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Comparative study on one shot Lipid A and Montanide™ ISA 70 adjuvanted Pasteurella Vaccines for Rabbits

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This study declared a trial for producing one shot Lipid A (AV) and Montanide™ ISA 70 (MV) adjuvanted *P. multocida* vaccines for rabbits and comparison between them. The vaccines were prepared using *P. multocida* serotypes A: 1, A: 3, A: 12, and D: 2 as antigens. Lipid A was extracted from *E. coli* by acid hydrolysis and evaluated through validated RP-HPLC. Twenty-one New Zealand rabbits were divided equally into 3 groups. First group and second group were vaccinated with a single dose of AV and MV respectively, and the third group kept as a control. At 21st day post vaccination, the three groups were challenged. Mortality percentages were as follow, 100% mortality for control group, and 28.6% mortality for the vaccinated groups. AV group lysozyme activity test results showed higher significant value on days 1, 3, and 5 than MV group, but MV was the highest on the 7th day. ELISA results of AV group also showed higher significant value than MV group on 1st and 2nd weeks post vaccination, but MV was the highest on the 3rd week. Briefly despite of the rapid value increase of the AV, the MV owned prevails.

Keywords: Lipid A, Montanide ISA 70, vaccines, *P. multocida*, Rabbit, HPLC.

INTRODUCTION

Pasteurellosis is one of the most important health problems in rabbits, which is considered as a common bacterial disease caused by *Pasteurella multocida* (*P. multocida*) and has been reported as a constant serious and highly contagious disease of domestic rabbits (Gracy, 1986). Several bacteria belonging to the family Pasteurellaceae are potential pathogens in rabbits. In particular, *P. multocida* is considered to be important, and outbreaks caused by this species result in considerable economic losses in rabbitries (Anina et al., 2009). *P. multocida* is non-motile, facultative anaerobic, Gram-negative

bacteria associated with a spectrum of animal diseases. Diseases caused by *P. multocida* include fowl cholera in birds; atrophic rhinitis in pigs; hemorrhagic septicemia in ungulates; enzootic pneumonia in cattle, sheep, and goats; and snuffles in rabbits (Rajeev et al., 2011). More than 50% of adult rabbits either die or are culled due to *P. multocida* infection. In postmortem of dead rabbits, the fluid and mucous accumulation in the trachea and lungs revealed snuffling sounds on auscultation and a morbidity rate of 35-40% with mortality of 23% were recorded (Suelam and Abdel Samie, 2011). There is a strong and continuing interest in the development of rabbit

industry in Egypt. Rabbit industry as one of the small livestock has a unique commercial that can play a role in solving the shortage in the meat after poultry industry. Obviously, the disease problem of rabbits differs greatly according to the age of the animal and the system of management (Mohammed et al., 2013). Development of vaccines to prevent human and poultry diseases has been a major accomplishment in the field of immunology. Some diseases have been eliminated through use of vaccines, and the prevalence of a large number of infectious diseases has been diminished (Charles et al., 1994). The prevention is the most likely means of controlling pasteurellosis disease, thus vaccines would be of great value in the protection of rabbits against pasteurellosis (Alexander et al., 1952). Montanide ISA Adjuvants [Seppic, Paris, France] are a group of oil/surfactant-based adjuvants, in which different surfactants are combined with mineral oil like Montanide™ ISA 70. Montanide adjuvant is not based on one mechanism but rely on different modes of action. As emulsion can have depot effect and induce a slow release of the antigen, they can recruit immunocompetent cells by micro diffusion of the droplets via the lymphatic system, or facilitate the antigen uptake by antigen presenting cells, or at least playing themselves the role of antigen presenting cells (APC). Other mechanism such as lymphocyte trapping and modification of the cell membranes, emulsion is the result of oil phase, established with a surfactant and able to modify the cell membranes or to draw together the antigen and APC (Aucotuier and Ganne 2002). Lipopolysaccharides (LPS) from Gram-negative bacteria exhibited adjuvant activity and detoxified LPS or related compounds such as lipid A have since been used as adjuvants in human studies (Johnson et al., 1956). The standard lipid A molecule can be chemically defined as a diglucosamine backbone that is hexa-acylated and bis-phosphorylated; consistent with the major lipid A species observed in the model organism *E. coli* (Raetzel et al., 2002) and (Trent et al., 2006). Lipid A of *E. coli* can be recognized by mammalian immune cells, leading to release of pro inflammatory cytokines (Han et al., 2013). As it is now known, lipid A harbors a certain structural heterogeneity that is either due to incomplete biosynthesis or generated by chemical degradation during the preparation of free lipid A by acid hydrolysis (Johnson et al., 1990). A variety of techniques have been used to evaluate Lipid A extract. Reversed phase high performance liquid chromatography (RP-HPLC)

