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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network



RESEARCH ARTICLE

BIOSCIENCE RESEARCH, 2019 16(4):3973-3986.

OPEN ACCESS

Preparation and evaluation of locally prepared inactivated combined vaccine of rabbit haemorrhagic disease virus, *Pasteurella multocida* and *Clostridium perfringens* type A

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Clostridium perfringens, *Pasteurella multocida* and rabbit hemorrhagic disease virus (RHDV) cause severe losses among rabbit populations. The efficacy of recently developed combined inactivated aluminium hydroxide gel and montanide oil vaccines against Enterotoxaemia, Pasteurellosis and RHDV were investigated. Doses exceeding 2¹⁰ Hemagglutination Unit (HAU) of viral antigen, 3.25 x 10¹⁰ Colony Forming Unit/ml (CFU/ml) of *P. multocida* antigens and 60 Minimum Lethal Dose (MLD) of *C. perfringens* alpha toxoid were sufficient to protect rabbits against Pasteurellosis, Enterotoxaemia and Rabbit hemorrhagic disease. Rabbits vaccinated with combined gel or oil vaccines showed high antibody titers against 3 organisms that continued till 6 months post vaccination (MPV). The *C. perfringens* antibody levels against combined gel and oil vaccines using toxin neutralization test (TNT) reached to 4 and 6.5IU/ml at 1st month post boosting and 2nd month post boosting, respectively. The antibody levels against *P. multocida* A and D antigens of combined gel and oil vaccines using indirect hemagglutination (IHA) reached to (1877, 1712) and (2090, 2059) at 6th week post vaccination and 8th week post vaccination, respectively. The antibody levels using hemagglutination inhibition (HI) against RHDV in rabbits at 6th week post vaccination and 10th week post vaccination reached to maximum levels of combined gel and oil vaccines (12) respectively. The target of preparation of combined inactivated aluminium hydroxide gel and montanide oil vaccines against Pasteurellosis, Enterotoxaemia and Rabbit hemorrhagic disease appears to be safe and enhancing the immune response of the vaccinated rabbits, in addition to combined speed and longevity of the immune response. Immune protection against *C. perfringens*, *P. multocida* and RHDV can be achieved with one manipulation to decrease the stress on animals and efforts.

Keywords: *P. multocida*, *C. perfringens* type A, RHDV, Efficacy monovalent vaccine, combined vaccine

INTRODUCTION

Clostridium perfringens type A has a great impact in rabbit farms due to great losses and high mortalities specially among weaned rabbits. It causes severe diarrhea, bloat and enterotoxaemia. Enterotoxaemia in rabbits is

considered one of the most economically important and financially crippling enteric diseases, causes the more commonly recognized fulminant infection which can result in outbreaks with mortality rates up to 50% (McDevitt et al., 2006). Toxoid vaccines are widely available

commercially and have been used extensively over the past decades for use in domesticated animals.

Pasteurella multocida is considered one of the most important bacterial pathogens of domestic rabbits. Pasteurellosis caused by *P. multocida* was regarded as one of the most important and significant bacterial diseases of rabbits and causes considerable economic losses in large production units all over the world (Takashima et al., 2001). In attempts to protect rabbits from infection with *P. multocida*, a variety of vaccines have been examined, including those composed of inactivated whole bacteria (Al-Lebban et al., 1989).

Rabbit hemorrhagic diseases virus (RHDV) is regarded as one of the most common and important contagious and fatal diseases affecting rabbits. There are many kinds of inactivated vaccines for Rabbit hemorrhagic disease virus have been developed including those that formalin handled and oil-adjuvanted. Daoud et al., (1998 a,b) prepared an inactivated RHDV vaccine from the local isolate of RHDV (Egypt 96) by 0.4% formalin at 37°C/48 hours adjuvant with aluminium hydroxide gel. Vaccines and other alternative products are central to the future success of animal agriculture because they can help minimizing the need for antibiotics by preventing and controlling infectious diseases in animal populations (El-Maghraby et al., 2019). Polyvalent vaccine strategies increase reactivity for many pathogens.

So, the main objective of this study is to prepare and evaluate combined vaccine containing *Clostridium perfringens* type A, *P. multocida* and Rabbit hemorrhagic disease virus either adjuvanted with aluminium hydroxide gel or montanide oil, where combined vaccines have the advantage of protecting against more than one disease at the same time, besides enhancing the immune response of the vaccinated rabbits, reducing vaccination expenses, decreasing the stress of vaccination for different vaccines.

MATERIALS AND METHODS

Bacteria and virus strains:

1- *Clostridium perfringens* type A strain: A locally isolated vaccinal *Clostridium perfringens* type A strain was obtained from Anaerobic Vaccines Research Department, (VSVRI), Abbasia, Cairo, Egypt.

2- A locally isolated vaccinal *P. multocida* strains (serotypes 5A, 9A, 8A and 2D) were obtained

kindly from Aerobic Bacterial Vaccines Research Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt, were used for preparation of *P. multocida* vaccines, serological tests and challenge of the tested rabbits.

3- Rabbit hemorrhagic disease virus (RHDV): A local Egyptian strain of RHDV designated as Giza/2006 (Salman, 2008) with a titer of $10^{4.65}$ LD₅₀/ml and of hemagglutination (HA) titer equal to 2¹⁴ HAU was used for vaccine preparation, challenge of vaccinated rabbits and in hemagglutination inhibition (HI) test and was obtained from Newcastle Vaccines Research Department, VSVRI, Abbasia, Cairo, Egypt.

Laboratory animals

Experimental rabbits:

A total number of three hundred, 4 weeks old, industrial hybrid Bosket rabbits their weights ranging from 2-2.5 kg were housed in disinfected metal cages in a well ventilated and disinfected room receiving commercial pellet ration and clean water *ad-libitum* in (VSVRI), Abbasia, Cairo. These rabbits were ascertained first to be free from *C. perfringens* type A, *P. multocida* and RHDV. They were used for preparation and evaluation of the prepared vaccines. Another 25 boskat rabbits from the same source were used in safety test of the prepared vaccines. Eight native rabbits, their body weight ranged between 1-1.5 Kg were used for the passage of local isolates of *P. multocida* types A and D.

