type "O" in a sealed V shaped-bottom micro-titer plate at 4°C to determine HAU used in HI test according to Capucci et al., (1996).

Haemagglutination inhibition (HI) test: It was carried out using 8 HA unit of RHDV and human RBCs type "O" to estimate specific RHDV antibodies in rabbit serum (Peshev and Christova, 2003).

Preparation of inactivated RHDV suspension: RHDVa and RHDV2 suspensions that incorporated into the vaccine were prepared according to OIE, (2018).

The viral inactivated suspension of both strains was assayed by HA test and it was found to be 2¹⁰ HAU for each one after inactivation as it is recorded by Kim et al., (1989). Abolishing viral infectivity was carried out using formaldehyde at 0.4% concentration at 37°C for 48 hours. During inactivation, the fluid was continuously agitated.

Preparation of the gel inactivated bivalent RHDV vaccine: The gel emulsion vaccine was prepared before distribution into neutral glass of 10 ml capacity vials (each
contains 5 ml of the vaccine). The vaccine was stored at 4°C till used.

Quality control: The prepared vaccine was subjected to sterility and safety following standard international protocols of British Pharmacopoeia Veterinary (2005).

Sterility test: The prepared vaccine was tested for the presence of viable bacteria, mycoplasma and fungi.

Safety: Safety test was carried out by S/C inoculation of 10 sero-negative rabbits with two times of the vaccinal dose. The rabbits were observed for 3 weeks post inoculation.

Vaccine efficacy: Vaccine efficacy assessment was based on antibody response measured by HI test and challenge test.

Experimental design: A total of 300 experimental rabbits were housed in disinfected metal cages in a well-ventilated and disinfected room receiving commercial pellet ration and clean water ad libitum. The rabbits proved to be sero-negative for specific RHDV antibodies of both strains. The rabbits were divided into 3 groups. Group (1) included 100 rabbits were vaccinated with the prepared bivalent inactivated RHDV vaccine once in a dose of 0.5 ml per rabbit injected S/C, group (2) included 100 rabbits were vaccinated twice with 14 days apart (booster dose) and group (3) included 100 rabbits were kept unvaccinated as negative control. Each rabbit group was housed separately under well hygienic measure and kept under daily observation till the end of experiment.

Humoral immune response: It was followed up for 6 months for all groups starting from 0 time. Blood samples were collected weekly till 4th WPV, every 2 weeks till 8th WPV and then monthly till 6th month post vaccination (MPV). Sera were separated and kept at -20°C till used to evaluate humoral immune response through HI test.

Challenge test: At the 1st, 2nd, 3rd WPV and 24 WPV, randomly chosen 20 rabbits from each group either vaccinated (group 1 and 2), or unvaccinated (group 3) were transported to experimental isolators where they were challenged as follow, 10 rabbits received 1ml suspension containing 10^3 LD_{50} virulent RHDV2a and the other 10 rabbits received 1ml suspension containing 10^3 LD_{50} virulent RHDV2. The challenged rabbits were kept under daily observation for 2 weeks post challenge. For clinical signs and/or deaths OIE, (2018).

RESULTS AND DISCUSSION

RHD is the major viral disease of rabbits. It is a highly fatal disease threatening rabbit population causing extreme economic losses (Barcena et al., 2000).

The disease was first reported in China in 1984 (Liu et al., 1984). The disease was endemic in most parts of Europe and Asia, in some African countries, in Australia and New Zealand (Grazioi et al., 2000). In Egypt, the RHD has been reported for the first time in 1991 in Sharkia governorate (Ghanem and Ismail, 1992). All RHDV isolates were antigenically related until Capucci et al. (1998) identified consistent genetic and antigenic differences in 1997 and this variant was named RHDV2.