used for evaluation of extracted Lipid A which have many advantages include its precision, speed and its ability to be automated. The developed method was accurate, precise and rapid for simultaneous estimation of lipid A.

The aim of this study was a trial for preparation one shot *P. multocida* vaccines for rabbits. Reducing the stress of multiple vaccine injection on animal. Production of economic vaccines as they reduce the vaccination cost and manipulation

MATERIALS AND METHODS

Experimental design:

Twenty-one Newzealand rabbits about 1.5-months age old divided into equal three groups (each contained seven animals) were used. The first group was vaccinated with Lipid A adjuvanted *P. multocida* vaccine (AV) and the second group was vaccinated with Montanide™ ISA 70 adjuvanted *P. multocida* vaccines (MV). The third group was kept as a negative control group. First and second groups of this experiment were vaccinated with 1 ml S/C one shot (no booster dose) and challenged with live *P. multocida* strains after 21 days. Control group (7 rabbits) was kept non-vaccinated but challenged at the same time of the other groups. Blood samples were collected for each group from the three groups separately on days 1, 3, 5, 7, 14, and 21. Determination of lysozyme activity was measured according to Schlitz, (1987) blood samples of days 1, 3, 5 and 7. Enzyme linked Immunosorbent Assay (ELISA) according to Briggs and Skeels (1984), were used for estimating antibodies titers on days 7, 14 and 21.

Bacterial strains

P. multocida local field isolates strains (serotypes A:1, A:3, A:12, D:2.), and *E. coli* O: 157 local field isolate strain were obtained kindly from Aerobic Bacterial Vaccines Researches Department, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt. These four *P. multocida* serotypes were used for preparation of vaccines under test, while *E. coli* strain was used for preparation of lipid A.

Preparation of vaccines antigens according to Ruzauskas (2005) with modifications:

Each serotype of *P. multocida* were cultured separately on Tryptone Soya broth (Oxoid). Yeast extract (Sigma) was added for the best growth of cultured bacteria. Eighteen hours later Formaldehyde (Sigma) was added in

concentration of 0.5% into the medium as inactivator then each bacterial suspension was centrifuged at 5000 rpm at 4°C for 30 minutes to pellet the bacterial strain. Then resuspension of the pellets to be at final concentration of 4×10^9 b.c./ml. We used thiomersal (Sigma) as preservative.

Preparation of Lipid A extract

According to El hamidi et al., (2005) with some modifications:

E. coli cells (10 mg) were suspended in 400 µl of (isobutyric acid(Merck)/ ammonium hydroxide (Merck)) (5:3, v: v), and were kept for 2 hours at 100°C in a screw cap test tube under magnetic stirring. The mixture was cooled in ice water and centrifuged (2000 g for 15 min). The supernatant was diluted with water (1:1 v: v). The sample was centrifuged (2000 g for 15 min). Finally, the insoluble lipid A was solubilized and extracted once in 100µl of a mixture of chloroform (Fischer Scientific): methanol: water (HPLC grade water) (3:1.5:0.25, v: v: v). The sample was centrifuged (2000 g for 15 minutes). The supernatant was used for HPLC.