Mice:

A total number of 200 Swiss white mice of about 18-20 gm body weights were obtained from the Laboratory Animals Department, VSVRI, Abbasia, Cairo, were used for determination of safety tests and evaluation of potency of *C. perfringens* type A and *P. multocida* prepared vaccines.

Adjuvants:

1-Rehydrigel LV (CHEM TRADE) Aluminium hydroxide gel:

Low viscosity gel. Stock No. 203120070602. It was supplied by Chem Trade- Berkeley Heights, New Jersey, and used according to manufacture instructions.

2-Montanide

ISA-70-VG.Montanide ISA 70 VG is a mineral oil based adjuvant which has been developed for

the manufacture of water in-oil (W/O) emulsion provided by France Seppic Company, France. It was used according to manufacture instructions.

Standard *C. perfringens* type A antitoxin:

C. perfringens type A antitoxin was obtained from National Institute for a Biological Standard Control, United Kingdom. It contains 270 IU/ml alpha antitoxic.

Toxin: Dried alpha toxin of *C. perfringens* type A was prepared according to Dixon and Webb 1979.

Positive and negative control serum of RHDV:

It was supplied in Rabbit hemorrhagic disease viral antibody (RHDV-Ab) used in HI test. It was supplied by Newcastle Vaccines Research Departement, (VSVRI), Abbasia, Egypt.

Erythrocyte 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.

Vaccines preparation:

Monovalent Rabbit enterotoxemia bloat vaccine:

The vaccine was prepared from the highly toxigenic isolates of *C. perfringens* type A according to methods of Gadalla et al., (1974) and Ahmed (1975) and inactivated by formalin in 0.5% concentration (Gadalla et al., 1969). 1ml of vaccine (equal to one vaccinal dose was adjusted to contain 60 MLD of *C. Perfringens* alpha toxoid. This inactivated culture and toxoid was divided into four parts. The first one used for preparation of monovalent *C. perfringens* type A montanide adjuvanted vaccine using montanide oil ISA-70-VG in a ratio of 70 adjuvant: 30 antigen (Barnett et al., 1996). The second part used for preparation of monovalent *C. perfringens* type A aluminium hydroxide gel adjuvanted vaccine using aluminium hydroxide gel added as concentration of 20% according to El-Sehamy et al., (2004). Finally, the thiomersal was added at a final concentration of 0.01%. The third and forth parts were used in preparation of combined vaccines emulsions.

Monovalent *P. multocida* Vaccine preparation according to Mukkur et al., 1982 and Borkowska et al., 1995): Each serotype of *P. multocida* (5A, 9A, 8A and 2D) was isolated from heart blood of

inoculated rabbits then propagated separately in tryptose phosphate broth at 37°C aerobically for 24 hrs to obtain a dense culture containing approximately 3.25×10^{10} CFU/ml of each strain. The culture was inactivated by addition of 0.5% formalin and incubated at 37°C for 24 hours. The inactivated culture was then cultured for the detection of viable pasteurilla by streaked onto nutrient agar medium, then incubated at 37° C for 24 hrs. Equal amounts of culture of each strain were mixed together in an emulsion. This inactivated culture was divided into four parts. The first one used for preparation of monovalent *P. multocida* montanide adjuvanted vaccine using montanide oil ISA-70-VG in a ratio of 70 adjuvant: 30 antigen according to manufacture instructions. The second part used for preparation of monovalent *P. multocida* aluminium hydroxide gel adjuvanted vaccine using aluminium hydroxide gel added as concentration of 20%. Finally, the thiomersal was added at a final concentration of 0.01%. The third and forth parts were used in preparation of combined vaccines emulsions.

Preperation of Monovalent inactivated RHDV vaccines according to OIE (2014):

The viral inactivated suspension was assayed by HA test and it was found that RHDV titer was 2^{10} HAU after inactivation as it was recorded by Kim et al., (1989). Also OIE (2014) recommended that HA titer of RHDV should be higher than 2^7 after inactivation for vaccine preparation and inactivated by formaline in 0.4% concentration at 37°C for 48 hours, during inactivation, the fluid was continously agitated. The equal amounts of culture were mixed and preserved in 0.01% of thiomersal and stored at 4°C until preperation of vaccine emulsion. This inactivated culture was divided into four parts as make in *P. multocida* and *C. perfringens* type A previously.

Preparation of inactivated combined *C. perfringens* type A, *P. multocida* and RHDV vaccine

Preparation of inactivated combined *C. perfringens* type A, *P. multocida* and RHDV vaccinewith aluminum hydroxide gel according to El-Sehamy et al., (2004). Equal parts (V/V) of the inactivated broth of *C. perfringens* type A, *P. multocida* (serotypes 5A, 9A, 8A and 2D), and RHDV were mixed using a magnetic stirrer. A forementioned suspension was adjusted its concentration to contain 60 MLD of *C. perfringens* alpha toxoid per dose of *C. perfringens* type A according to Gadalla et al., (1974) and $3.25 \times$

10^{10} CFU/ml of each strain of *P. multocida* according to Mukkur et al., (1982) and with a titer of $10^{4.65}$ LD₅₀/ml and 2^{14} HAU/ml for RHDV. Equal amounts of aforementioned inactivated culture was divided into two parts. The first one was adjuvanted with montanide oil ISA-70-VG (in a ratio of 70 adjuvant: 30 antigen), the second one was adjuvanted with aluminium hydroxide gel added as concentration of 20%. Finally, the thiomersal was added at a final concentration of 0.01%.

Quality Control of the Prepared Vaccines:

The prepared monovalent and combined oil emulsion and gel vaccines were tested for sterility, safety, complete time of inactivation and potency test according to the Standard International Protocols as described by the OIE, (2017).

Sterility test:

It was carried out according to British Veterinary Codex, (2007). The prepared vaccines candidate were tested for sterility (freedom from any bacterial, fungul, Mycoplasma contaminants) by culturing on thioglycolate broth, MacConkey and nutrient agar, incubated at 37°C for 72 hrs., and inoculation of 1 ml of the prepared vaccines on sabaroud agar and incubated at 25°C for 15 days. This procedure was carried out before and after emulsification of the prepared vaccines.

