Some Studies on Rabbit Hemorrhagic Septicemia Vaccines in Egypt

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ABSTRACT

In this study, a trivalent autogenous inactivated *P.multocida* vaccine was prepared. The prepared vaccine contained three different *P. multocida* strains which were previously isolated from different localities at Sharkia Governorate and identified by biochemical and molecular methods. The protective effect of the prepared vaccine was evaluated through estimation of the humeral immune response using indirect heamagglutination test (IHA); challenge infection and field evaluation. *Pasteurella multocida* strains were inactivated with formalin (0.5%) and adjuvanted with montanide oil (ISA -70). Repeated immunization raised up the specific antibody level eight times in vaccinated rabbits. It conferred protection against challenge with homologous strains in commercial rabbits. In the field study a positive effect of vaccination on nasal colonization with *P. multocida* was found.

INTRODUCTION

Rabbit breeding became an intensive and known as Rabbit industry in Egypt, thus facing several disease problems. Rabbit Pasteurellosis is one of the most economic diseases causing great economic losses in rabbits. Rabbit Pasteurellosis is an acute, sub acute and chronic contagious disease affecting rabbits at different ages and characterized by several clinical forms, snuffles, hemorrhagic septicemia, enzootic pneumonia, otitis media, conjunctivitis, pyometra / orchitis and subcutaneous abscesses. Stress factors are predisposing to the disease such as pregnancy, parturition, lactation, enteritis and improper hygiene *(1-6)*.

Since most or all rabbits carry *Pasteurella multocida* in the nasal cavity, management measures are aimed to control the clinical disease expression. Some antimicrobials are used to control pasteurellosis, although in most cases, they are effective only for a short period and resulted in recurrence of the disease. Although some manufacturers produce a vaccine against this disease, the antigenic structure of the antigens not always identical to the antigenic structure of bacteria which are spread in different areas. For these reasons auto vaccines should be used *(8, 9)*.

Effective vaccines against haemorrhagic septicaemia are formalin-killed bacterins or dense bacterins with adjuvants. The latter enhance the level and prolong the duration of immunity *(10)*. The objective of this study was preparation and evaluation of autogenous trivalent *P.multocida* vaccine and compared with commercial H.S. vaccine used in Egypt.

MATERIAL AND METHODS

Bacterial Strains

P.multocida strains type PS/R/BB/3.1/D/009; PS/R/ZG/26.1/A/010 and PS/R/HH/ 28.2/A/011 were isolated from local outbreaks of rabbit pasteurellosis at Sharkia Governorate (Minia El Kamh; El-salheia Al-gededa; Belbis; Hehia, Abou-Hammad and Zagazig). These strains were identified by biochemical and molecular methods. These

serotypes were used for preparation of autogenous vaccine, preparation of antigen used for measuring the level of specific *P.multocida* antibodies in rabbit sera and challenge of mice in active mouse protection test.

Experimental animals

Mice

Five hundred healthy Swiss albino mice of 4 weeks - old (each of 18-24g) were used for pathogenicity test; purification of *Pasteurella* microorganisms and LD_{50}.These mice were obtained from Laboratory Animal Unit; Faculty of Vet. Medicine, Zagazig University, Zagazig, Egypt.

Rabbits

One hundred and twenty five; New-Zealand white rabbits; aged two month, were obtained from a private rabbitary neither vaccinated nor affected with Rabbit heamorrhagic septiceamia. These rabbits were kept and reared in an isolating unit for preparing and testing inactivated autogenous *Pasteurella multocida* vaccine.

Montanide ISA (ISA-70): oil adjuvant, permitting increased immune system response. It was supplied by SEPPIC, Cosmetic/ Pharmacy Division, Paris, France and used as adjuvant for the inactivated autogenous *P.multocida* vaccine.

Commercial vaccine: Formalized polyvalent Rabbit Pasteurellosis vaccine was kindly supplied from: Vet. Ser. and Vac. Res. Inst., Cairo, Egypt. Batch no. 35.

Preparation of inactivated autogenous *Pasteurella multocida* vaccine ***(9, 10)***

Bacterial isolates selection

1) Selection of *P. multocida* field strains was performed according to their properties for vaccine development. These strains must be capsulated and highly pathogenic to animals. Their growth, antigenic, biochemical and pathogenic properties must be stable for the whole time of observation.
2) Three strains of *P. multocida* were prepared for vaccine development. Two of them belonged to capsular type A and another one belonged to capsular type D.