Apparatus and analysis of Lipid A extract by HPLC according to (Plante et al., 2011)

Agilent Series 1200 quaternary gradient pump, Series 1200 auto sampler, Series 1200 UV detector, and HPLC 2D Chemstation software (Hewlett-Packard, Les Ulis, Germany). The HPLC conditions as the chromatographic separation was performed with a reversed-phase column (Dionex Acclaim™ 120, C18(150 × 4.6 mm, 5 µm). Freshly washed *E. coli* cells extracts were analyzed isocratically using Chloroform: methanol: water (65:35:8) (v: v: v) mixture as the mobile phase. The column temperature adjusted at 45 °C at a flow rate of 1.0 mL/min to achieve the optimum resolution of the Lipid A. The injection volume was maintained at 10 µL for both the sample and standard solutions. A UV detector set at 210 nm was used to monitor the effluent. A stock standard solution of 1 mg /ml was prepared by dissolving 10mg of lipid A standard in 10 ml of mixture chloroform :MEOH(3:1)v:v (Raetz et al., 2006). The adjuvanted vaccines was adjusted to be at final concentration 4×10^9 b.c./ml (The dose is 1ml for each rabbit). For AV, each ml of the vaccine contained 25 ug of Lipid A adjuvant according to Garg and Subbarao (1992). For MV, the final vaccine form was obtained by diluting 30g of the aqueous antigenic part into 70g the Montanide™ ISA 70 VG at room temperature

under vigorous stirring according to manufacture recommendation (SEPPIC Co.). Tests for vaccines sterility were done according to Hanna et al.,(2014) and safety were done according to Ruzauskas (2005).

Lipid A validation

Validation method: It is the evaluation process used to ensure that the performance characteristics of an analytical procedure are to demonstrate that it is suitable for its intended purpose.

System Precision:

It was conducted using five replicates of the caffeine standard solution with acceptance criteria of Relative Standard Deviation (RSD) $\leq 1\%$ according to the International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use (ICH, Q2 (R1),

Harmonised tripartite guideline, Validation of analytical procedures:

text and methodology International Conference Harmonization ICH, Geneva, Nov 2005).

Linearity and range:

Linearity was performed by preparing a minimum of five different concentrations of drug standard (Lipid A standard) and defined by the squared correlation coefficient, which should be 0.99 (r^2) according to ICH.

Precision method:

It was conducted using six replicates of Lipid A standard solution (1µg/ml) with acceptance criteria of RSD $\leq 1\%$ according to ICH.

Selectivity and specificity:

Verification of selectivity was conducted by evaluating the standard response in the presence of known concentrations of excipient. Regarding the acceptance criteria, there is no interference between the pure standard and peaks of any impurities or extracted solvents according to ICH.

Accuracy and recovery:

Addition of known quantities of Lipid A to a sample of known concentration. Those samples were analyzed against standard solutions of the corresponding concentrations. The method was accurate according to the calculated test results from the% recovery.

Limit of detection and limit of quantification: LOD and LOQ were calculated using the following equations designated by International Conference on Harmonization (ICH) guidelines. $LOD = 3.3 \times \sigma/S$ $LOQ = 10 \times \sigma/S$

Where, σ is the standard deviation of intercept S is slope of the calibration curve.

Robustness:

It was determined by observing how an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Acceptance criteria: pooled RSD is not more than 6% in every change item.

RESULTS AND DISCUSSION

The natural history of infection with *P. multocida* in domestic rabbits was studied prospectively at a commercial rabbitry. At weaning, about 25% of rabbits had nasal infections with *P. multocida* (Deeb et al.,1990). The bacterial species *P. multocida* includes many different strains, which vary in their virulence. Rabbits can be affected by many strains of the bacteria, but the ones most commonly isolated are types A and D. Type A is most commonly found, but type D is more pathogenic than type A and is associated with more severe disease (Mead et al., 2008). Vaccines are the most powerful biologicals which have modulated the economic, social and cultural life of human beings. Certain diseases have haunted humanity for centuries but are now extinct due to vaccines (Singh, 2009).