Safety Test:

The prepared vaccines candidate were tested for safety by s/c inoculation of 5 seronegative rabbits with double times the recommended vaccinal dose for each prepared vaccine. The rabbits were observed for 3 weeks post inoculation for any possible local or systemic adverse reaction of each vaccine.

Experimental design:

Two hundred, 4 weeks old industrial hybrid Bosket rabbits were divided into 10 groups (30 rabbits for each). All rabbits were injected s/c with 2 doses of 1ml of the prepared vaccines 3 weekesapart (Diab et al., 2003) except the control +ve and -ve groups were left unvaccinated.

Group 1: vaccinated with monovalent aluminium hydroxide gel *C. perfringens* type A vaccine.

Group 2: vaccinated with monovalent montanide oil *C. perfringens* type A vaccine

Group 3: vaccinated with monovalent aluminium hydroxide gel *P. multocida* vaccine

Group 4: vaccinated with monovalent montanide oil *P. multocida* vaccine.

Group 5: vaccinated with monovalent aluminium hydroxide gel RHDV vaccine.

Group 6: vaccinated with monovalent montanide oil RHDV vaccine.

Group 7: vaccinated with combined aluminium hydroxide gel vaccine.

Group 8: injected with combined montanide oil vaccine.

Group 9: kept as non-vaccinated challenged (control +ve) group.

Group 10: kept as non-vaccinated non challenged (control -ve) group

Blood samples were collected from the ear vein 3 weeks after 1st dose of vaccination for all vaccines and weekly after 2nd dose of vaccination for *P. multocida* and RHDV vaccines and monthly for *C. perfringens* vaccines. The collected blood samples were allowed to coagulate and centrifuged (2500 rpm for 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 to detect the specific antibodies.

Evaluation of the potency of the vaccines

Toxin Neutralization Test for *C. perfringens* type A:

Determination of test dose of alpha toxin of *C. perfringens* type A, then determination of the potency of unknown sera as described by Gadalla et al., (1971).

ELISA for *C. perfringens* type A:

It was carried out according to Mattar et al., (2002) and Bruce et al., (1984).

Indirect Hemagglutination Test (IHA) for *P. multocida*:

This test was conducted to determine the antibody titer against *P. multocida* as mentioned by Carter (1955) and Sawada et al., (1982).

Passive mouse protection test (Tabatabaei et al., 2007).

Challenge test for *P. multocida*:

After the 3rd week post boosting, randomly chosen 10 rabbits from each group either vaccinated (from 3rd to 8th) groups or unvaccinated (9th) group were transported to experimental isolators where they were challenged by s/c inoculation with 0.1 ml of the virulent *P. multocida* cell suspension containing 100 LD₅₀ of virulent *P.*

multocida serotypes A and D for 15 days post-boostering. Observation period was 15 days post-challenge and mortality was recorded according to OIE (2012).

Hemagglutination (HA) test for RHDV:

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

Hemagglutination inhibition test (HI) for RHDV:

It was carried out according to Pu *et al.*, (1985) and Peshevet *et al.*, (1989), using 8 HAU of RHDV and human RBCs type "O" to estimate specific RHDV antibodies in rabbit sera. The antibody titer was the end-point dilution showing inhibition of HA.

Challenge test for RHDV according to OIE, (2014):

After the 3rd week post boosting randomly 10 rabbits chosen from each group either vaccinated (from 5th to 8th) or unvaccinated (9th) were transported to experimental isolators where they were challenged by I/M inoculation of 1ml of virulent suspension RHDV ($10^{4.65}$ LD₅₀/ml- 2¹⁴ HAU) containing at least 100 LD₅₀ or presenting a HA titer higher than 2⁸. The challenged rabbits were kept under daily observation for 2 weeks post challenge.

Ethics Statement:

Care of laboratory and experimental animals were conducted in accordance with animal ethics guidelines and approved protocols of reference laboratory for veterinary quality control on poultry production (NLQO). It was reviewed and supervised by the Ethical Committee of Veterinary Serum and Vaccine Research Institute (VSVRI).

RESULTS AND DISCUSSION

Regarding to quality control of the prepared vaccine, the results of safety test revealed that all the inoculated rabbits with different vaccine formulas were survived all over the observation period and neither signs of local reactions as abscess formation, irritation nor systemic reaction, that indicated that all prepared vaccine formulas were safe. The obtained results of sterility tests revealed that all the prepared vaccines candidates (either monovalent or combined) were sterile and free from any aerobic, anaerobic, fungus and

mycoplasmal contaminants.

Enterotoxaemia in rabbits is considered one of the most economically important and financially crippling enteric diseases, causes the more commonly recognized fulminant infection which can result in outbreaks with mortality rates of up to 50% (McDevitt *et al.*, 2006). The protective effect of the prepared *C. perfringens* type A vaccine in rabbits was detected in Tables (1, 2) revealed that the mean *C. perfringens* type A alpha antitoxin titers as measured by SNT in rabbits vaccinated with aluminium hydroxide gel adjuvanted monovalent *C. perfringens* type A and combined vaccines were (2, 3 IU/ml, respectively) at 2 weeks post 1st vaccination, and were (3, 3.5 IU/ml, respectively) at 2 weeks post boosting, and reached the maximum titers (3.5, 4 IU/ml respectively) at 1st month post boosting. The titers decreased slightly from the 2nd month till 6th month post boosting. These results agree with El-Sehamy *et al.*, (2004). However, the antibody titer of all groups were more than the minimum protective level of *C. perfringens* type A alpha antitoxin all over the period of the experiment for 6 months, where the minimum protective level of *C. perfringens* type A alpha antitoxin is (0.1 IU/ml) as stated by (Tytell *et al.*, 1947 and Diabet *et al.*, 2003). While the Mean *C. perfringens* type A alpha antitoxin titers in the sera of rabbits vaccinated with montanide oil adjuvanted monovalent *C. perfringens* type A and combined vaccines were (3.5, 4.5 IU/ml respectively) at 2 weeks post 1st vaccination and were (4.5, 5.5 IU/ml respectively) at 2 weeks post boosting, and reached the maximum titers (5.5, 6.5 IU/ml respectively) at 2nd month post boosting. The titers were still stable at the 3rd month and declined gradually till the 6th month post boosting.