In Egypt this variant (RHDV2) was emerged and isolated at 2006 (Salman, 2007). Indirect control of the disease in Egypt has been achieved by vaccination using inactivated RHDV vaccine which has been manufactured from the classic RHDV strain. (Daoud et al., 1998 and Salman, 1999) then the classic RHDV strain has been replaced by variant RHDV2 strain starting from 2008 until now. (Salman, 2007). RHDV2 was first recorded in France, 2010 (Le Gall-Recule et al., 2011). It was considered to be a newly emerged virus of RHDV due to its unique genetic and antigenic profile (Le Gall-Recule et al., 2013). Emergence of variant RHDV2 was reported in some Egyptian governorates with high mortality rates especially in suckling rabbits in Egypt during 2018 and early, 2019. Ten isolates were consistently found and all were RHDV2 after sequence and submission to Gen Bank with the following accession numbers: Benha2018 VSVRI (MK991768), M.Ghamr2019 VSVRI (MK991769), Menofia2019 VSVRI (MN007210), KaI2018 VSVRI (MN007207), Daaqah2019 VSVRI (MN007208), K Sheik2018 VSVRI (MN007209), Sempel2019 VSVRI (MN007211), Alex2019 VSVRI (MN007212), Dosaq2018 VSVRI (MK991770), Mahala 2019 VSVRI (MK736667) (Unpublished Data).

Table 1: Geometric mean of RHDV specific HI antibody titers (log2) in serum of vaccinated rabbit groups by prepared bivalent RHDV vaccine and unvaccinated rabbits.

<table>
<thead>
<tr>
<th>Time post vaccination</th>
<th>Geometric mean of RHDV specific HI antibody titers (log2)</th>
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<tbody>
<tr>
<td></td>
<td>Group (1)</td>
</tr>
<tr>
<td></td>
<td>Antigen used in HI test</td>
</tr>
<tr>
<td></td>
<td>RHDV2a</td>
</tr>
<tr>
<td>0 Day</td>
<td>0</td>
</tr>
<tr>
<td>1st WPV</td>
<td>6</td>
</tr>
<tr>
<td>2nd WPV</td>
<td>8.9</td>
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<tr>
<td>3rd WPV</td>
<td>10.7</td>
</tr>
<tr>
<td>4th WPV</td>
<td>11.5</td>
</tr>
<tr>
<td>6th WPV</td>
<td>11</td>
</tr>
<tr>
<td>8th WPV</td>
<td>10.92</td>
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<tr>
<td>10th WPV</td>
<td>10.85</td>
</tr>
<tr>
<td>12th WPV</td>
<td>9.82</td>
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<tr>
<td>16th WPV</td>
<td>9.5</td>
</tr>
<tr>
<td>20th WPV</td>
<td>9</td>
</tr>
<tr>
<td>24th WPV</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Protective HI antibody titers ≥20 HI units; RHDV= Rabbit Haemorrhagic Disease viruses; HI= Haemagglutinating inhibiting; WPV= Week Post Vaccination; Group (1) = Vaccinated; Group (2) = Vaccinated by booster dose. Group (3) = Un vaccinated control.
There is cross protection immunity between classic RHDV and RHDVa (Capucci et al., 1998), while no cross protection immunity between RHDVa and RHDV2 (Bárceca et al., 2015). An inactivated vaccine against RHDV2 was evaluated with its simultaneous administration with a classical RHDV vaccine. The results suggested that the simultaneous administration of classical RHD vaccine did not interfere with inactivated RHDV2 vaccine. Montbrau et al. (2016) also the use of combined vaccination with both antigenic types (RHDVa & RHDV2) was highly advisable as mentioned in OIE (2018).

It should be taken into consideration that both vaccines were prepared in separate vials and introduced simultaneously in rabbits. What was unique in the current study? It was that a single bivalent vaccine protect against both strains; the variant RHDVa and RHDV2. The prepared bivalent vaccine control both viruses in one shot consequently saving time, efforts of labor and avoiding stress of rabbits during vaccination. Inactivated RHDV content in the vaccine was 2<sup>10</sup> HAU in accordance with Kim et al. (1989) and more than 2<sup>10.3</sup> as recommended by OIE (2018).

