Original article

A novel bivalent Pasteurellosis-RHD vaccine candidate adjuvanted with Montanide ISA70 protects rabbits from lethal challenge

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ABSTRACT

In the present study, a bivalent vaccine against *Pasteurella multocida* and rabbit hemorrhagic disease virus (RHDV) was formulated with Montanide™ ISA70 oil adjuvant (Seppic, Paris, France). Its efficacy was evaluated and compared to similar monovalent preparations and commercially available monovalent vaccines. White new Zeeland rabbit groups (n = 10) received 2 successive doses of the tested vaccines and were challenged 2 weeks after 2nd dose with *Pasteurella multocida* and RHDV or either pathogens according to their vaccination schedule. Challenged not-vaccinated group of rabbits (n = 10) was included as a control. The bivalent and monovalent ISA70 preparations were found stable, safe, sterile, pure and of low viscosity. Group 3 (GP3) which received bivalent vaccine showed the highest antibody geometric mean titers against *Pasteurella multocida* and RHDV evaluated by ELISA and hemagglutination inhibition (HI) respectively. Following virulent challenge; Gp3 rabbits were 90% protected from challenge over other groups that showed 80% protection. Detection of either pathogen in the livers of dead and euthanized rabbits had failed except for non-vaccinated controls. The bivalent vaccine candidate was fully protective. Immunization against both pathogens can be achieved by single vaccination.

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1. Introduction

Rabbit production is a source of income improvement and high biological-value animal-sourced protein for low income families in Egypt (Abdel-Kafy et al., 2017). Three decades ago, rabbit production represented 7.1%of Egypt's poultry production (Galal and Khalil, 1991) and it seemed to achieve almost similar growth rate as that of poultry as the central agency for public mobilization and statistics reported that rabbits represented 6.25% of Egypt's poultry production in 2015 (Annual Bulletin of statistics livestock, 2015). It is believed that 90 percent of rabbits bred in Egypt are held by small and medium sized producers; only ten percent of the production is in large scale industrial farms (Galal and Khalil, 1991). Hence, the concept of real cuniculture (industrial rabbit farming) is not present in Egypt and most of these rabbits are bred in sub-optimal or poor conditions.

Similar to other animal species, rabbits are susceptible to many infections and infestations leading to varying degrees of economic losses (Eid and Ibraheem, 2006). From these multiple agents; *Pasteurella multocida* and rabbit hemorrhagic disease virus (RHDV) are the most devastating; leading to great economic losses in production rabbits (Ismail et al., 2018, Soliman et al., 2015). *Pasteurella multocida*, are transmissible virulent non-motile Gram-negative coccobacilli. It is the etiological agent of snuffles, one of the most

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significant bacterial diseases of rabbits that causes considerable economic losses in all rabbit production settings world-wide (Carter, 1967, Soriano-Vargas et al., 2012). Pasteurellosis can cause high morbidity and mortality up to 100%butit is most often characterized by subclinical infections of the respiratory, genital, and nervous systems. Clinical signs in rabbits include rhinitis with purulent nasal discharge, pneumonia, otitis media, pyometra, orchitis, abscesses, ocular conjunctivitis, and septicemia (Nassar, 2013). Direct and indirect cont act are the prime routes of *Pasteurella multocida* transmission mainly via aerosol (Kumar and Purushothaman, 2009). Based on genetic bases; genotype A: L3: *ST9*was found to be the most dominant in rabbit *Pasteurella multocida* isolates (76.47%). A refers to capsular type genotype, L3 refers to lipopolysaccharide genotype and *ST9* refers to multilocus sequence type (MLST) (Peng, 2018).