On the other hand, Tables (3,4) showed the protective effect of the prepared *C. perfringens* type A vaccine in rabbits revealed that the mean *C. perfringens* type A alpha antitoxin titres as measured by ELISA in rabbits vaccinated with aluminium hydroxide gel adjuvanted monovalent *C. perfringenstype* A and combined vaccines were (1.087, 1.18 IU/ml respectively) at 2 weeks post 1st vaccination, and were (1.183, 1.21 IU/ml respectively) at 2 weeks post boosting, and reached the maximum titres (1.307, 1.33 IU/ml, respectively) at the 1st month post boosting. The titers decreased slightly within the minimum protective level (0.1 IU/ml) from the 2nd month till the 6th month post boosting.

Table 1: Mean *C. perfringenstype* A alpha antitoxin titers in the sera of rabbits vaccinated with aluminium hydroxide gel adjuvanted monovalent and combined vaccines measured by SNT(IU/ml)

Period post vaccination	Mean <i>C. perfringenstype</i> A alpha antitoxin titer of	
	Aluminium hydroxide gel adjuvanted monovalent vaccine	Aluminium hydroxide gel adjuvanted combined vaccine
Pre-vaccination	0	0
Post 1st Vaccination		
2weeks post 1 st dose	2	3
Post Boostering		
2 WPB	3	3.5
1 st MPB	3.5	4
2 nd MPB	2.5	3.5
3 rd MPB	1.5	3
4 th MPB	1.5	2.5
5 th MPB	1.2	2
6 th MPB	0.9	1.5

Table 2: Mean *C. perfringenstype* A alpha antitoxin titers in the sera of rabbits vaccinated with montanide oil adjuvanted monovalent and combined vaccines measured by SNT(IU/ml)

Period post vaccination	Mean <i>C. perfringenstype</i> A alpha antitoxin titre of	
	Montanide oil adjuvanted monovalent vaccine	Montanide oil adjuvanted combined vaccine
Pre-vaccination	0	0
Post 1st Vaccination		
2 weeks post 1 st dose	3.5	4.5
Post Boostering		
2 WPB	4.5	5.5
1 st MPB	5	6
2 nd MPB	5.5	6.5
3 rd MPB	5	6.5
4 th MPB	4	4.5
5 th MPB	2.5	3
6 th MPB	1.5	2

Table 3: Mean *C. perfringenstype*A alpha antitoxin titers in the sera of rabbits vaccinated with aluminium hydroxide gel monovalent and combined vaccines by ELISA

Period post vaccination	Mean <i>C. perfringenstype</i> A alpha antitoxin titre of	
	Aluminium hydroxide gel adjuvanted monovalent vaccine	Aluminium hydroxide gel adjuvanted combined vaccine
Pre-vaccination	0	0
Post 1st Vaccination		
2 weeks post 1 st dose	1.087	1.18
Post Boostering		
2 WPB	1.183	1.21
1 st MPB	1.307	1.33
2 nd MPB	0.947	0.99
3 rd MPB	0.892	0.90
4 th MPB	0.884	0.880
5 th MPB	0.694	0.79
6 th MPB	0.682	0.687

While the Mean *C. perfringens* type A alpha antitoxin titers in the sera of rabbits vaccinated with montanide oil adjuvanted monovalent *C. perfringens* type A and combined vaccines were (1.419, 1.45 IU/ml, respectively) at 2 weeks post 1st vaccination and were (1.424, 1.489 IU/ml, respectively) at 2 weeks post boosting, and reached the maximum titers (1.611, 1.627 IU/ml, respectively) at 2nd month post boosting. The titers decreased from the 3rd month gradually till the 6th month post boosting.

P. multocida is an important bacterial pathogen of domestic rabbits and leads to a great economic losses. Control of that disease is still as a subjected of interest and usually attracts attention of many researchers. Vaccination is still considered as one of the major tools for controlling the disease. Humoral immune response of *P. multocida* was determined by IHA test in Tables (5,6) which detected the mean IHA antibody titers of *P. multocida* serotypes A and D in the sera of rabbits vaccinated with aluminium hydroxide gel adjuvanted monovalent *P. multocida* and combined vaccines were (395, 368) and (469, 470) respectively at the 2 weeks post 1st vaccination, and increased till reached the maximum level (1621,1578) and (1877,1712) respectively at the 6th week post boosting and decreased gradually from the 8th week till the end of the experiment. While the mean IHA antibody titre of *P. multocida* in the sera of rabbits vaccinated with montanide oil adjuvanted monovalent *P. multocida* and combined vaccines were (448, 428) and (597, 512) respectively at the 2 weeks post 1st vaccination and increased gradually till reached the maximum level (2048, 2048) and (2090, 2059) at 8th week post boosting and decreased slightly from the 10th week till the end of the experiment. These results agree with Abd El-Aziz et al., (2015) who concluded that the inactivated *P. multocida* vaccine adjuvanted with montanide ISA-70-VG induced early and high immune response with long duration measured by IHA test. Also, Ahmed et al., (2010) concluded that the inactivated *P. multocida* vaccine adjuvanted with montanide ISA70 induced high and protective antibody titers measured by IHA. Youssef and Tawfik (2011) reported that the inactivated rabbit pasteurellosis vaccine adjuvanted with Montanide ISA-50 induced protective antibody titer against *P. multocida* and gave high and long duration of antibody level measured by IHA test.

Harper et al., (2016) reported that *P. multocida* LPS is a primary stimulator of the host

immune response and a critical determinant of bacterin protective efficacy. Also *P. multocida* is a Gram-negative pathogen and the causative agent of fowl cholera and the major outer membrane component LPS is both an important virulence factor and a major immunogen Harper et al., (2013).

These data were explained by Harper et al., (2012) who reported that the capsule and LPS of *P. multocida* constitute the major components of the bacterial cell surface. They play key roles in a range of interactions between the bacteria and the hosts they colonize or infect. Both polysaccharides are involved in the avoidance of host innate immune mechanisms, such as resistance to phagocytosis, complement-mediated killing, and the bactericidal activity of antimicrobial peptides; they are therefore essential for virulence. In addition, LPS is a major antigen in the stimulation of adaptive immune responses to infection.