Method validation results

The HPLC system was found precise because the RSD of 5 replicates of caffeine standard solution was 0.01%. Linearity existed within range of 0.005 and 5 $\mu\text{g}/\text{ml}$ within a correlation coefficient ($r^2 = 0.99995$) Fig (1). The method for separating Lipid A was precise as the RSD for both intra-day precision and inter day precision as shown in table (1). The Lipid A standard was added to known sample quantity(0.01 $\mu\text{g}/\text{ml}$) as shown in table (2) at three levels which were 0.05,0.1 and 0.2 $\mu\text{g}/\text{ml}$ table (3). There is no interference between the pure standard and peaks of any impurities or extracted solvents as shown in Fig(2) and the retention time of the standard and sample peak was 6.51min. The method was accurate according to the calculated test results from the % recovery which ranged

from 98.82-101.06%. LOD of Lipid A standard was 0.00155 $\mu\text{g}/\text{mL}$ and LOQ was 0.005 $\mu\text{g}/\text{mL}$. The results of robustness indicated that changing the mobile phase composition and changing the detection wavelength had slight effect on the chromatographic behavior of Lipid A. However, the alteration in the column temperature had no significant effect. The RSD % of robustness testing under different altered conditions were 1.2%,1.32% and 0.072% respectively, indicating that the current method is robust.

In this study, the protection rate for both vaccines (A.V. group and M.V. group) was 71.4%, while 100% mortality for control group as shown in table (4). Ringler *et al.*, (1985) extracted antigens from a virulent isolate of *P. multocida* with potassium thiocyanate, and a vaccine was prepared and challenged. None of the six immunized rabbits died, whereas five of six of the nonimmunized rabbits died 2 to 4 days post challenge. Zyan *et al.*, (2004) made a challenge test for evaluating the efficacy of aluminum hydroxide gel (ALV) rabbit Pasteurella vaccine in rabbits in comparison to the classical aqueous formalized (AV) and oil adjuvant rabbit Pasteurella vaccines and found that; two doses of ALV and one dose of AV + oil adjuvant bacterins induced good protection levels (83.3 %) in comparison with 33.3 % in vaccinated rabbits with one dose of ALV while it was 0% in unvaccinated controls. Ruzauskas (2005) when made a laboratory trial of the *Pasteurella* spp. Vaccine against rabbit pasteurellosis using rabbits as experimental and at the same time as target animals revealed that 100% of vaccinated rabbits survived after *P. multocida* infection when the vaccine concentration was 4×10^9 b.c./ml by immunization then booster dose of rabbits with no less than 1 ml of the vaccine. Youssef (2011) prepared inactivated formalized *P. multocida* for different serotype of *P. multocida* and the protection percentages ranged from 50 – 100% according to the used serotype, and 100% deaths to the control group. Youssef and Tawfik (2011) when used two doses of Montanide ISA50 as an adjuvant for preparation of inactivated rabbit pasteurellosis vaccine gave a protection percentage 90% and 100% according to the type of antigen. Abdel-Samie (2014) when estimated the *P. multocida* vaccines for rabbits by challenge test to compare between Commercial vaccine (Oil adjuvanted formalized 0.5% inactivated *P. multocida*) and sonicated *P. multocida* vaccine found that; the sonicated vaccine gave a protection of 100% while the Commercial vaccine

gave 80%.

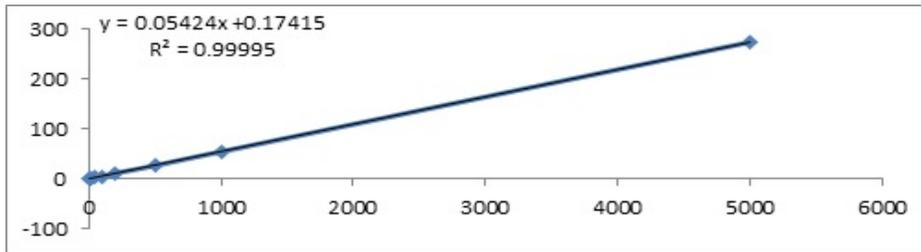


Figure (1): Standard curve of Lipid A.