The prepared inactivated bivalent vaccine was cultured on different synthetic media for detection of bacterial and fungal growth. It was found that, the vaccine was sterile (no growth of micro-organisms on nutrient agar, blood agar and Sabaroud agar) and this went with British Pharmacopoeia Veterinary (2005). The prepared inactivated bivalent vaccine was proved to be safe (The ten inoculated rabbits S/C with two times of the vaccinal did not show notable signs of disease or local reaction and remained healthy during the 3 weeks observation period).

Evaluation of the vaccine was based on Humoral immune response and challenge. The prepared bivalent inactivated RHDV vaccine was evaluated immunogenically in 3 groups of experimental rabbits, experimental Group (1) vaccinated with the prepared bivalent inactivated RHDV vaccine once in a dose of 0.5 ml per rabbit injected S/C, Group (2) vaccinated twice with 14 days apart (booster dose) and Group (3) unvaccinated negative control.

Humoral response was assessed by HI test. Estimated mean specific RHDV HI antibodies were recorded and shown in Table (1). All rabbits vaccinated and unvaccinated were seronegative before vaccination. Huang, (1991) and Smid et al., (1991) reported that immunity to RHD after vaccination was rapidly developed in the vaccinated rabbits and persisted for more than 6 months. and OIE (2018) reported that inactivated and adjuvanted vaccinated animals quickly produce solid protective immunity against RHD infection within 7-10 days. In the current study, the specific RHDV HI antibodies began to be detected from the 1<sup>st</sup> WPV in agreement with Wei et al. (1987); Haralambiev et al., (1990); Popovic, (1990) and Smid et al., (1991). The vaccine induced fast immunity in the vaccinated rabbits manifested by the mean titers for specific RHDV HI antibodies at 1st WPV for the vaccinated groups ranged from 2<sup>6</sup>, 2<sup>5.75</sup> for RHDVa and RHDV2 respectively in group (1). While in group (2) which vaccinated with booster dose they were 2<sup>5.5</sup>, 2<sup>6</sup> for RHDVa and RHDV2 respectively and these results agree with Montbrau et al., (2016) who reported that the administration of inactivated classic (RHDVa) and inactivated variant RHDV2 vaccine simultaneously supported the onset of immunity from 7 days post vaccination. Mean titers for RHDV HI antibodies increased gradually in the two vaccinated groups reaching 2<sup>9.7</sup>, 2<sup>10.3</sup> in group(1) for RHDVa and RHDV2 respectively, while in group (2) they were 2<sup>10.3</sup>, 2<sup>10.4</sup> for RHDVa and RHDV2 respectively at 3<sup>rd</sup> WPV, then quickly produced strong humoral immune response against RHDVa and RHDV2 as represented by elevated and sustained HI titers peak (11,11,12,11) log<sub>2</sub> at 6<sup>th</sup> WPV then decrease but still high at 12,16,20, 24 weeks post vaccination. Furthermore, rabbits vaccinated twice with two weeks interval revealed nearly similar humeral immune response. From the aforementioned results, it could be concluded that using of prepared of bivalent vaccine with single dose protect rabbits from disease.

Following challenge test, the prepared bivalent inactivated RHDV vaccine gave protection of vaccinated rabbits against challenge with virulent RHDVa and RHDV2 (10<sup>5</sup> LD50/ml) after observation period beginning from 1WPV and this agree with Montbrau et al., (2016), who found that RHDV2 vaccine was effective in protecting rabbits against challenge with virulent RHDV-2 strain seven days after vaccination.

Protection percent was 70% when challenged with RHDV2 and it was 80% to RHDVa at 1WPV These results support the onset of immunity to established from 7 days post vaccination, this protection increase to 90% to RHDV2 and full protection was achieved for RHDVa (100%) at 2<sup>nd</sup> WPV while full protection was gained for both viruses.