The potency of the vaccines was evaluated by passive mouse protection test as tabulated in tables (7 and 8) against the challenge with the virulent strains of *P. multocida* types A and D in rabbits vaccinated with monovalent and combined *P. multocida* vaccines. the protection percentage (P%) against the challenge with virulent strain of *P. multocida* type "A" post 1st vaccination for rabbit groups vaccinated with monovalent and combined *P. multocida* vaccines were 80% and 100%, respectively in comparison with 0% for control group. While, post boosting and 8 weeks post challenge the P% were 100% for rabbit groups vaccinated with monovalent and combined *P. multocida* vaccines in comparison with 0% for control group. While, the protection% against the challenge with virulent strain of *P. multocida* type "D" were 100% post 1st vaccination for rabbit groups vaccinated with monovalent and combined *P. multocida* vaccines in comparison with 0% for control group. Also, post boosting and 8 weeks post challenge the P% were 100% for all vaccinated groups in comparison with 0% for control group. These data were in the same manner with those of Fatma Fathy (2018) and El-Bayomy and Daoud (2004) who found that there was an elevation in protective values of *P. multocida* adjuvanted vaccines against challenge with virulent strains of *P. multocida* types A and D.

The results of challenge assay against *P. multocida* tabulated in Table (9) proved that combined vaccine adjuvanted with aluminium hydroxide gel gave protection 90% against serotypes A and D.

Table 4: Mean *C. perfringens* type A alpha antitoxin titers in the sera of rabbits vaccinated with montanide oil adjuvanted monovalent and combined vaccines measured by ELISA

Period post vaccination	Mean <i>C. perfringens</i> type A alpha antitoxin titre of	
	Montanide oil adjuvanted monovalent vaccine	Montanide oil adjuvanted combined vaccine
Pre-vaccination	0	0
Post 1st Vaccination		
2 weeks post 1 st dose	1.419	1.45
Post Boostering		
2 WPB	1.424	1.489
1 st MPB	1.511	1.526
2 nd MPB	1.611	1.627
3 rd MPB	1.43	1.445
4 th MPB	1.211	1.226
5 th MPB	0.989	1.098
6 th MPB	0.887	0.977

Table 5: Comparative results of Anti- *P. multocida* antibodies in sera of rabbits vaccinated with monovalent and combined inactivated adjuvanted with Aluminum hydroxide gel by IHA test

Weeks	Type of vaccines / type of Antigen					
	Combined inactivated adjuvanted with Aluminum hydroxide gel		Monovalent inactivated vaccine adjuvanted with Aluminum hydroxide gel		Control group	
	A	D	A	D	A	D
Pre-vaccination	8	8	8	4	2	2
Post 1st Vaccination						
1 st WPV	260	275	235	205	4	2
2 nd WPV	335	298	318	277	8	6
3 rd WPV	469	470	395	368	8	7
Post Boostering						
4 th WPB	853	789	731	693	8	8
5 th WPB	1453	1389	1376	1368	9	9
6 th WPB	1877	1712	1621	1578	10	10
8 th WPB	1621	1578	1493	1472	10	10
10 th WPB	1387	1376	1195	1067	9	9
12 th WPB	939	811	789	715	9	8
14 th WPB	683	597	554	555	9	8
16 th WPB	469	427	448	405	9	9
20 th WPB	427	389	403	341	7	8
24 th WPB	277	235	224	203	7	6

Table 6:Comparative results of Anti- *P. multocida* antibodies in sera of rabbits vaccinated with monovalent and combined inactivated adjuvanted with montanide oil ISA70 by IHA test

Weeks	Type of vaccines / type of Antigen					
	Combined inactivated of vaccine adjuvanted with montanide oil ISA 70		Monovalent inactivated vaccine adjuvanted with montanide oil ISA 70		Control group	
	A	D	A	D	A	D
Pre- vaccination	8	8	8	8	2	2
Post 1st vaccination						
1 st WPV	395	368	261	267	8	7
2 nd WPV	405	384	373	366	8	7
3 rd WPV	597	512	448	428	8	7
Post Boostering						
4 th WPB	939	859	789	747	8	8
5 th WPB	1536	1472	1453	1370	9	9
6 th WPB	1950	1877	1749	1706	10	10
8 th WPB	2090	2059	2048	2048	9	8
10 th WPB	1878	1792	1707	1712	9	8
12 th WPB	1710	1706	1622	1578	9	9
14 th WPB	940	896	812	790	9	8
16 th WPB	533	437	510	410	9	9
20 th WPB	512	427	405	395	7	8
24 th WPB	403	363	341	297	7	8

Table 7: Passive mouse protection test against the challenge with *P. multocida* types " A" in rabbits vaccinated with monovalent and combined *P. multocida* vaccines

Time intervals	Total No. of mice	Types of vaccines									
		G1		G2		G3		G4		G5	
		S/T	P%	S/T	P%	S/T	P%	S/T	P%	S/T	P%
Pre-vacc	5	0/5	0%	0/5	0%	0/5	0%	0/5	0%	0/5	0%
Post 1 st vaccination											
2 weeks	5	4/5	80%	5/5	100%	4/5	80%	5/5	100%	0/5	0%
Post boostering											
2 weeks	5	5/5	100%	5/5	100%	5/5	100%	5/5	100%	0/5	100%
Challenge											
2 weeks	5	5/5	100%	5/5	100%	5/5	100%	5/5	100%	0/5	100%
4 weeks	5	5/5	100%	5/5	100%	5/5	100%	5/5	100%	0/5	100%
6 weeks	5	5/5	100%	5/5	100%	5/5	100%	5/5	100%	0/5	100%
8 weeks	5	5/5	100%	5/5	100%	5/5	100%	5/5	100%	0/5	100%

Table 8: Passive mouse protection test against the challenge with *P. multocida* types "D" in rabbits vaccinated with monovalent and combined *P. multocida* vaccines