Table (1) Percision results:

Intraday Percision			Inter day percision		
Ser.no.	Conc.level (µg/ml)	Peak area	Ser.no.	Conc.level (µg/ml)	Peak area
1	1	54.51	1	1	54.924
2	1	54.426	2	1	55.061
3	1	54.73	3	1	55.014
4	1	54.31	4	1	55.152
5	1	54.213	5	1	55.104
6	1	54.814	6	1	54.867
Mean	54.5005		55.02		
SD	0.2348		0.10846		
RSD%	0.4307		0.197133		

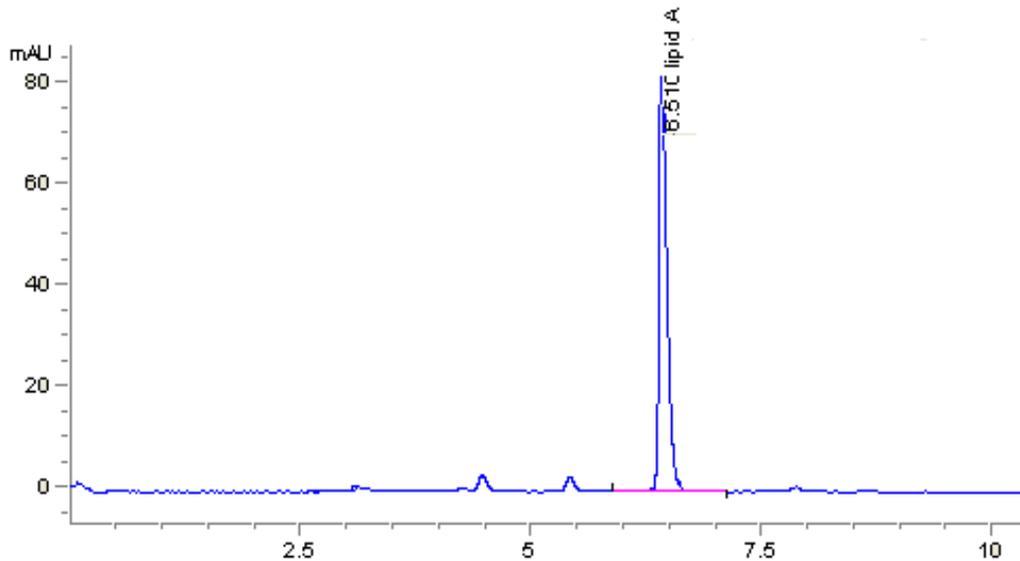


Figure (2): Liquid chromatogram of 1 µg/ml Lipid A standard

The lysozyme activity test results (fig 3) showed an increase in lysozyme activity in rabbit serum of A.V. group than the M.V. group at 1st day, 3rd day, and 5th day, but at 7th day, the M.V. group had a higher increase than the A.V. group. On the other hand, the two tested groups showed a highly significant increase in lysozyme activity in rabbit sera in compare with the control group. Youssef and Tawfik (2011) Evaluated the cell mediated immune response in different rabbits of *P. multocida* vaccinated group adjuvanted with Montanide ISA50 in comparison to group adjuvanted with mineral oil by macrophage activity using *candida albicans* expressed by phagocytic percentage and phagocytic index, and the results

were as follow; for 1st dose on 7, 14, and 21 days the Montanide ISA51 was the predominant except 7th day was lower. Also, for 2nd dose on 7, 14, and 21 days the Montanide ISA51 was the predominant except 7th day was lower.

ELISA test results declared that, antibodies titers of A.V. group were higher than antibodies titers of M.V. group in 1st and 2nd week, but antibodies titers of M.V. group became the higher in 3rd week (Fig.4). Control group had no significant changes in their titers.