In 1991, rabbit hemorrhagic disease (RHD) was first reported in Egypt with storm of mortalities and economic losses reached 90% in Nile delta region then spread to upper Egypt in the winter and spring of 1993 causing another wave of mortalities in all rabbits above 4 weeks of age (El-Mongy, 1998; El-Zanaty, 1994). RHDV, the causative agent of RHD, is a non-enveloped, icosahedral, single-stranded plus polarity RNA lagovirus with 7.2 kb viral genome and 32–44 nm capsid diameter. Like the majority of calici-viruses, RHDV is non-cultivable in vitro (Verdaguer et al., 2004; Abrantes et al., 2012). RHDV was destructive for rabbit production in Egypt as rabbit population was naïve to the agent; outbreaks continued to cause losses (Fahmy et al., 2010). RHDV transmission can occur through oral, nasal, parenteral, or conjunctival routes. Virus shed in feces and bodily secretions; contact with shed virus is the main route of RHD transmission (Abrantes et al., 2012).

Pasteurella multocida and RHDV infections could be controlled via biosecurity and prevented by immunoprophylactic vaccination. Because of RHDV uncultivability; commercial vaccines are prepared from infected homogenized rabbit livers after inactivation (Arguello Villares, 1991). Adjuvants represent a key factor for inactivated vaccine success (Dupuis et al, 2006). The present study aimed to prepare a potent bivalent vaccine against rabbit Pasteurellosis and RHD using Montanide™ ISA70 oil adjuvant (Seppic, Paris France). To our knowledge, the prepared combined *Pasteurella multocida* RHDV vaccine candidate is the first ISA70-adjavanted bivalent rabbit vaccine in Egypt.

2. Material and methods

2.1. Ethics statement

All animal related procedures in the study were approved ethically by Cairo University institutional animal care and use committee (CU-IACUC). Approval document number is CU-11-F-66-17.

2.2. Micro-organisms and seeds preparation

Fully identified Serotype (A) rabbit-origin virulent local *Pasteurella multocida* strain was obtained from Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza. Virulent local RHDV strain was obtained from Animal Health Research Institute, Dokki, Giza, Egypt. Both strains were verified by PCR and RT-PCR in the Departments of Microbiology and Virology –Faculty of Veterinary Medicine, Cairo University using Oligonucleotide Primers mentioned in Table 1.

Pasteurella multocida was propagated aerobically in Tryptic Soy Broth (Merck, Darmstadt, Germany) at 37 °C for 24 h. Colony forming unit titer (cfu/ml) was evaluated (CFR, 2018). Inactivation was done by addition of 0.25% formalin (Merck, Darmstadt, Germany). Inactivated culture was tested for purity, safety and sterility

Table 1
Oligonucleotide primers sequences.

Gene	Primer sequence 5′-3′	Amplified Segment (bp)	Reference
Kmt1	ATCCGCTATTTAAGTGG GCTGTAAACGAACTCGCCAC	460	Townsend et al., 2001
VP60	CCACCACCAACACTTCAGGT CAGGTTGAACACGAGTGTGC	538	Guittre et al., 1995

(Mukkur et al., 1982) then, it was preserved in 0.01% of thiomersal (Merck, Darmstadt, Germany) at 4 °C until preparation of vaccine emulsion.

Infected livers and spleens were collected from rabbits that died between 24 and 96 h following an oral RHDV inoculation. Organs were homogenized for 10 min in 1/10 (w/v) sterile PBS, pH 7.3. Three −20 °C freeze-thaw cycles were applied to liberate virus particles. Homogenate was treated with 2% chloroform (Sigma) for18 h at 4 °C, followed by centrifugation at 6000g for 1 h at 4 °C. The supernatant was collected was subsequently inactivated. Virus hemagglutination (HA) titer was tested before and after inactivation (Le Gall-Recule et al., 2001, OIE, 2015). Prepared virus was used directly for vaccine preparation after being tested free from myxoma virus (OIE, 2015).

2.3. Preparation of inactivated vaccines

For each vaccine preparation, the precise amount of diluent was calculated to reach the final concentration of each antigen per vaccine dose, taken in consideration that oil will occupy 70% of each preparation volume. Montanide™ ISA70 oil (Seppic, Paris, France) was emulsified with each different antigen preparation, water phase, under aseptic condition. Homogenization is performed at low speed (1200 rpm) with ultra-homogenizer in an ice bath according to manufacturer instructions. Mixing continued till obtaining stable, milky, and low viscosity emulsion. Floating drop technique was used to assess viscosity. Single dose of inactivated *Pasteurella multocida* vaccine was adjusted to contain 10^8 CFU. Viral vaccine dose was adjusted to contain 2^8HAU inactivated RHD virus. Bivalent vaccine dose was adjusted to contain minimum 2^8HAU inactivated RHDV &10^8 CFU inactivated *P. multocida*.