Time intervals	Total No. of mice	Types of vaccines									
		G1		G2		G3		G4		G5	
		S/T	P%	S/T	P%	S/T	P%	S/T	P%	S/T	P%
Pre-vacc	5	0/5	0%	0/5	0%	0/5	0%	0/5	0%	0/5	0%
Post 1 st vaccination											
2 weeks	5	5/5	100%	5/5	100%	4/5	80%	5/5	100%	0/5	0%
Post boostering											
2 weeks	5	5/5	100%	5/5	100%	5/5	100%	5/5	100%	0/5	100%
Challenge											
2 weeks	5	5/5	100%	5/5	100%	5/5	100%	5/5	100%	0/5	100%
4 weeks	5	5/5	100%	5/5	100%	5/5	100%	5/5	100%	0/5	100%
6 weeks	5	5/5	100%	5/5	100%	5/5	100%	5/5	100%	0/5	100%
8 weeks	5	5/5	100%	5/5	100%	5/5	100%	5/5	100%	0/5	100%

G1: Monovalent aluminium hydroxide gel *P. multocida* vaccine G2: Combined *P. multocida*aluminium hydroxide gel vaccine
 G3: Monovalent montanide oil *P. multocida* vaccine G4: Combined montanideoil *P. multocida* vaccine G5: Control group S/T: Survive /Total No. P%: Protection %

Table 9: Protective efficacy in rabbits vaccinated with monovalent and combined vaccines adjuvanted with aluminium hydroxide gel and montanide oil against virulent *P. multocida* antigens

Groups	Challenge antigen	Total No. of rabbits	No. of survived rabbits/ Total No. of rabbits	Protection %
Monovalent vaccine adjuvanted with aluminium hydroxide	A	10	8/10	80%
	D	10	8/10	80%
Combined vaccine adjuvanted with aluminium hydroxide	A	10	9/10	90%
	D	10	9/10	90%
Monovalent vaccine adjuvanted with montanide oil	A	10	9/10	90%
	D	10	9/10	90%
Combined vaccine adjuvanted with montanide oil	A	10	10/10	100%
	D	10	10/10	100%
Control group	A	5	0/5	0%
	D	5	0/5	0%

Table 10: Mean hemagglutination inhibition antibody titer of RHDV in the sera of rabbits vaccinated with aluminium hydroxide gel adjuvanted monovalent and combined vaccines as measured by HI test

Weeks	Types of vaccines	
	Combined inactivated of vaccine adjuvanted with Aluminum hydroxide gel	Monovalent inactivated vaccine adjuvanted with Aluminum hydroxide gel
Pre-vaccination	2	0
Post 1 st vaccination		
1 st WPV	6.8	6
2 nd WPV	8.4	8
3 rd WPV	8.9	9
Post Boostering		
4 th WPB	9	9
5 th WPB	11	10
6 th WPB	12	10
8 th WPB	11	9
10 th WPB	10	9.5
12 th WPB	10.5	9
14 th WPB	10	8.5
16 th WPB	9	9
20 th WPB	8.5	9
24 th WPB	8	8

Table 11: Mean hemagglutination inhibition antibody titer of RHDV in the sera of rabbits vaccinated with Montanide oil adjuvanted monovalent and combined vaccines by HI test

Weeks	Types of vaccines	
	Combined inactivated of vaccine adjuvanted with montanide oil ISA 70	Monovalent inactivated vaccine adjuvanted with montanide oil ISA 70
Pre vaccination	1	1
Post 1 st vaccination		
1 st WPV	5.4	5
2 nd WPV	6.75	6.4
3 rd WPV	8	8.5
Post Boostering		
4 th WPB	8.5	9
5 th WPB	10.5	9.5
6 th WPB	11.5	10
8 th WPB	12	10.5
10 th WPB	12	11.5
12 th WPB	11	11
14 th WPB	11	10.5
16 th WPB	11	10
20 th WPB	10.5	9
24 th WPB	10	9

Table 12: Protective efficacy in rabbits vaccinated with RHDV monovalent and combined vaccines adjuvanted with aluminium hydroxide gel and montanide oil against virulent RHDV strain

Groups	Total No. of rabbits	survived rabbits/ Total No. of rabbits	Protection %
Monovalent RHDV vaccine adjuvanted with aluminium hydroxide	10	10/10	100%
Combined RHDV vaccine adjuvanted with aluminium hydroxide	10	10/10	100%
Monovalent RHDV vaccine adjuvanted with montanide oil	10	10/10	100%
Combined vaccine adjuvanted with withmontanide oil	10	10/10	100%
Control group	10	0/10	0%

On the other hand, the combined vaccines adjuvanted with montanide oil gave 100% protection. The monovalent vaccine adjuvanted with aluminium hydroxide gel gave protection 80% against serotypes A and D. On the other hand, the monovalent vaccines adjuvanted with montanide oil give 90% protection. There was an elevation in protective values of fowl cholera adjuvanted vaccines against challenge with virulent strains of *P. multocida* types A and D for the serum of the vaccinated chicken group. These results were in agreement with, Amal et al., (2005) , Ahmed et al., (2010) and Elham and Hoda (2011) Abd El-Aziz et al., (2015) and Akhtar et al., (2016).

Rabbit hemorrhagic disease (RHD) is a highly contagious, highly fatal, peracute and acute viral

disease of both wild and domestic rabbits caused by rabbit hemorrhagic disease virus (RHDV). Tables (10,11) showed the mean HI antibody titre of RHDV in the sera of rabbits vaccinated with aluminium hydroxide gel adjuvanted monovalent RHDV and combined vaccines were (8, 8.4) at the 2 weeks post 1st vaccination, while the RHDV antibody titre in the sera of rabbits vaccinated with montanide oil were (6.4, 6.75) at the 2 weeks post 1st vaccination, and increased gradually till reached maximum level at the 6th week post boosting (10, 12) for aluminium hydroxide gel monovalent and combined RHDV vaccines, while the RHDV antibody titers reach the maximum level (11.5, 12) at the 10th week post boosting respectively, and decreased gradually from the 11th week till the end of the experiment. So, the

mean HI titer reached maximum levels of combined Aluminium hydroxide gel at the 6th week post boosting and combined montanide oil vaccines at the 10th week post boosting, respectively and these agree with Von Claudia (2018).

