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IMMUNOGENICITY OF EXPERIMENTAL *PASTEURELLA MULTOCIDA* VACCINE IN RABBITS

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ABSTRCT

Previously *Pasteurella multocida* was isolated and genotyped by the author from different outbreaks in rabbits at Sharkia Governorate, Egypt. In this work, development of avaccine was carried out in the laboratory by sonication of the isolated bacteria genotype A, B and D. Two experiments were conducted for evaluation of the trialed vaccine. The first one was carried out for determination of the protective dose of the vaccine and the second experiment for testing of its immune competency with commercial vaccine. Immunogenic response was evaluated serologically by ELISA and protection percentage on challenge against infection. Results revealed that sonicated vaccine containing $4x10^9$ colony forming unit (cfu)/ml provoked high level of immunity. On comparison with commercial vaccine, geometric mean antibody titre of the prepared vaccine was statistically higheralong 4 weeks post vaccination. Moreoverit produced 100% protection against challenge while commercial vaccine revealed 80% protection. Unvaccinated control grouphad zero protection. It is concluded that immunization of rabbits with vaccine prepared by sonication of genotyped field *P multocida* isolate would besupportive means for avoidance of pasteurollosis in rabbitaries.

Key words: *Pasteurella multocida – Vaccine – Sonication – Immunogenicity – Rabbit*

INTRODUCTION

Pasteurellosis caused by *Pasteurella multocida* is one of the most significant bacterial diseases of rabbits and causes considerable economic losses in large production units throughout the world (Takashima *et al.*, 2001). In most cases, the likely site of initial infection is the upper respiratory tract. Transmission occurs readily through direct contact of susceptible rabbits with carrier animals, and airborne transmission does not occur after exposure periods of 3-8weeks. Stressors such as crowding, transportation, and high ammonia concentrations in the air often stimulate latent *P. multocida* to proliferate and cause disease (Al-lebban *et al.*, 1989, DiGiacomo *et al.*, 1991 and Manning *et al.*, 1989).

The disease is characterized by various clinical symptoms, including respiratory distress, genital infections, abscesses, otitis, and septicemia, but infection by *P. multocida* can also appear without manifesting any clinical signs (DeLong and Manning, 1994). Serogroup A and, to a lesser extent, serogroup D strains of *P. multocida* have been considered as causative agents of rabbit pasteurellosis (Kawamoto *et al.*, 1990 and Dabo *et al.*, 1999). PCR – fingerprinting assessment separated the field isolates of *P. multocida*into 3 genotypes. The circulating *P. multocida* strain was genotype I conserved in 3 of the 4 examined outbreaks. So, the local vaccine strain should be prepared from the circulating strain in the region (Suelam and Abdel Samie, 2011).

Presently, prevention of pasteurellosis is carried out by vaccination using whole-cell bacterin, which confer serotype-specific protection, or with live vaccines composed of attenuated strains, which protects against both homologous and heterologous serotypes (Glisson *et al.*, 1993 and Wang and Glisson, 1994). Publications on the specific prophylaxis of rabbit pasteurellosis are limited. 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).

Although control measures such as optimizing the environment of the rabbitary and culling of symptomatic animals reduce morbidity,6 endemic infections represent a source of considerable potential loss. Results demonstrate the possibility of using vaccination to greatly limit infection and disease under field conditions. Several vaccines have

been tested under laboratory conditions, field trials of vaccines for the control of *P. multocida* in rabbits are few (Suckow *et al.*, 2008).

The main objective of the study was preparation of vaccine from field isolates of *P. multocida* that genotyped by PCR and determination of its efficiency for protection of rabbits from infection.

MATERIALS and METHODS

P. multocida: Field isolates from 4 different outbreaks were previously identified and genotyped by PCR (Suelam and Abdel Samie, 2011). Genotype I (A, B and D) was used for development and assessment of the vaccine. The virulence of the bacterial isolates was tested by intra-peritoneal injection of 6 month old mice which died 24 hours post inoculation.

Commercial vaccine: Oil adjuvant formalin 0.5% inactivated *P. multocida* (A1, A3, A12 and D2) vaccine containing $4x10^9$ c.f.u/ml produced by Veterinary Serum and Vaccine Research Institute, Cairo, Egypt.