2.4. Quality control evaluation of the prepared vaccines

Safety, sterility, purity, and stability of the prepared vaccines were all evaluated according to the Code of American Federal Regulation (CFR, 2018).

2.5. In-vivo efficacy and challenge of the prepared vaccines

Sixty healthy New Zealand male White Rabbits with an average body weight of 1.3 to 1.7 kg were supplied from laboratory animal unit (VACSERA, Dokki, Egypt) under strict hygienic measures in which RHDV and *Pasteurella multocida* infections were not evident. They were kept in specific rabbit housing rooms, fed and watered ad-libitum. Experimental design was shown in Table 2.

2.6. Evaluation of humoral immune response among the vaccinated groups

Homemade ELISA (Briggs and Skeeles, 1984) using homologous antigen was used to evaluate anti-*Pasteurella multocida* antibodies in vaccinated rabbits while hemagglutination inhibition (HI) was used to evaluate anti-RHDV antibody response (OIE, 2015) pre and post-challenge.

Table 2
Experimental Design used for evaluation of vaccines

Time of injection and serum collected	Group 1 (GP1)	Group 2 (GP2)	Group 3 (GP3)	Group 4 (GP4)	Group 5 (GP5)	Group 6	
						Group 6a (GP6a)	Group 6b (GP6b)
Day 0 injection	10 rabbits were injected S/C with the prepared P. multocida vaccine	10 rabbits were injected S/C with commercial P. multocida vaccine	10 rabbits were injected S/C with the prepared bivalent vaccine against P. multocida and RHDV	10 rabbits were injected S/C with commercial RHDV vaccine	10 rabbits were injected S/C with the prepared RHDV vaccine	5 rabbits were used as P. multocida non-vaccinated control	5 rabbits were used as RHDV non-vaccinated control
15 days post inoculation	1st collection of blood (T1) from rabbits and injection of booster dose.					1st collection of blood from rabbits	
30 days post inoculation	2nd collection of blood (T2) from rabbits. Rabbits were challenged orally with 2^8HAU/dose for RHDV and/ or 2^8CFU/dose for Pasteurella multocida in accordance with the used vaccine.					challenged orally, with 2^8CFU/dose for P. multocida	challenged orally, with 2^8HAU /dose for RHDV
45 days post-inoculation	3rd collection of blood (T3) from rabbits						
60 days post inoculation	4th collection of blood (T4) from rabbits						

2.7. Post challenge protection percent verification

All rabbits were kept under observation. Clinical signs &mortality rates were recorded. Remaining rabbits were euthanized after experiment. Livers were collected from all rabbits and tested by PCR and culture for *Pasteurella multocida* and only PCR for RHDV.

2.8. Statistical analysis

Simple one-way ANOVA was used to analyze ELISA and HI test results, Duncan's multiple range test was used to detect significance. The recorded data $p < 0.05$ was considered as statistically significant (Snedecor and Cochran, 1989). Software used was SPSS version 22.0.

3. Results

3.1. Seed verification, titration, and inactivation

Pasteurella multocida was verified by PCR using *KMT1* primers producing a 460 bp amplicon at the expected molecular size compared to positive control and molecular size marker. Colony forming unit titer was 10^9 CFU/ml. inactivated bacterial culture failed to exhibit growth in tryptic soy agar.

Compared to molecular size marker, the 538 bpb and consistent with partial amplification of RHDV VP60 gene was obtained from original RHDV strain as well as from RHDV in the clarified liver homogenate collected from inoculated rabbits after death. HA titer of propagated RHDV reached 2^{13}HAU/50 μl. One log decrease in the HA activity following chloroform treatment was observed while no change in the titer was observed after formalin inactivation of the virus. Inactivated virus failed to induce clinical signs in orally infected rabbits.