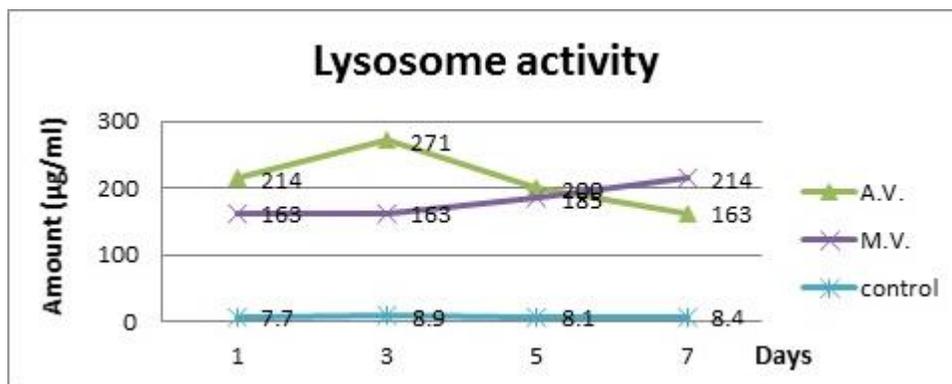


Figure (3): The effect of Lipid A adjuvanted *P. multocida* vaccine and Montanide™ ISA 70 adjuvanted *P. multocida* vaccine on Lysozyme activity in the serum.



Figure (4) Antibody titers of rabbits vaccinated with Lipid A adjuvanted *P. multocida* vaccine and Montanide™ ISA 70 adjuvanted *P. multocida* vaccine measured by enzyme-linked immunosorbent assay (ELISA).

Table (2) Recovery studies:

Concentration in sample(0.01µg/ml) with triplicate readings	Mean±SD	Recovery%
0.010012 0.009879 0.009989	0.00996±7.10845E-05	99.6%

Table (3) Accuracy and Recovery studies:

Actual conc. In sample	Added conc. At 3 levels	Resulted conc. Levels	Found conc.	Mean±SD	RSD%	Recovery%	Average Recovery%±SD
0.00996	0.04	0.05	0.049302	0.0494±0.0001	0.2112	98.604	98.822±0.2
			0.04951			99.02	
			0.049421			98.842	
	0.09	0.1	0.099081	0.0993±0.0002	0.2011	99.081	99.30167±0.1997
			0.09947			99.47	
			0.099354			99.354	
	0.19	0.2	0.20233	0.20213±0.0006	0.3014	101.165	101.0667±0.3046
			0.20262			101.31	
			0.20145			100.725	

Table (4): Protective value of different rabbit pasteurellosis vaccines (Lipid A adjuvanted *P. multocida* vaccine (AV) and Montanoide ISA 70 adjuvanted *P. multocida* vaccine (MV) in rabbits.

Group	Mortality	Protectivity %
A.V.	2/7	71.4
M.V.	2/7	71.4
Control	7/7	0

Ringler et al., (1985) used ELISA test to evaluate a Pasteurella vaccine prepared by extracting antigens from a virulent isolate of *P. multocida* with potassium thiocyanate, and according to the ELISA test results there was a high increase in the antibodies titers in comparison to the control group. Suckow (2000) used ELISA test in rabbit Pasteurella vaccines evaluation by comparing between a commercial swine *P. multocida* bacterin-toxoid vaccine and inactivated heat-labile toxin of *P. multocida* (from rabbits) vaccine and found that the commercial swine vaccine stimulates antibody activity to and protective immunity against *P. multocida* heat labile toxin in rabbits. Youssef and Tawfik (2011) when compared between ELISA test results of *P. multocida* vaccinated group adjuvanted with Montanide ISA50 to group adjuvanted with mineral oil, the Montanide ISA50 group was predominant. Abdel-Samie (2014) when estimated the *P. multocida* vaccines for rabbits by ELISA

test to compare between Commercial vaccine (oil adjuvanted formalized 0.5% inactivated *P. multocida*) and sonicated *P. multocida* vaccine found that; the sonicated vaccine gave a higher titer of antibodies than the Commercial vaccine gave 80%. Ashraf et al., (2014) extracted the Lipopolysaccharides (LPS) from the whole cell of the causative organism (*P. multocida*), then inoculated it subcutaneously into rabbits to stimulate antibody production. The antibody titer was determined by applying indirect ELISA and concluded that with an increase in the dose of LPS antigen, the antibody titer increased and the combination of LPS of *P. multocida* with yeast produced more immunity when compared to LPS alone in rabbits.