One of the very important parameters of a good vaccine is the induction of a long-lasting immunity without the need of repeated booster vaccination (Castellino et al., 2009). All vaccinated rabbits were completely protected 6 months after vaccination. These results agree with Von Claudia (2018).

The results of challenge assay against RHDV tabulated in Table (12) proved that combined vaccine adjuvanted with Aluminium hydroxide gel gave protection 100%. These results agree with Daoud et al., (1998a; 1998b), El-Sehamy and wanis (2005) and Abd El-Motelib et al., (1998) . On the other hand, the combined vaccines adjuvanted with montanide oil gave 100% protection these results agree with Peshive and Christova (2003). The monovalent vaccine adjuvanted with aluminium hydroxide gel gave 100% protection. On the other hand, the montanide oil adjuvanted monovalent vaccines give 100% protection.

CONCLUSION

The combined vaccines against Pasteurellosis, Enterotoxaemia Bloat and RHDV appears to be safe and combines speed and longevity in the immune response. Immune protection against *C. perfringens*, *P. multocida* and RHDV can be achieved with one manipulation to decrease the stress on animals and efforts

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEDGEMENT

This work was supported by the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abbasia, Cairo and Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt

AUTHOR CONTRIBUTIONS

ASM designed the concept of the review article. ASM wrote the manuscript. All the authors designed and performed the experiments and reviewed the manuscript. All authors read and approved the final version..

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REFERENCES

- Abd El-Aziz HMG, El-Enbaawy MIH, Afifi M, Ibrahim SI, Omar L and Koudier MH. 2015. Efficacy of Montanide ISA-70-VG as adjuvant to fowl cholera vaccine. *J. Vet. Adv.*, 5(3): 848-852.
- Abd El-Motelib TY, Abd El-Gawad AM, Azzaz HA. 1998. Viral haemorrhagic disease of rabbits. Comparative study between the immune response of local and imported vaccine. *Assiut Vet. Med. J.*, 40.
- Ahmed AY. 1975. Factors concerned with the growth and lecithinase production of *Clostridium welchii* type A. M.V.Sc. Thesis (Microbiology) Fac. Vet. Med. Cairo University.
- Ahmed ES, Mahmoud MS. and Ghoniemy WA. 2010. Immunological studies on a modified adjuvanted fowl cholera vaccine. *Minufiya Vet. J.*, 7(2): 325-330.
- Akhtar M, Rahman MT, Ara MS, Rahman M, Nazir KHMNH, Ahmed S, Hossen ML and Rahman MB. 2016. Isolation of *Pasteurellamultocida* from chickens, preparation of formalin killed fowl cholera vaccine, and determination of efficacy in experimental chickens. *J. Adv. Vet. Anim. Res.*, 3(1): 45-50.
- Al-Lebban ZS, Kruckenber S. and Coles EH. 1989. Rabbit pasteurellosis: respiratory and renal pathology of control and immunized rabbits after challenge with *Pasteurellamultocida*. *Histol.Histopathol.*, 4:77-84.
- Amal IM, Daoud AM and El-Bourdyn F. 2005. Preparation of inactivated infectious bronchitis virus disease vaccine using new oil adjuvant. *Vet. Med. J. Giza*, 53(2): 329-339.
- Barnett PV, Pullen L, Williams L, Doel, TR. 1996. International bank for Foot and Mouth Disease vaccine assessment of Montanide ISA25 and ISA 206, two commercially