Preparation of *Pasteurella multocida* bacterin

P.multocida isolate grew on 5% defibrinated blood agar at 37° C for 24 hours then one or two colonies from each isolate 24 hours old cultures were inoculated separately into 10 ml of trypticase soya broth (Difco) containing 0.3% yeast extract (TSBY) and incubated at 37°C for 24 hrs. This was then transferred to one liter of TSBY medium in a 2 liter flask and incubated at 37°C for 24 hrs. Purity was checked by Gram's staining and part of the culture was diluted and plated on tryptic soya agar containing 0.3%yeast extract (TSAY) to check its purity and also to determine the number of colony-forming units (CFU/ml) which equivalent to 5 X 10^9 CFU/ml of each isolate *(8)*. The culture was inactivated with 0.5% formalin at room temperature for 24 hrs. Sterile oil adjuvant (ISA-70) (sterilization by autoclaving) was added to give a final concentration of 7/3 (v/v).

A preservative, thiomersal (Merthiolate) was finally added at a dilution of 1/10.000 (V/V) during filling and before distribution into bottles. Then the final vaccine product was filled in clean and sterile bottles. The prepared vaccine was kept at 4°C until use.

Evaluation and quality control of the prepared vaccine

The vaccine was tested for sterility test, safety and Potency according to *(11)*

1- Sterility test.
2- Safety test.
3-Active mouse protection test (Potency test in mice).
4- Potency of the prepared vaccine (Potency test in rabbits).
5- Field evaluation.

Challenge test for *P.multocida* LD_{50} in mice using active mouse protection test (Potency of the prepared vaccine in mice) *(12)*

Experimental design for testing potency of locally prepared autogenous P.M vaccine is shown in Table 1.

The median lethal dose (LD_{50}) was calculated for each subgroup of mice, vaccinated and control groups based on the accumulated deaths on 7th day using Reed and Muench (1938) method. A requirement of 2 logs of protection is necessary to qualify the prepared vaccine as an index *(9, 10)*.

Table 1. Experimental design for testing potency of the locally prepared autogenous *p.multocida* vaccine in mice

Mice groups No.	No. of mice	Vaccination		Booster Vaccination (14 - days later)		Challenge 21days post second vaccination by 0.1 0.1ml (10^8 CFU) / S/C	Mice subgroups (50mice for each)	Strain of challenge
		Dose	Route	Dose	Route			
1	150	0.2ml	S/C	0.2ml	S/C		1a	PS/R/BB 3.1/D/009
							1b	PS/R/ZG 26.1/A/010
							1c	PS/R/HH 28.2/A/011
2	150	Control (not vaccinated)		-			2a	PS/R/BB 3.1/D/009
							2b	PS/R/ZG 26.1/A/010
							2c	PS/R/HH 28.2/A/011

PS/R/BB/ 3.1/D/009: *Pasteurella multocida*/Rabbit /Belbis/3.1/ capsular type D/2009.
PS/R/ZG/ 26.1/A/010: *Pasteurella multocida*/Rabbit /Zagazig/26.1/ capsular type D/ 2010.
PS/R/HH/ 28.2/A/011: *Pasteurella multocida*/Rabbit /Hehia/28.2/ capsular type D /2011.

Potency of the locally prepared autogenous inactivated *Pasteurella multocida* vaccine in rabbits

Determination of the humeral immune response by passive haemagglutination test, antibodies titer against *Pasteurella multocida* in the vaccinated rabbits were estimated as follows:

1-Experimental design in rabbits.
2-Passive heamaglutination test (IHA).

Experimental Laboratory evaluation of autogenous and commercial vaccines (Table 2).

Blood samples (individually from ear vein of all rabbits) were collected before initial immunization from all rabbit groups then weekly till 4th week post vaccination (WPV) then monthly till 24th WPV, sera were prepared to evaluate the humeral immune response by using passive heamaggulutintion test.

Passive or indirect heamagglutination test (PHA)

Anti *P. multocida* antibodies were measured by passive heamagglutination test *(14)*. The test was performed by two fold dilution of the sera in the wells of U shape microtitre plates (the initial dilution was 1/20).