3.2. Quality control of the prepared vaccines

Emulsion quality of the prepared vaccines was good, milky white in color and of low viscosity. The prepared vaccines were stable for 12 months like their commercial counterparts when stored at 4 °C. No evidence of any bacterial (aerobic or anaerobic contaminants), fungal growth or even Mycoplasmal growth has been detected after prolonged incubation (14 days) with different inoculated media. No deaths, local or general reactions were observed in rabbits vaccinated with either monovalent inactivated *Pasteurella multocida* vaccine, monovalent inactivated RHD vaccine and bivalent vaccine up to 1 month of injection except for slight subcutaneous tissue reaction.

3.3. Evaluation of humoral immunity among rabbits vaccinated with the prepared vaccines

Antibodies that developed against *Pasteurella multocida* in rabbits vaccinated with monovalent inactivated *Pasteurella multocida* vaccine, bivalent vaccine and commercial inactivated *Pasteurella multocida* vaccines were evaluated by ELISA. Antibody mean titer, group differences and statistical significance were calculated using one-way ANOVA, Table 3. Antibody titer increased in GP3 at different time points with means of 2.333, 1.321, 0.982, and 0.725, respectively than both GP1 and GP2.

Overall mean antibody titer differed significantly among the three groups with p-value 0.000. GP3 showed the highest antibody titer with an overall mean of 0.668 and standard error of 0.018.

Humoral response against RHDV was assessed by comparing serum HI antibody titers after immunization. Chloroform-treated RHD virus was diluted to 3 log2 and used as an antigen for HI

Table 3
Comparative results of anti-*Pasteurella multocida* antibodies in sera of vaccinated rabbits measured by ELISA.

Time	GP1	GP2	GP3	GP6a	p- value
T1	0.162 ± 0.019[a]	0.159 ± 0.0236 [a]	2.333 ± 0.0282[b]	_____	0.000*
T2	0.151 ± 0.020 [a]	0.182 ± 0.032 [a]	1.321 ± 0.000[b]	_____	0.000*
T3	0.190 ± 0.029 [a]	0.237 ± 0.038 [a]	0.982 ± 0.036 [a]	0.070 ± 0.002[c]	0.526
T4	0.173 ± 0.025 [a]	0.176 ± 0.021 [a]	0.725 ± 0.033[b]	0.071 ± 0.002[c]	0.395
Overall Mean ± SE	0.169 ± 0.012 [a]	0.188 ± 0.0150 [a]	0.668 ± 0.018[b]	0.122 ± 0.012 [a]	0.000*

Table 4
Comparative results of anti RHDV antibodies in sera of rabbits vaccinated with RHDV vaccines and bivalent vaccine measured by HI test.

Time	GP3	GP4	GP5	GP6b	p- value
T1	7 ± 0.000[a]	7 ± 0.000[a]	4.77 ± 0.333[b]	___	0.000*
T2	7 ± 1.000 [a]	6.33 ± 0.882 [a]	5.33 ± 0.333 [a]	___	0.387
T3	11.3 ± 0.667 [a]	12.0 ± 0.000 [a]	5.33 ± 0.333[b]	3.33 ± 0.333[c]	0.000*
T4	6.67 ± 0.667 [a]	6.00 ± 0.000 [a]	5.67 ± 0.333 [a]	4.0 ± 0.000[b]	0.006
Overall Mean ± SE	8.00 ± 0.651[a]	7.83 ± 0.757 [a]	4.91 ± 0.313[b]	3.67 ± 0.21[c]	0.001*

(Table 4). Two weeks after vaccination (T1), GP3 and GP4 induced almost typical immunological responses that were stronger than GP5 with a mean of 7log2HIU/50 μl serum. At the second collection time (T2), GP3 showed increased antibody titer with a mean of 7 log2 HIU/50 μl serum, higher than other groups but, statistically insignificant. At day 45 of experiment (T3), rabbits in GP3 and GP4 showed great increase in the antibody titer (post-challenge titer) higher than rabbits in GP5. By the end of experiment, all groups showed decrease in antibody titer, GP3 maintained to be the highest with a mean of 6.67log2HIU/50 μl serum but statistically insignificant.