- available oil adjuvants vaccines. *Vaccine*, 14:1187- 1198.
- Borkowska–Opacka B, Jurga IR, Truszczynski M and Zymski W. 1995. Effect of various adjuvants on the efficacy of the vaccines against pasteurellosis in rabbits. *Bulletin of the Vet. Institute in Pulawy*, 39(2): 115-119.
- British Veterinary Codex 2007. The pharmaceutical Press, London.
- Bruce A, MCCLANE and ROBERT J. STROUSE Feb. 1984. Rapid Detection of *Clostridium perfringens* Type A Enterotoxin by Enzyme-Linked Immunosorbent Assay. *Journal of clinical microbiology*, 112-115.
- Capucci L, Fusi P, Lavazza A, Pacciarini ML and Rossi C. 1996. Detection and preliminary characterization of a new rabbit calicivirus related to rabbit hemorrhagic disease virus but non-pathogenic. *J. Virol.*, 70: 8614–8623.
- Carter GR. 1955. Studies on *Pasteurellamultocida*. I. A hemagglutination test for the identification of serological types. *Am. J. Vet. Res.*, 16:481-484.
- Castellino F, Galli G, Del Giudice G, Rappuoli R. 2009. Generating memory with vaccination. *Eur. J. Immunol.*, 39(8):2100-5.
- Daoud AM, Khodeir MH, Abbas AM, Ibrahim SI and Gergis SM. 1998b. Preliminary study for preparation of rabbit pasteurellosis and rabbit hemorrhagic disease virus combined vaccine. In: 4th Veterinary Medicine Zagazig Congress. Hurghada, August 26-28. Hurghada (Egypt): Zigazig University. p. 191-198.
- Daoud AM, Khodeir MH, Abbass AM. and Ibrahim SI. 1998a. Preparation of a specific inactivated vaccine against RHDV. 4th Sci. Cong., Fac. Vet. Med. Zag. Univ., P. 230-234.
- Diab RA, El-Sehemy MM, Nadia ME, Fatheia S. and Hussein AZ. 2003. Enterotoxaemia in rabbits and trial for preparing vaccine from the isolated strains. *Journal of Veterinary Medical Association*, 63(2): 59-64.
- Dixon M. and Webb EC. 1979. *Enzymes*. Academic Press, Inc., New York.
- El-Bayomy AA. and Daoud AM. 2004. Efficacy of double adjuvant system on the immune response of fowl cholera vaccinated chickens. *J. Egypt. Vet. Med. Assoc.*, 64(2): 215-329.
- Elham AY and Hoda ET. 2011. Improvement of rabbit pasteurellosis vaccine using montanide ISA-50. *Egypt J. Agric. Res.*, 89(2).
- El-Maghraby AS, Wafaa RA, Abd El-Moneam MM and Helal AM. 2019. Efficacy of locally prepared inactivated combined vaccine of *Salmonella enteritidis*, *Salmonella typhimurium*, Avian influenza (H9N2) and Newcastle disease viruses. *Bioscience Research*, 16(2):1668-1678.
- El-Sehemy MM, Diab RA, Hussien AZ, FathiaShafie, Roukia M. Osman. 2004. Immunological studies on rabbit enterotoxaemia vaccine. 6th Sci. Conf., Egypt. Vet. Poult. Assoc., 25-27.
- FatmaFathy Ibrahim Hassan. 2018. Preparation and Evaluation of a Combined Bivalent Vaccine Against Avian Pasteurella and Mycoplasma Infections in Chickens. Ph. D. Thesis in Veterinary Medical Sciences Microbiology. Faculty of Vet. Med., Cairo Univ.
- Gadalla MS, Farrag I, El-Shahat F, El-Bendary T. and Moustafa R. 1969. Studies on polyvalent vaccine against some clostridial diseases in sheep. *J. Vet. Sci., U.A.R.*, 6: 1-14.
- Gadalla MS, Farrag I, Lotfy OM, El-Danaf NA, Sharaf D, Hussein M. 1971. The immunogenicity of alum precipitated multicomponent clostridial vaccine. *J. Egypt. Vet. Med. Ass.*, 31 :135-150.
- Gadalla MS, Farrag, I., Sharf, D. 1974. Effect of growth requirement on the improvement of clostridial vaccine. *J. Egypt. Vet. Med. Ass.*, 34: 19-28.
- Harper M, Boyce JD. and Adler B. 2012. The key surface components of *Pasteurellamultocida*: Capsule and lipopolysaccharide. *Curr. Top. Microbiol. Immunol.*, 361: 39–51.
- Harper M, John M, Edmunds M, Wright A, Ford M, Turni C, Blackall PJ, Cox A, Adler B. and Boyce JD. 2016. Protective efficacy afforded by live *Pasteurellamultocida* vaccines in chickens is independent of lipopolysaccharide outer core structure. *Vaccine*, 34(14): 1696-1703.
- Harper M, Michael FSt, John M, Vinogradov E, Steen JA, Dorsten LV, Steen JA, Turni C, Blackall PJ, Adler B, Cox AD. and Boyce JD. 2013. *Pasteurellamultocida* Heddleston serovar 3 and 4 strains share a common lipopolysaccharide biosynthesis locus but display both inter- and intrastrain lipopolysaccharide heterogeneity. *J. Bacteriol.*, 195(21): 4854–4864.
- Matter MA, Cortinas TI and Guzman AM. 2002. Immunogenic protein variations of *C. chauvoei* cellular antigens associated with

- the culture growth phase'. FEMS Immunol. Med. Microbiol., 33: 9-14.
- McDevitt RM, Brooker JD, Acamovic T. and Sparks NHC. 2006. Necrotic enteritis; a continuing challenge for the poultry industry. Worlds Poul. Sci. J., 62 (2), 221–247.
- Mukkur TKS, Pylitis NA and Bones A. 1982. Possible immunological synergism among the protective antigens of *P. multocida* type A. J. Comp. Pathol., 92: 249-260.
- OIE. 2012. Avian mycoplasmosis (*Mycoplasma gallisepticum*, *M. synoviae*), Ch. 2.3.5. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 7th ed., France, 1: 455-469.
- OIE. 2014. Avian Influenza OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Ch. 2.3.4. OIE, Paris, France.
- OIE. 2017. Terrestrial Animal Health Code. Chapter: 6.5. Prevention, Detection and Control of Salmonella in poultry.
- Peschive R, Petkove A, Belemzove P, Bostandjieva R and lyutskanove M. 1989. An acute contagious disease of domestic rabbits accompanied by hemorrhagic syndrome. Veterinariasbrika, 3: 34-36 (in BULGRIAN).
- Peshev R. and Christova L. 2003. The efficacy of a bivalent vaccine against pasteurellosis and rabbit hemorrhagic disease virus. Vet. Res. Comm., 27(6): 433-444.
- Pu BQ, QIAN HH. And CUI SJ. 1985. Micro HA test for the detection of antibody titres to so-called "haemorrhagic pneumonia" in rabbits. Chinese Journal of Veterinary Medicine, 11: 16-17.
- Salman OGA, Khelfa DEDG, Shakal MA, Salwa AA. El-Assily, Seham AE. El-Zeedy, Dalia AM. Abd El-Moaty and Yousif AA. 2008. The use of VP60 RT-PCR to overcome limitations with the current diagnostic approaches to rabbit hemorrhagic disease virus. Egyptian J. Virol., 5(2): 217-237.
- Sawada T, Rimler RB and Rhoades KR. 1982. Indirect hemagglutination test that uses glutaraldehyde-fixed sheep erythrocytes sensitized with extract antigens for detection of pasteurella antibody. J. Clin. Microbiol., 15(5): 752-756.
- Tabatabaei M, Moazeni-Jula GR, Jabbri AR. and Esmailzadeh M. 2007. Vaccine efficacy in cattle against haemorrhagic septicemia with live attenuated *aroA* mutant of *Pasteurellamultocida*B:2 strain. Journal of Cell and Animal Biology, 1 (4): 062-065.
- Takashima H, Sakai H, Yanai T. and Masegi T. 2001. Detection of antibodies against *Pasteurellamultocida* using immunohistochemical staining in an outbreak of rabbit pasteurellosis. J. Vet. Med. Sci., 63:171–174.
- Tytell AA, Logan MA, Alice G. and Tepper J. 1947. J. of Immunology, 55: 233. Von Claudia Müller aus Freiberg 2018. A new RHDV-2 vaccine based on recombinant baculovirus - Generation and characterization of induced immunity in rabbits. Ausdem Veterinärwissenschaftlichen Department der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität München Lehrstuhl für Virologie.
- Youssef EA. and Tawfik HE. 2011. Improvement of rabbit pasteurellosis vaccine using Montanide ISA-50. Egypt. J. Agric. Res., 89(2): 697-708.