Three weeks post second vaccination, 15 rabbits from each experimental group(A,B,C) were subdivided into three subgroups (1,2,3), each subgroup contained 5 rabbits. Rabbits of subgroup 1; subgroup 2 and subgroup3 were challenged with PS/R/BB/ 3.1/009; PS/R/ZG/ 26.1/010 and PS/R/HH /28.2/011 respectively. The dose of challenge for each strain was 10^8 CFU/ml via subcutaneous route. The percentage of survivors was calculated as a proportion of dead rabbits and survivors after challenge with three pathogenic strains of *Pasteurella multocida* which were used in vaccine preparation (Table 2). Trials for *P.multocida* reisolation from freshly dead challenged rabbits and P.M examination were performed.

Table 2. Challenge infection of rabbits vaccinated with both commercial and autogenous prepared vaccines

Rabbit groups	No. of rabbits	21 days post second vaccination	Rabbit subgroups	No. of rabbits/ subgroup	Challenge		
					Strain of challenge	Dose	Route
A	20		1A	5	PS/R/BB 3.1/D/009	0.1ml LD_{50}	S/C
			2A	5	PS/R/ZG 26.1/A/010	0.1ml LD_{50}	S/C
			3A	5	PS/R/HH 28.2/A/011	1ml LD_{50}	S/C
B	20		1B	5	PS/R/BB 3.1/D/009	0.1ml LD_{50}	S/C
			2B	5	PS/R/ZG 26.1/A/010	0.1ml LD_{50}	S/C
			3B	5	PS/R/HH 28.2/A/011	1ml LD_{50}	S/C
C	20		1C	5	PS/R/BB 3.1/D/009	0.1ml LD_{50}	S/C
			2C	5	PS/R/ZG 26.1/A/010	0.1ml LD_{50}	S/C
			3C	5	PS/R/HH 28.2/A/011	1ml LD_{50}	S/C

PS/R/BB/ 3.1/D/009: *Pasteurella multocida*/Rabbit /Belbis/3.1/ capsular type (D) /2009.
PS/R/ZG / 26.1/A/010: *Pasteurella multocida*/Rabbit /Zagazig/26.1/ capsular type (A) /2010.
PS/R/HH / 28.2/A/011: *Pasteurella multocida*/Rabbit /Hehia/28.2/ capsular type (A)/2011.

Field evaluation of the locally prepared autogenous vaccine in rabbits *(8)*

Field evaluation of the locally prepared autogenous *P.multocida* vaccine were performed using 3 farms located at El-salheia, Kafr El Hamam and Bordin located at Sharkia Governorate, Egypt. El-Salheia farm was containing 150 does, 30 bucks and 500 kids. Kafr El Hamam farm was containing 100 does, 20 bucks and 300 kids. Bordin farm was containing 50 does, 10 bucks and 150 kids. These farms containing different breeds of rabbits. Before vaccination, in all farms about 15-20% of rabbits (adult and young rabbits) were suffered from clinical signs of Pasteurellosis and the diagnosis was confirmed by bacteriological investigations. All rabbits in all farms were vaccinated with locally prepared autogenous vaccine at a dose of 1 ml (containing 10^9 CFU) via subcutaneous route and revaccinated after 14 days. Females were revaccinated in the middle of every third pregnancy. Young rabbits were vaccinated after weaning. Breeding males were revaccinated every 4 months. All rabbits were vaccinated except rabbits that suffered intense clinical signs which were condemned prevaccination. The frequency of clinical signs of rabbit pasteurellosis was observed pre and post vaccination on a regular basis for 12 months. In addition, *P.multocida* antibodies monitoring (Serum analysis) monthly for 6 months using passive hemagglutination test *(8)*.

RESULTS AND DISCUSSION

In this study a trial for preparing an autogenous trivalent inactivated vaccine of excellent potency and safety using montanide oil (ISA-70) which enhances immune response was carried out. The evaluation of the immune response of vaccinated rabbits with autogenous and commercial vaccine either experimentally or in the field was carried out. The experimentally prepared vaccine proved to be sterile (no growth of microorganisms on nutrient agar, blood agar and Sabaroud dextrose agar) and safe to the target animal species. A slight increase of the rabbit's body temperature was observed on the first day after vaccination. A mild transient reaction at the site of injection was observed during the period of the investigations. Similar results were recorded by previous studies *(10, 11)*.