Vaccine preparation: The bacteria were grown in brain heart infusion agar for 18h at 37°C. Cells were harvested, washed then centrifuged (8000 x g for 10 min. at 4°C), the supernatant was dialyzed extensively against a buffer containing 0.01 M Tris-HCl, 0.32 M NaCl, and 0.01% NaN_3 (pH 8.0). The pellet was concentrated at $4x10^9$ cfu/ml. The number of bacteria was determined by the optical density of the solution at 480 nm (Spectronic 70, Bausch and Lomb, USA) and compared with a calibrated standard. Then the bacterial suspension, was sonicated using ultrasonic disintegrator (MSE) at 1.2A for 15 min until a transparent solution was obtained, the lysate was filtered through a 0.45 sterile filter. Total protein concentration was 1.9 mg/ml. Sterility of the filtrates was confirmed by plating in blood agar containing 5% sheep blood. The Safety of the vaccine was carried outaccording to (OIE, 2008). These procedures took place in Biotechnology Lab., Faculty of Veterinary Medicine, Cairo University.

Preparation of challenge organisms: Isolated *P. multocida* cultured on blood agar containing 5% sheep blood at 37°c for 24 hours, harvested into broth and diluted to 10^8 organisms per 1ml. The challenge dose was 1ml per rabbit inoculated intranasal at 4weekspost last immunization for determination of vaccine efficacy. Protection% measurement was based on number of survivals and dead or rabbits show clinical signs.

ELISA: Commercial *Pasteurella multocida* ELIS Akit (ProFLOK, Snybiotic) was tested and used for screening of antibody level post vaccination in rabbits. Sera obtained from vaccinated and control rabbits were examined for the presence of *P. multocida* IgG antibodies on weekly intervals for one month. The titre was calculated according to the manufacturer Log_{10} titer = (1.464 x Log_{10} SP) + 3.197,

$$\text{SP value} = \frac{\text{OD value of sample} - \text{Avg. OD Normal Control}}{\text{OD value of positive control} - \text{Avg. OD Normal Control}}$$

Result interpretation: titer levels 0 (Negative), 148 and higher (Positive).

Animals: 65 weaned apparently healthy New Zealand white rabbit (Veterinary Hospital, Zagazig University) were deployed. Rabbits were divided into two experimental groups. All rabbits were housed in cages and fed on non medicated commercial feed. Tape water and feed were provided ad-libitum.

Experimental design:
Experiment 1: twenty rabbits of 60 days old were equally divided in four labeledcages. Rabbits of **cage 1**werevaccinated twice subcutaneously with 0.5 ml (2×10^9 cfu), **cage 2** with 1 ml (4×10^9 cfu.) and **cage 3** with 2 ml (8×10^9 cfu) of the prepared vaccine with 2weeks interval while last **cage4** was not vaccinated control group. After 3 weeks post last immunization sera were harvested from all animals for serological test then inoculated intranasal with 1ml of *P. multocida* broth contain 10^8 cfu and monitored for 14 days for morbidity and mortality.

Experiment 2: forty five rabbits of45 days old equally divided into three groups in separate cages. **Group A** was immunizedt wice with sonicated vaccine, **group B**immunized with commercial vaccine at the same age while **group C** was not vaccinated control. Immunized groups were vaccinated subcutaneously with1ml containing $4x10^9$ cfu at 45 and 60 days old. Blood samples were collected from all groups on weekly basisstarted with one week post last vaccination and lasted4 weeks for immune-assay. All groups were challenged intranasal with 1ml (10^8 cfu) of *P. multocida* at 88 days old.

Statistical analysis: Data were collected, organized and analyzed using one-way analysis of variance (ANOVA) through the general linear models (GLM) procedure of the Statistical Package for Social Sciences version 17.0 (SPSS for Windows 17.0, Inc., Chicago, IL, USA). Duncan multiple range test were used to separate means at $p < 0.05$.

RESULTS

All experimental rabbits were tested negative for *P. multocida* before immunization. Experiment 1 results showed neither morbidity nor mortality among

rabbits of cage 2 and 3 reflecting 100% protection against challenge with virulent *P. multocida*. Cage 1 showed 2 mortalities and 1 with respiratory manifestation in the form of rhinitis, conjunctivitis and sneezing reflecting 40% protection while cage 4 had zero protection as all rabbits died. 3 weeks post last immunization, seroconversion results by ELISA recorded average antibody titer 738.40±24.74[a] and 760.20±25.82[a] for cage 2 and 3 respectively, with no statistically difference while cage 1 recorded 158.80±97.36[b] (Fig 1). The results of experiment 1 interpretive that dose of 1ml containing $4x10^9$ cfu of sonicated *P. multocida* vaccine provide 100% protection and higher dose provoked the same degree of immunity while lower dose was unable to provide sufficient protection for immunized rabbits (Table 1).