CONCLUSION

In the light of this study, we concluded that Montanoid ISA70 adjuvanted vaccine(MV) and Lipid A adjuvanted *P. multocida* vaccine have the same protection ratio. In spite of Lipid A

adjuvanted *P. multocida* vaccine (AV) expressed higher immune response in the first, Montanoid ISA70 adjuvanted vaccine(MV) overcome at the last as one shot vaccine.

Statistical analysis

The obtained results were subjected to Statistical analysis as all the parameters were analyzed by one- way ANOVA for comparison between groups using a complete randomized design (Snedecor and Cochran ,1989). The differences between groups means were further compared by Duncan's multiple range test. Differences with a *P* value below 0.05 were considered statistically significant

CONFLICT OF INTEREST

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

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AUTHOR CONTRIBUTIONS

MTAI prepared the vaccines, vaccination of rabbits, challenge test, collection of blood samples for lysozyme test and ELISA and performed ELISA. MIE designed experiments and reviewed the manuscript. EME cultivated and harvested *E. coli* strain and performed Lysozyme test. MAF extracted Lipid A from *E. coli*, performed Lipid A validation steps and data statistical analysis. MTAI and MAF wrote the manuscript. All authors read and approved the final version.

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REFERENCES

- Abdel-Samie LK, 2014. Immunogenicity of experimental *Pasteurella multocida* vaccine in rabbits. Assiut Vet. Med. J. 60 (143): 104-108.
- Anina BJS, Richard KH, Peter K., Bozena MK, 2009. Phenotypic and genetic characterization of *Pasteurella multocida* and related isolates from rabbits in Switzerland. J Vet Diagn Invest 21:793–802.
- Ashraf A, Mahboob S, Al-Ghanim K, Huma T, Shah MS, 2014. Immunogenic activity of lipopolysaccharides from *Pasteurella multocida* in rabbits. The Journal of Animal & Plant Sciences 24(6): 1780-1785.
- Aucoutuier J, Ganne V, 2002. Adjuvants designed for vet use. Seppic incorporation limited company.
- Briggs DJ, Skeels JK, 1984. As enzyme linked immunosorbent assay for detecting antibodies to *P. multocida* in chicken. Avian Dis. 28 (1): 208-215.
- Charles SD, Hussein I, Nagraja KV, Sivanadan V, 1994. Adjuvanted subunit vaccines for the control of *Salmonella enteritidis* infection in turkeys. Am. J. Vet. Res. 55 (5): 636-642.
- Deeb BJ, Digiacomio RF, Bernard B.L, Silbernagel SM, 1990. *Pasteurella multocida* and *Bordetella bronchiseptica* infections in rabbits. J. Clin. Microbiol. 28: 70-75.
- El Hamidi A, Tirsoaga A, Novikov A, Hussein A, Caroff M, 2005. Microextraction of bacterial lipid A: easy and rapid method for mass spectrometric characterization. J Lipid Res 46: 1773-1778.
- Garg M, Subbarao B, 1992. Immune responses of systemic and mucosal lymphoid organs to Pnu-Imune Vaccine as a function of age and the efficacy of Monophosphoryl Lipid A as an Adjuvant. Infection and immunity 2329-2336.
- Gracy JF, 1986. Infection of rabbits and horses. Meat Hygiene, Ed 8. Bailliere Tindall, East Sussex, UK, pp. 482–486.
- Han Y, Li Y, Chen J, Tan Y, Guan F, Wang X, 2013. Construction of Monophosphoryl Lipid A producing *Escherichia coli* mutants and comparison of immuno-stimulatory activities of their lipopolysaccharides. Mar. Drugs J. 11: 363-376.
- Hanna TN, El-Sayed MG, Ebeid MH, Houmda FK, Elmeneisy AA, Gorgy SF, 2014. The effectiveness of bivalent clostridial and pasteurella combined vaccines in the laboratory animals. Benha Veterinary Medical Journal 27(2):302-308.
- ICH 2005, Q2 (R1), Harmonised tripartite guideline, Validation of analytical procedures: text and methodology International Conference Harmonization ICH, Geneva
- Johnson AG, Gaines S, Landy M, 1956. Studies