The result of challenge test in vaccinated and control mice illustrated in Table 3,Active mouse protection test has been described as satisfactory for measuring immunity in vaccinated animals *(15)*. From the results of the Present study it could be seen that

autogenous vaccine adjuvanted with montanide oil (ISA-70) was protective against challenge with three virulent *P.multocida* strains where log protection in three strains was more than log 2. Montanide ISA 70 vaccine shown to be efficacious in eliciting early and higher protective immune response in mice *(16)*.

Results in Table 4 showed no antibodies in the serum samples from all vaccinated and control rabbits against *P.multocida* before vaccination. All vaccinated rabbits in groups 1and 2 induced systemic humeral antibodies as measured by indirect heamagglutination test. It was evident from the results that group 1 (vaccinated with commercial formalized *P. multocida* vaccine) gave lowest *P. multocida* antibody titer (40) at 1st wpv while group 2(vaccinated with autogenous oil emulsified vaccine) gave earlier and higher antibody titer. *P.multocida* antibody titer at 2nd wpv was stable in group1 while increased in group 2. This enhancement was continued at 3rd , 4th and 8th wpv where *P. multocida* antibody titer increased to 5120 in group 2 and 320 in group 1 then decline in both groups 1 and 2 till the end of observation period(24th wpv).

A 100% and 80% rabbits' survival was observed when the rabbits vaccinated with trivalent autogenous vaccine while rabbits vaccinated with commercial vaccine showed variable degree of protection ranging from 60% and 80%. All rabbits of the control group were died 2-3 days post challenge. Post mortem lesions of freshly dead rabbits were nasal discharge; septiceamia and frothy exudate in trachea. *P. multocida* was reisolated from freshly dead challenged rabbits (Table 5 and Fig. 1).

During field evaluation of the experimentally prepared vaccine, 12 months following the active immunization of adult and young rabbits, the number of rabbits that had clinical signs of pasteurellosis significantly decreased. After 12 months, only 2% of rabbits had sneezing and nasal discharges from all farms. The vaccine had no adverse effects on rabbits except sterile swelling at the site of injection. It is common when animals are vaccinated with inactivated vaccines with oil adjuvants.

Non of serum samples from all vaccinated rabbits (El-salheia, Kafr El Hamam and Bordin rabbit farms) showed presence of antibodies against *P.multocida* before vaccination. All vaccinated rabbits induced systemic humoral antibodies as measured by indirect heamagglutination test. Titers of antibodies were gradually increased in all vaccinated rabbits till the 4th month post vaccination then decline at the 5th and 6th months post vaccination (Table 6 and Fig. 2).

Table 3. Results of challenge of vaccinated and control mice against *P. multocida*

Group No.	Challenge Strain	LD_{50} after challenge		Log protection
		Vaccinated	Control	
1	PS/R/BB/ 3.1/D/009	$10^{-5.54}$	$10^{-8.09}$	2.55
2	PS/R/ZG/ 26.1/A/010	$10^{-6.57}$	$10^{-8.59}$	2.02
3	PS/R/HH /28.2/A/011	$10^{-5.19}$	$10^{-7.45}$	2.26

Group (1): mice vaccinated with autogenous vaccine and challenged with *P.multocida* (PS/R/BB/ 3.1/D/009).
Group (2): mice vaccinated with autogenous vaccine and challenged with *P.multocida* (PS/R/ZG/ 26.1/A/010).
Group (3): mice vaccinated with autogenous vaccine and challenged with *P.multocida* (PS/R/HH /28.2/A/011).
N.B: Less than log2 is not protective post challenge result.

Table 4. Specific *P. multocida* antibody titers determined by indirect haemagglutination test (IHA) in vaccinated and non vaccinated rabbit groups

Rabbit Group	Type of Vaccine	Weeks post vaccination / PHA titer									
		0	1	2	3	4	8	12	16	20	24
A	commercial vaccine	0	40	40	80	160	320	320	320	160	160
B	autogenous vaccine	0	80	160	640	640	5120	320	160	160	160
C	Non vaccinated	0	0	0	0	0	0	0	0	0	0

Group A: vaccinated with commercial vaccine (inactivated formalized *P. multocida* vaccine)
Group B: vaccinated with experimentally prepared autogenous *P.multocida* vaccine (inactivated trivalent oil emulsified vaccine with ISA 70)
Group C: Non vaccinated control.