Experiment 1 results revealed that rabbits immunized with sonicated *Pasteurella multocida* vaccine provoked not only higher antibody titre but also significantly difference than other rabbits immunized with commercial gel adjuvant formalized vaccine along 4 weeks post last vaccination (Table 2). The mean antibody titre of group A was 440.47±7.44[a], 582.80±8.26[a], 744.53±14.28[a] and 564.80±18.63[a] at 1st week, 2nd week, 3rd week and 4th week post second vaccination respectively, while it was 410.10±7.48[b], 563.90±8.50[a], 710.53±7.64[b] and 494.87±12.21[b] in group B respectively. Group C had negative results for antibody titre. At the end of the experiment challenge of all groups with pathogenic genotype of *P. multocida* reflected 100% protection in group A while it was 80% where 3 rabbits of group B showed signs of respiratory manifestation in the form rhinitis, lacrimation with conjunctivitis. All rabbits of group C died.

Fig. 1: Showing Mean plot of sero-conversion of different doses of sonicated vaccine

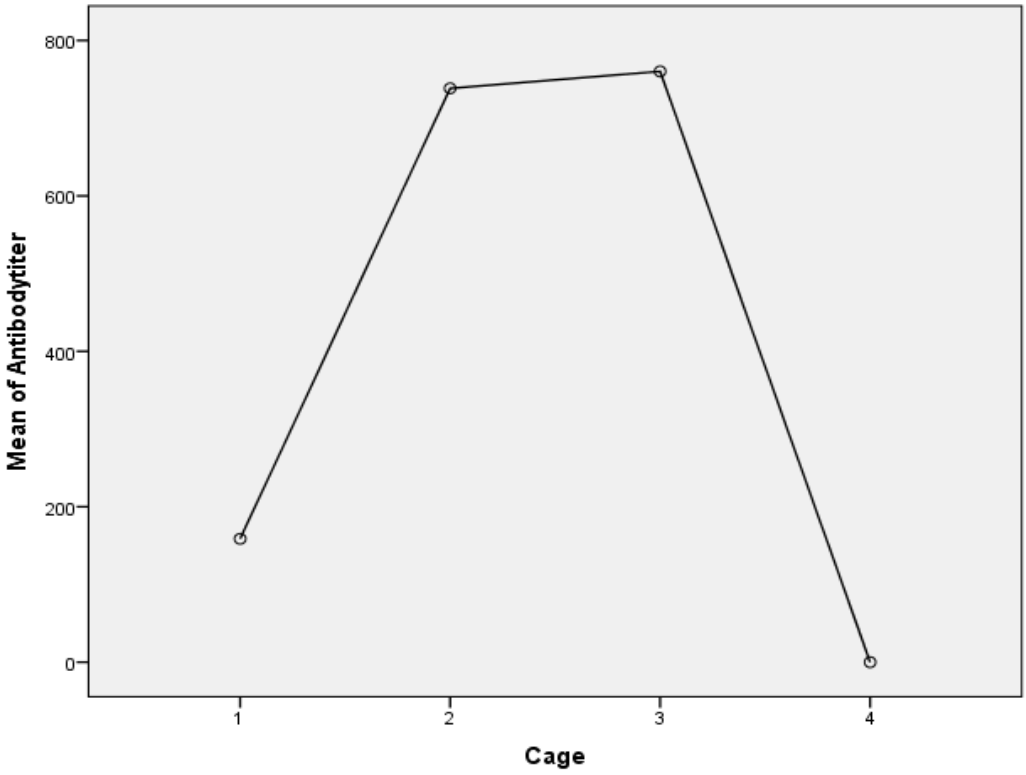

Table 1: Results of experiment 1.