Overall mean antibody titer differed significantly among different groups with p-value of 0.001, GP3 showed the highest overall mean of 8.00 and standard deviation of 0.651a.

3.4. Results of challenge test among the vaccinated animals

No local reaction was found in all vaccinated rabbits (intramuscular injection) compared to control group. There were significant differences between clinical signs of vaccinated and unvaccinated groups. Pneumonia, recumbency, loss of appetite, and deaths were observed in unvaccinated challenged groups (Table 5).

The protection rate was 70% among rabbits vaccinated with monovalent *Pasteurella* vaccine GP1, commercial *Pasteurella* vaccine GP2, monovalent RHDV vaccine GP5 and 60% among commercial RHDV vaccineGP4. While it was 90% among rabbits vaccinated with bivalent vaccine GP3 (Table 5).

Liver samples from dead and euthanized rabbits were negative for RHDV using RT-PCR and for *Pasteurella multocida* using PCR and culture methods, indicating that deaths in vaccinated groups may be attributed to causes other than RHD virus or *Pasteurella multo-*

Table 5
Deaths and protection percentages of different rabbit groups in the study, GP6b showed sever signs of bacterial infection and purulent discharge typical for Pasteurella infection, they were euthanized and considered as deaths.

Groups	Deaths	Protection percent
GP1	3/10	70%
GP2	3/10	70%
GP3	1/10	90%
GP4	4/10	60%
GP5	3/10	70%
GP6a	5/5*	0%
GP6b	5/5	0%

cida infection. Only liver samples from GP6b successfully amplified RHDV RNA by RT-PCR, other groups were negative.

4. Discussion

Pasteurella multocida is the most common bacterial pathogen isolated from rabbits. Its prevalence has been reported to be between 7% and nearly 100% (Kawamoto et al., 1990a, 1990b). It is the cause of a highly contagious disease of rabbits, snuffles, which primarily affects the upper respiratory tract with potential fatal consequences including otitis media; enzootic pneumonia, conjunctivitis; pyometra; orchitis; abscesses and septicemia (Wilson and Ho, 2013).

Rabbit hemorrhagic disease (RHD) is characterized by 100% morbidity and mortality rates that reach 90% in adult European rabbits. The etiological agent, RHDV, is a single-stranded Plus polarity RNA virus belonging to the genus Lagovirus, of the calicivirus family (Parra and Prieto, 1990). Virus is believed to be endemic in countries where wild European rabbits are present (Abrantes et al., 2012). Infection occurs in all rabbits exceeding 4 weeks of age, but clinical disease is only present in older animals (Prieto et al., 2000, Shien et al., 2000).

The present investigation aimed to prepare a bivalent vaccine against pasteurellosis and the virus of rabbit hemorrhagic disease using Montanide® ISA70 oil as an adjuvant. Successful vaccination depends on antigen(s) association with potent adjuvant. Good adjuvant can activate specific mediators of the immune system and strengthen the humoral and /or cellular responses against antigens. Adjuvants are a group of vaccine additives that are added mainly to inactivated antigens for the purpose of enhancing and prolonging immune response. Suitable adjuvant should have lower toxicity and high safety margin (Abd El-hamied and Hussein, 2015). Adjuvants may reduce required antigen/dose thus improving vaccine economy (Aucouturier et al., 2001, Dupuis et al., 2006). SEPPIC Montanide™ ISA-W/O adjuvants are ready to use mineral oil-based continuous oil phase emulsions (SEPPIC, 2019). These adjuvants are suitable for inactivated and recombinant antigens, safe, efficient and able to stimulate both humeral and cellular immunities. Different Montanide™ ISA adjuvants were used and proved successful with rabbit vaccines; ISA50 and ISA70 with monovalent Pasteurella multocida vaccine (Ismail et al., 2018, Youssef and Tawfik, 2011), ISA70, ISA206, and ISA201 with monovalent RHDV vaccines (Bárcena et al., 2015, Elkady et al., 2016, Miao et al., 2019). All tested adjuvants were able to induce fast,

strong and prolonged protection against candidate agents. The success of Montanide™ ISA70 adjuvant has been extended for human vaccine candidates trials (Khabazzadeh Tehrani et al., 2016).