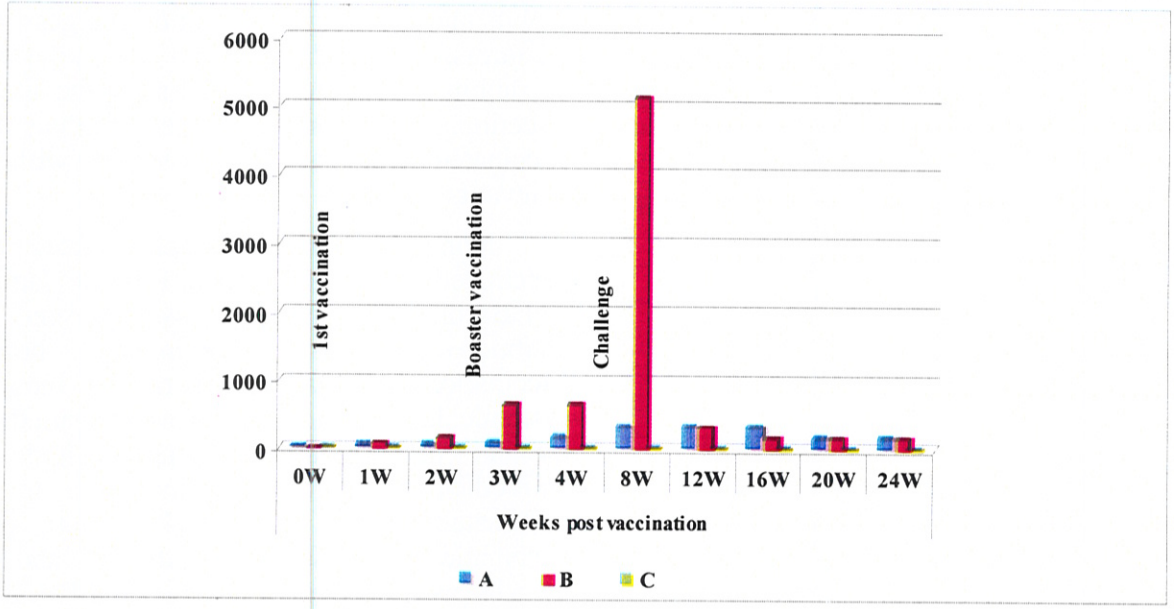

Fig. 1. *P.multocida* antibodies titers determined by passive heamagglutination test in vaccinated (commercial and autogenous locally prepared vaccine) and non vaccinated rabbits