- on the O-antigen of *Salmonella typhosa* V. Enhancement of antibody response to protein antigens by the purified lipopolysaccharide. J. Exp. Med. 103: 225–46.
- Johnson RS, Her G-R, Grabarek J, Hawiger J, Reinhold V, 1990. Structural characterization of monophosphoryl lipid A homologs obtained from *Salmonella minnesota* Re595 lipopolysaccharide. J Biol Chem 265:8108-8116.
- Mead MHC, DVM CVA and Meredith A. (2008): Pasteurella: A Ubiquitous Bacteria—Its Health Effects in Rabbits. The magazine for bunny mad people, Issue 7.
- Mohammed HA, Eid AAM, El-Bakrey RMM. 2013. A review of rabbit diseases in Egypt. WARTAZOA 23 (4) :185-194.
- Plante M, Bailey B, Acworth I, Clark D, 2011. Analysis of lipids by HPLC-CAD. ESA-Dionex Inc.
- Raetz CR, Garrett TA, Reynolds CM, Shaw WA, Moore JD, Smith DC, Ribeiro AA, Murphy RC, Ulevitch RJ, Fearn C, Reichart D, Glass CK, Benner C, Subramaniam S, Harkewicz R, Bowers-Gentry RC, Buczynski MW, Cooper JA, Deems RA, Dennis EA, 2006. Kdo2-Lipid A of *Escherichia Coli*, a defined endotoxin that activates macrophages via Tlr-4. J Lipid Res., 47: 1097-1111.
- Rajeev R, Panda SK, Acharya AP, Singh AP, Gupta MK, 2011. Molecular diagnosis of Haemorrhagic Septicaemia. A Review. Veterinary World 4: 189-192.
- Ringler DH, Peter GK, Chrisp CE, Keren DF, 1985. Protection of rabbits against experimental Pasteurellosis by vaccination with a Potassium Thiocyanate extract of *Pasteurella multocida*. Infect immun 49(3):498-504.
- Ruzauskas M, 2005. Development and assay of inactivated Pasteurella vaccine for rabbits. BIOLOGIJA.2: 35–39.
- Schlitz LA. (1987): "Veterinary Haematology". Ed 3. Lea and Febiger.; 39(2): 217-222.
- Sendecor GW, Cochran WG, 1989. Statistical methods, Ed 9. Iowa state Univ.Press.,Ames, Iowa,USA.
- Singh BR, 2009. *Salmonella* vaccines for animals and birds and their future perspective. The Open Vac. J. 2: 100-112.
- Suckow MA, 2000. Immunization of rabbits against *Pasteurella multocida* using a commercial swine vaccine. Lab Anim 403-408.
- Suelam IIA, Abdel Samie LK, 2011. Molecular diversity of *Pasteurella multocida* isolated from different rabbit outbreaks at Zagazig Suburbs, Egypt. Global Veterinaria 6 (2): 208-212.
- Youssef, 2011. Study of pathogenicity and immunogenicity of *Pasteurella multocida* recently isolated from infected rabbits. Egypt. J. Agric. Res. 89 (2):731-742
- Youssef EA, Tawfik HE, 2011. Improvement of rabbit pasteurellosis vaccine using Montanide ISA50. Egypt. J. Agric. Res., 89 (2): 697-708.
- Zyan KA, Saad AE, Fad HA, Abd EL-Maksoud HAA, 2004. Field trial to evaluate the efficacy of aluminum hydroxide gel (ALV) rabbit Pasteurella vaccine in rabbits. First Scientific Conference of Fac. Vet. Med.; Moshtohor, Sept. 1-4, Benha-Ras sedr.
- Raetz CR, Whitfield C, 2002. Lipopolysaccharide endotoxins. Annu Rev. Biochem. 71:635–700.
- Trent MS, Stead CM, Tran AX, Hankins JV, 2006. Diversity of endotoxin and its impact on pathogenesis. Journal of endotoxin research 12:205–223.