Grp	No of ravbbit	Dose of the vaccine	Survivals	Death	Protection %	IgG titer*
Cage 1	5	0.5 ml (2×10^9 cfu)	2	3	40	158.80±97.36[b]
Cage 2	5	1 ml (4×10^9 cfu.)	5	0	100	738.40±24.74[a]
Cage 3	5	2 ml (8×10^9 cfu.)	5	0	100	760.20±25.82[a]
Cage 4	5		0	5	0	0.00±0.00[c]

* Means within the same column carrying different superscript are significantly different at P value<0.05

Table 2: Results of experiment 2

Grp	No of rabbit	Type of vaccine	Mean antibody titer*				Protection %
			1st W	2nd W	3rd W	4th W	
A	15	Sonicated	**440.47±7.4[a]**	**582.80±8.3[a]**	**744.53±14.3[a]**	**564.80±18.6[a]**	**100**
B	15	Adj formalized	**410.10±7.5[b]**	**563.90±8.5[a]**	**710.53±7.6[b]**	**494.87±12.2[b]**	**80**
C	15	Non	0	0	0	0	0

* Means within the same column carrying different superscript are significantly different at P value<0.05

DISCUSSION

This study investigated the immunogenic effect of sonicated vaccine prepared from field isolates of *Pasteurella multocida* in rabbits. The first Out comereflected that the minimal dose of the vaccine that provoked the best immuneresponse in rabbits was1 ml containing 4×10^9 cfu, in agreement with results (Ruzauskas, 2005) who recorded thatin laboratory trial of the inactivated vaccine against rabbit pasteurellosis using rabbits as experimental and at the same time as target animals revealed that 100%of vaccinated rabbits survived after *Pasteurella multocida* infection when the vaccine concentration was4×10^9 b.c./ml by immunization of rabbits with noless than 1 ml of the vaccine. On the contrary of (Arif *et al.*, 2013) who prepared *P. multocida* vaccine containing 2×10^6cell./ mlsupplemented with 10% outer membrane protein and the total protein contents were maintained at 1mg/mlused for immunizing rabbits producing 88% protection on challenge at 21 days post vaccination.

On evaluation of the prepared vaccine, results showed that it was competent with other commercial vaccine that widely used in the Egyptian market, mean level of IgG was significantly higher and produced better protection against challenge. In spite of different methodology (Lee *et al.*, 2007) who prepared outer membrane protein (OmpHs) *P.multocida* vaccine by sonication of the bacterial envelope and extracted the outer membrane and estimated its immunogenicity. Theyrecorded similar results on matching with other vaccines noting that the levels of anti body response in commercial vaccine and formalin-killed whole cell vaccinates were relatively lower than those of the Omp Hs. This may be attributed to the use of genotyped bacterial isolates at certain locality in vaccine preparation and homologous challenge.

A 3rd week post initial vaccination antibodies was measured by ELISA in our experiment, unlikeresult of (Suckow *et al.*, 2008) recorded that after vaccination of rabbits with potassium thiocyanate extract (PTE) produced from *P.multocida*, ELISA was performed on samples obtained before, 5 and 24 week after initial vaccination. Both IN and SC vaccination with PTE stimulated humeral immunity by 5 week after initial vaccination this response decreased by 24 week afterward.

It concluded that methods of vaccine preparation and type of bacterial isolates responsible to great extend for its immunogenicity. Uses of sonicated vaccine from *P.multocida* isolated and genotyped from specific area would be a useful means for prevention of the disease in rabbits and improvement of its immune complex.

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مناعية لقاح البستريلامالتوسيدا المعملى فى الأرانب

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فى بحث سابق تم عزل ميكروب البستريلا مالتوسيدا من أرانب مصابة بعدة أماكن بمحافظة الشرقية جمهورية مصر العربية وتصنيفها جينيا بإستخدام PCR. فى البحث الحالى أستخدمت العترات المعزولة فى تحضير لقاح معملى بطريقة تفكيك بروتين الخلايا البكتيرية بواسطة الموجات فوق الصوتية وقد أجريت تجربتان الأولى لتحديد جرعة اللقاح المناسبة لتنشيط الجهاز المناعى للأرانب لتكوين أجسام مضادة قادرة على صد الأصابة التجريبية وقد كانت ١٠x٤ ^{٩} /سم والتجربة الثانية للتحقق من فعالية اللقاح المعملى ومقارنته بلقاح متداول تجاريا فى الأسواق المصرية. أثبتت النتائج السيرولوجية أن اللقاح المعملى أستطاع أن يحمى الأرانب بنسبة ١٠٠% وأن مستوى الأجسام المناعية المضادة أعلى بنسبة معنوية أحصائيا عن تلك التى قيست فى المجموعة المحصنة باللقاح التجارى. هذا يدل على أن تحضير لقاح بهذه الطريقة ممكن أن يساعد فى الوقاية من هذا الوباء فى الأرانب.