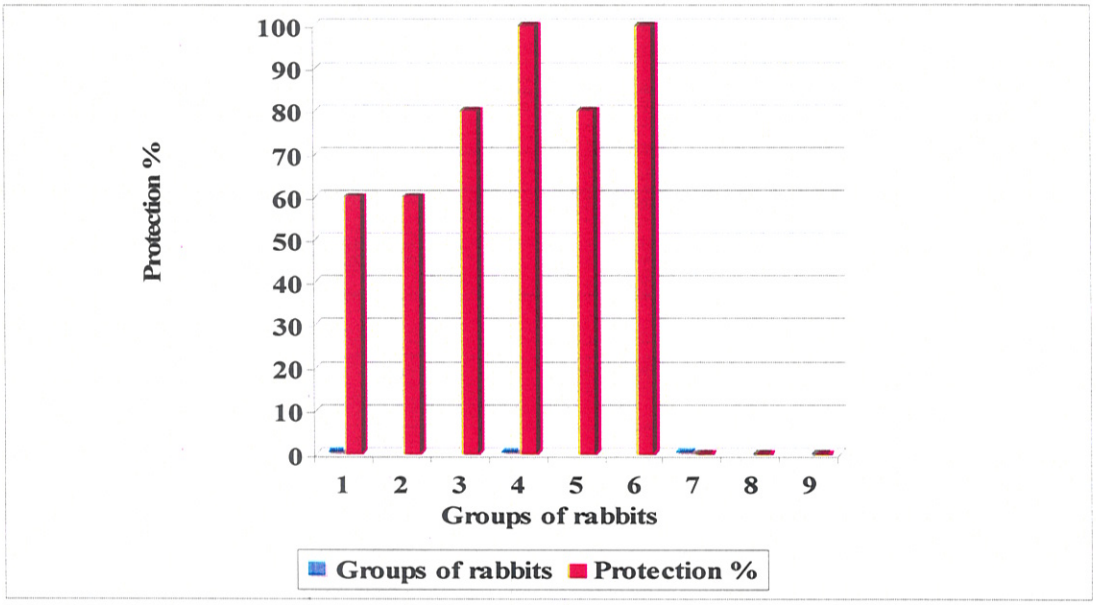

Fig. 2. Protection percent in vaccinated and control rabbits after challenged with local isolated, virulent *P.multocida* strains

Table 5. Protection percent in vaccinated and non vaccinated rabbits after challenge with three virulent *P.multocida* strains

Group No.	Type of vaccine	Rabbit subgroups	No. of rabbits	Age	Strains of challenge	Challenge dose	Route	No. of dead rabbits	Protection percent
A	Commercial vaccine	1A	5	2ms	PS/R/BB 3.1/D/009	0.1ml LD_{50}	S/C	2	60%
		2A	5	2ms	PS/R/ZG 26.1/A/010	0.1ml LD_{50}	S/C	2	60%
		3A	5	2ms	PS/R/HH 28.2/A/011	1ml LD_{50}	S/C	1	80%
B	Trivalent autogenous vaccine	1B	5	2ms	PS/R/BB 3.1/D/009	0.1ml LD_{50}	S/C	1	100%
		2B	5	2ms	PS/R/ZG 26.1/A/010	0.1ml LD_{50}	S/C	0	80%
		3B	5	2ms	PS/R/HH 28.2/A/011	1ml LD_{50}	S/C	0	100%
C	Control (unvaccinated)	1C	5	2ms	PS/R/BB 3.1/D/009	0.1ml LD_{50}	S/C	5	0%
		2C	5	2ms	PS/R/ZG 26.1/A/010	0.1ml LD_{50}	S/C	5	0%
		3C	5	2ms	PS/R/HH 28.2/A/011	1ml LD_{50}	S/C	5	0%

Group A: vaccinated with commercial vaccine (inactivated formalized *P.multocida* vaccine).
Group B: vaccinated with experimentally prepared autogenous vaccine (inactivated trivalent oil emulsified vaccine with ISA 70).
Group C: Non- vaccinated control.

Table 6. Specific *P. multocida* antibody titers determined by indirect haemagglutination test (IHA) in field rabbits vaccinated with autogenous vaccine

Localities of Serum samples	Months post vaccination / PHA titer						
	0	1	2	3	4	5	6
El-salheia	0	80	160	320	640	320	160
Kafr El Hamam	0	40	80	160	320	320	160
Bordin	0	80	160	320	320	160	80