### Table 2: Potency of bivalent inactivated RHDV vaccines to RHDVa and RHDV2.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Viruses used in challenge</th>
<th>Time of challenge</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>1WPV</td>
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<tr>
<td>Group 1</td>
<td>RHDVa</td>
<td>SR/CR</td>
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<tr>
<td></td>
<td></td>
<td>P%</td>
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<tr>
<td></td>
<td>RHDV2</td>
<td>SR/CR</td>
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<tr>
<td></td>
<td></td>
<td>P%</td>
</tr>
<tr>
<td></td>
<td>RHDVa</td>
<td>SR/CR</td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td>P%</td>
</tr>
<tr>
<td></td>
<td>RHDV2</td>
<td>SR/CR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P%</td>
</tr>
<tr>
<td></td>
<td>RHDVa</td>
<td>SR/CR</td>
</tr>
<tr>
<td>Group 3</td>
<td></td>
<td>P%</td>
</tr>
<tr>
<td></td>
<td>RHDV2</td>
<td>SR/CR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P%</td>
</tr>
</tbody>
</table>

SR/CR= Survived rabbits/Challenged rabbits; P%=Protection percent WPV = Week Post Vaccination. Group (1) = Vaccinated, Group (2) = Vaccinated by booster dose, Group (3) = Unvaccinated control, RHDV= Rabbit Haemorrhagic Disease viruses.
(100%) at 3rd WPV which continued till the end of the experiment (24th WPV) in vaccinated groups (Table 2).

The challenge resulted in 100% protection in vaccinated groups and this was identical with that recorded by Shevchenko, (1994) who showed that RHDV vaccine resulted in 100% protection of rabbits, Salman, (1999) who found that the protection percentage against the challenge with 10^3.5 LDs of RHDV was 100% in the vaccinated rabbits. Also the challenge result agree with the result of Smid et al. (1991) and Daoud et al. (1998) who recorded that rabbits developed full protection against RHDV infection 3 weeks after the administration of a single dose of inactivated RHDV vaccine.

Protection against clinical disease was expected with the specific RHDV HI antibody titer, induced in vaccinated rabbits, and proved that all the prepared inactivated RHDV vaccines having sufficient amount of RHDV antigen that may have the potential to induce higher level of protection against infection than was currently realized, This result comes in contact with those of Stone et al. (1983).

The obtained results also agreed with those of Nowotny et al., (1993) who found that the adult rabbits with RHDV-antibody titers ranging from 2^6 to 2^13 remained clinically healthy after inoculation with virulent RHDV and Simon et al. (1993) who concluded that a titer > 20 HIU was protective.

The mortality rate obtained in the unvaccinated (control) group of RHDV was 100% and the mortality rate obtained in the un vaccinated (control) group of RHDV was ranged from 50-70% and these results were similar to pervious reported results by many authors when experimental infections were made with the variant RHDV. as Le Gall-Reculé (2013) who obtained a mortality rate of 46%; Parra and Dalton (2013) who registered mortality rates of 50-55% 72 hours after challenge. These results confirmed that mortality rate when challenge with RHDV was lower than the rate when challenge was caarried out with RHDV. These results agree with OIE (2018) which reported that RHD was characterised by high morbidity and a mortality of 70–90% for RHDV/RHDV and 5–70% for RHDV2.

The prepared bivalent vaccine was proved to be sterile, safe and potent protecting rabbits against 2 viruses (RHDV and RHDV2) in one shot consequently saving time, efforts of labor and avoiding stress of rabbits during vaccination.

Finally, it was recommended that further continuous periodic investigation should be applied with effective disease surveillance for early detection and reporting Egyptian circulating field RHD viruses either RHDV and/or RHDV2 in order to use proper, safe, effective and superior long term vaccines efficacy.

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