Local rabbit-origin *Pasteurella multocida* strain serotype (A) was verified by PCR using *KMT I* gene primers. In comparison with molecular size marker (100 bp), band size was about 460 bp specific for *Pasteurella multocida* (Townsend et al., 2001, OIE, 2015).

The RHDV strain was verified by PCR and produced single specific band in accordance with (Guittre et al., 1995) and titrated by hemagglutination test (HA) (OIE, 2015). The number of HA units from initial titration was $2^{13}/50$ µl.

Rabbit polyvalent vaccines combined with RHDV have been tested successfully in the People's Republic of China. The combinations included bivalent vaccines (RHDV/pasteurellosis, RHDV/clostridiosis and RHDV/ bordetellosis) (Tong, 1987–44). Bivalent *Pasteurella multocida*-RHDV vaccine had been tested with marked success in Bulgaria (Peshev and Christova, 2003) and in Egypt (Eid et al., 2014) but none of the studies combined both antigens with Montanide™ ISA70 adjuvant.

In this study, a laboratory trial for preparation of an ISA70-adjuvanted inactivated bivalent vaccine against *Pasteurella multocida* and RHDV was conducted. The prepared vaccine candidate contained 10^8 CFU/dose *Pasteurella multocida* and 2^8 HU/dose RHDV; 0.5 ml dose. Monovalent vaccines with ISA70 oil adjuvant were also prepared. Prepared vaccine candidates were compared to their commercial counterparts adjuvanted with mineral oil.

Prepared emulsions (vaccines) were found to be milky white in color with low viscosity based on floating drop test results, stable for the whole experiment duration (9 months) and safe. Little to moderate tissue reaction was observed in rabbits inoculated subcutaneous double-dose safety testing; previous reports about ISA70 in rabbits didn't report such reaction (Ismail et al., 2018, Bárcena et al., 2015). This reaction may be due to high antigenic mass of the preparations; specially inactivated virus load (256HAU/dose) while it was reported that doses as low as 4 and 8 HAU/dose were protective for rabbits (Peshev and Christova, 2003). Rabbit group which received bivalent vaccine (GP3) showed earlier and higher antibody response against *Pasteurella multocida* than GP1(monovalent inactivated Pasteurella *multocida)* and GP2 (commercial monovalent inactivated Pasteurella *multocida*)measured by ELISA (Table 3). These data were as reported earlier (Peshev and Christova, 2003). Anti-RHDV antibody response, evaluated by HI, were the highest in GP 3 unlike GP 4 and GP5 vaccinated with monovalent inactivated RHDV vaccines (Table 4). Two weeks Post challenge, GP3 and GP 4 showed immunological response higher than GP 5. All groups showed slight decrease in the antibody response at 60 day of vaccination (time4). All vaccinated groups showed higher level of antibodies than GP6a and GP6b (unvaccinated control groups) (Tables 3 and 4) agreeing with (Peshev and Christova, 2003). Protection percent based on live/dead ratios (Table 5) was different form protection percent after testing rabbit carcasses with PCR and RT-PCR. All vaccinated groups were fully protected (100%) as rabbit carcasses except for unvaccinated controls were negative to either *P. multocida* DNA or RHDV RNA according to their respective vaccination schedule (Table 2). bivalent and monovalent viral vaccine candidates completely inhibited virus shedding. Such potent vaccine is required in Egyptian conditions as experimental efficacy will always be lower under field conditions. These results agreed with (Ismail et al., 2018; Peshev and Christova, 2003).

5. Conclusion

The study reveals that the bivalent vaccine candidate not only provided lower stress to rabbits by decreasing the required manip-

ulation by 50% but also induced better protection and higher antibody response for both antigens. To our knowledge, the presented data is the first report combines Montanide™ ISA70 oil adjuvant in bivalent vaccine candidate against *Pasteurella multocida* and RHDV in rabbits.

Author contributions

All authors contributed to the reagents/materials/analysis tools, collected the material, analyzed the data and wrote and revised the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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