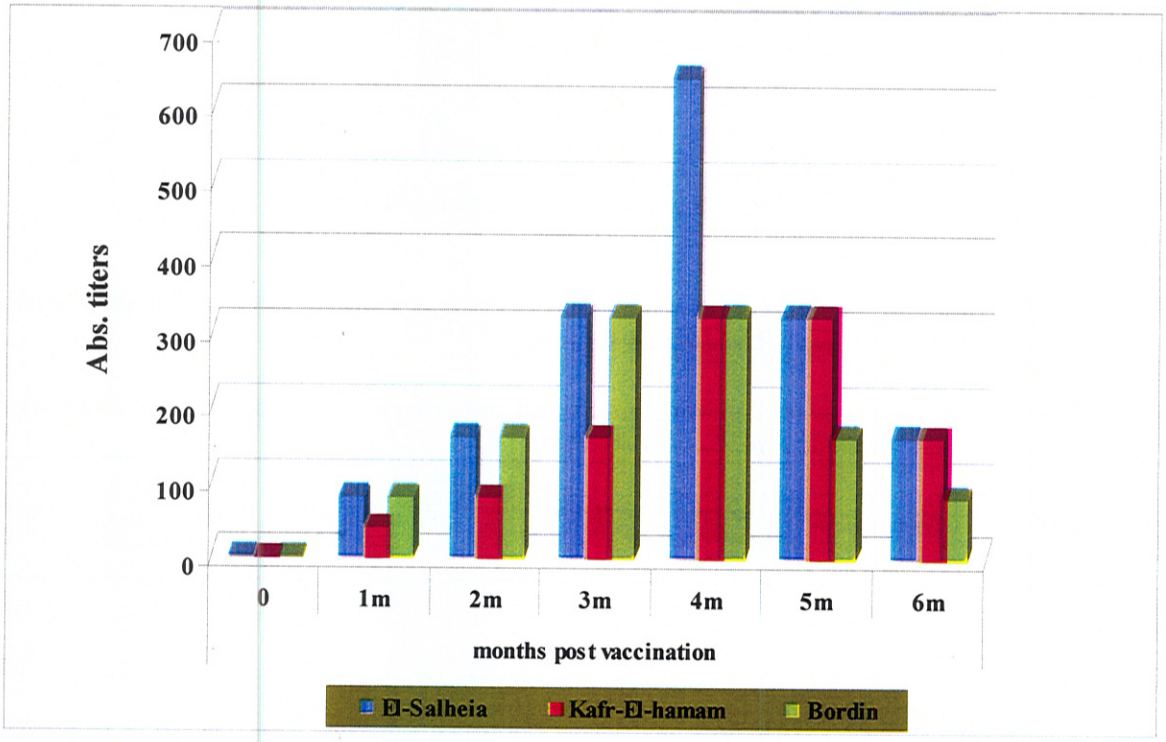

Fig. 3. Specific *P. multocida* antibody titers determined by indirect haemagglutination test (IHA) in field rabbits vaccinated with autogenous vaccine.

Conclusion

Finally it could be concluded that autogenous trivalent *P.multocida* vaccine adjuvanted with montanide ISA-70 gave faster and higher immune response than commercial formalized vaccine due to its low viscosity

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الملخص العربي

بعض الدراسات عن لقاحات التسمم الدموي في مصر

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مرض التسمم الدموي البكتيري من أهم الأمراض التي تصيب الأرانب في مصر وتسبب خسائر اقتصادية كبيرة بين القطعان. علما بأنه يتم تحصين الأرانب باللقاح التجاري الفورمالييني المصنع في معامل المصل واللقاح بالعباسية وبالرغم من ذلك يحدث المرض بصوره المختلفة. لذا كانت هذه المحاولة لتحضير لقاح محلي ثلاثي ذ اتي ضد التسمم الدموي البكتيري في الأرانب باستخدام زيت المونتانيد (ي س أ ٧٠). وتم تقييم هذا اللقاح الذاتي من خلال حقنه في أرانب وفئران وقياس مستوي الأجسام المضادة باستخدام اختبار تجمع الدم الغير مباشر وكذلك اختبار تحدي العدوي في الأرانب المحصنة.وأيضا تم مقارنته باللقاح التجاري الفورمالييني المستخدم في السوق المصري ضد مرض التسمم الدموي البكتيري. وقد وجد أن اللقاح الذ اتي هو الأفضل من حيث مستوي الأجسام المناعية للأرانب والفئران المحقونين باللقاحين الذاتي والتجاري وكذلك من حيث مستوي تحدي العدوي في الأرانب.حيث وجد زيادة تدريجية في مستوى الاجسام المناعية في الارانب المحصنة باللقاحين المحضر والتجاري ولكن هذه الزيادة كانت مرتفعة في الارانب المحصنة باللقاح المحلي أكثر من الأرانب المحصنة باللقاح التجارى. وكذلك اختبار تحدي العدوي في الأرانب أظهر اختلافا واضحا بين اللقاحين . حيث أن مستوي التحدي في الارانب المحصنة باللقاح المحضر كانت ٨٠-١٠٠% بينما في الأرانب المحصنة باللقاح التجاري كانت ٦٠-٨٠% وذلك بسبب الاختلاف في مستوي الأجسام المناعية الناتج عن كلا اللقاحين وكذلك لأن عترات التحدي هي نفس عترات اللقاح المحضر .